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Award Number: W81XWH-06-1-0694

TITLE: The SDF1-CXCR4 Axis Functions through p38-MAPK Signaling to Drive Breast Cancer Progression and Metastasis

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REPORT DATE: September 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-Sep-2007		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 30 Aug 2006 - 29 Aug 2007	
4. TITLE AND SUBTITLE The SDF-1 - CXCR4 axis functions through p38-MAPK signaling to Drive breast cancer progression and metastasis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0694	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Lyndsay Vanhoy				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tulane University New Orleans, LA 70112				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research & Material Command Fort Detrick Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The primary long-term objective of this research is to understand how chemokine signaling through MAPK influences progression of breast carcinoma cells to a hormone-independent, endocrine therapy resistant and metastatic phenotype. Our preliminary evidence demonstrates that overexpression of CXCR4 in breast carcinoma cells leads to a hormone independent phenotype in vivo. The ability of CXCR4 expression to lead to estrogen independent breast tumor growth in immunocompromised mice is correlated with CXCR4 mediated activation of downstream signaling events and enhancement of estrogen receptor mediated gene expression. We hypothesize that altered expression of CXCR4 and activation by its ligand SDF1 directly controls progression to hormone independence in breast carcinoma cells. We specifically hypothesize that the SDF1-CXCR4 axis functions through G-protein coupled signaling through the downstream MAPK pathway to regulate gene expression. Our research will provide strong evidence that combining SDF1/CXCR4 blockade with current endocrine therapy strategies will be synergistically effective in treatment for endocrine resistant and metastatic breast cancer. As a novel mechanism of hormone independence this could prove to be a unique approach to breast cancer treatment. New treatment options for patients are necessary for improved patient care.					
15. SUBJECT TERMS Breast cancer, chemokine, SDF-1					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 18	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

The development of resistance to anti-estrogens and the progression to hormone-independence are still poorly understood problems in the treatment of ER-positive breast cancers. The function of the ER-coregulated proteins and crosstalk with ER signal pathway, growth factor mediated signaling, and other kinase networks could be responsible for this resistance. It has been shown that some chemokines lead to the initiation of migration. One such chemokine, stromal-derived growth factor 1 (SDF1/CXCL12) along with its receptor chemokine X receptor 4 (CXCR4) expression is a critical component in the ability of cancer cells to invade and metastasize. It has been demonstrated that estrogen-estrogen receptor (ER) is able to mediate the upregulation of the SDF-1 thereby establishing a link between hormone and chemokine signaling in proliferation of breast carcinoma cells. This SDF1-CXCR4 axis functions to stimulate proliferation, promote cell motility/invasion, and suppress apoptosis through activation of specific downstream signaling pathways. One such pathway includes phosphatidylinositol 3-kinase (PI3K)/AKT and members of mitogen-activated protein kinase (MAPK) family such as Erk1, JNK and p38. Interestingly, both the MAPKs and PI3K-AKT have been implicated in development of resistance to endocrine therapy in breast carcinoma. It has been reported that unlike other transmembrane G-protein coupled receptors, SDF1 has the same affinity for CXCR4 in both the coupled and uncoupled state with G-protein. So the possibility exists that the interaction of SDF1 with CXCR4 can induce two distinct signal cascades, one requiring G-protein mediated signaling (our focus) and another where G-protein independent signal pathway activated through other membrane receptors. While the ability of CXCR4 or other chemokine receptors to regulate the ER has not been examined, the ability of GPCRs such as the chemokine receptor CXCR4 to regulate estrogen receptor signaling and progression of cancer cells to hormone-independence represents an important area of research. We believe our research will provide very strong evidence to resolve endocrine therapy resistance in breast cancer.

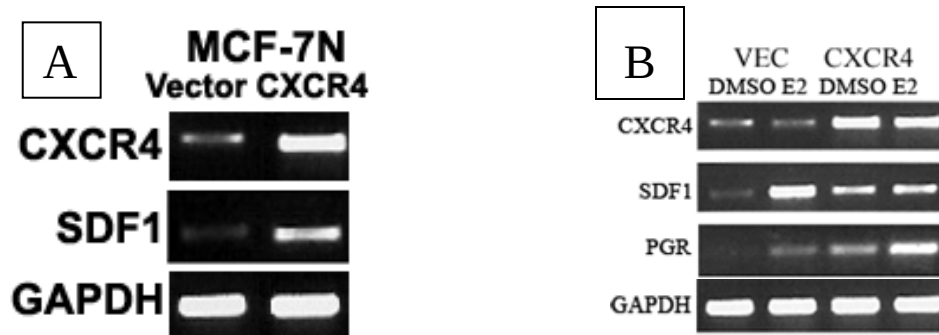
BODY

We have broken the work performed during year one of this proposal into tasks:

Task 1. Establish crosstalk between CXCR4/G-protein signaling and estrogen receptor (ER).

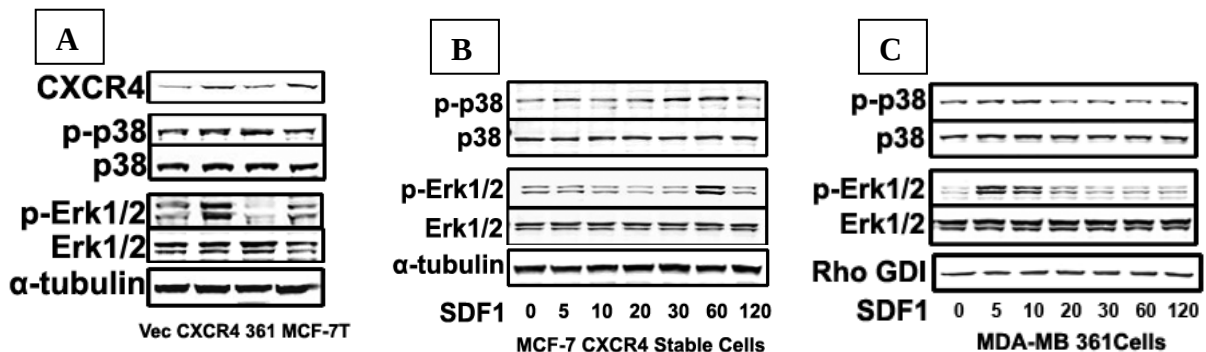
1.A Determine CXCR4 activation leads to increased ER-mediated gene expression.

Experiments to test CXCR4/SDF-1 ability to regulate ERE-luciferase activity in MCF-7-VEC, MCF-7-CXCR4, and MDA-MB-231 when stimulated with estrogen, Tamoxifen, or ICI have been initiated but have not produced results at this time.



A. MCF-7-vector or MCF-7-CXCR4 overexpressing stable cell lines were harvested for RNA isolation and RT-PCT to test for SDF-1 gene expression. SDF-1 is an ER regulated gene. As the figure above demonstrates, overexpression of CXCR4 induces SDF-1 expression. B. The same cell lines were treated with estrogen and harvested for RNA isolation and RT-PCR to test for ER responsive genes. Cells stimulated with estrogen exhibit an increase in SDF-1 mRNA illustrating a role for CXCR4/ER crosstalk in regulation of ER gene expression.

1.B Determine if CXCR4 activates p38.



A. Western blots of MCF-7-VEC, MCF-7-CXCR4, MDA-MB-361, MDA-MB-231 for basal levels of p38 and ERK signaling. **B.** MCF-7-CXCR4 cells or **C.** MDA-MB-361 cells were treated with SDF-1 in a timecourse manner and a Western blot was performed in order to determine the phosphorylation of p38 and ERK1/2. We have shown that SDF-1 increases phospho-p38 levels in these cell lines as well as phosphor-ERK.

1.B.2 Determine the role of CXCR4 regulation of ER by p38 signaling using p38 luciferase.

This task has not yet been started. We plan to use phospho-specific ER ELISA kits for confirmation of transcription factor activation by CXCR4.

1.C Determine if p38-MAPK signaling is required for CXCR4 mediated breast cancer progression.

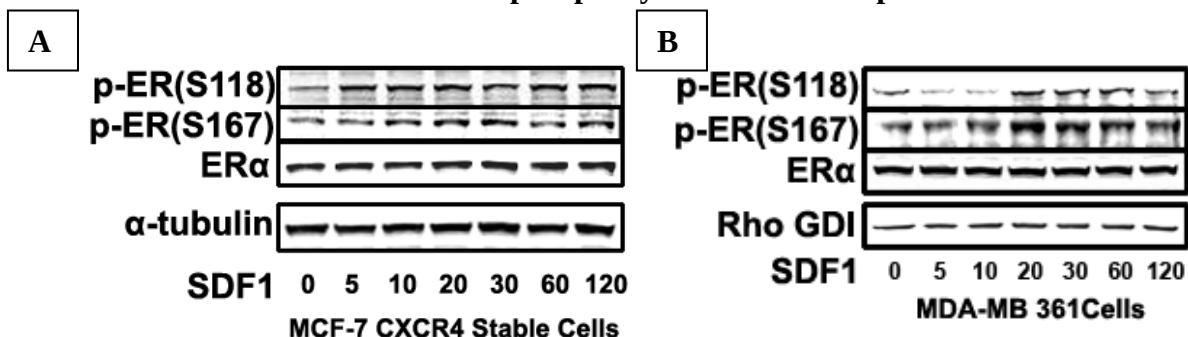
We plan to use cells treated with either shRNA to p38, chemical inhibitors of p38, or DN-p38 with ERE-luciferase to determine CXCR4 mediated gene expression. This will determine if p38-MAPK signaling is required for CXCR4 mediated cancer progression. These experiments are in progress but have not produced reportable data at this time.

Task 2. CXCR4/SDF-1 axis regulates ERE-mediated gene expression through phosphorylation of the ER.

2.A Determine which transactivation domain (AF1 or AF2) CXCR4 signaling targets on ER.

Western blots were performed to determine the total phosphorylation of ER α AF1 and AF2 sites after stimulation with SDF-1. Our first blot showed that the AF1 domain was the site of phosphorylation following treatment with SDF-1. However, subsequent blots have not been able to reproduce these results. We will not draw any conclusions as the transactivation domain involved in CXCR4 signaling until we work out our antibody issues.

2.B Determine if CXCR4 phosphorylates the ER at specific sites.



Western blots were carried out on cells after treatment with and without SDF-1 using phosphor-specific antibodies to the ER. We determined that SDF-1 treated cells induce phosphorylation at both serine 118 and serine 167 on the ER in both **A.** MCF-7-CXCR4 and **B.** MDA-MB-361 cell lines.

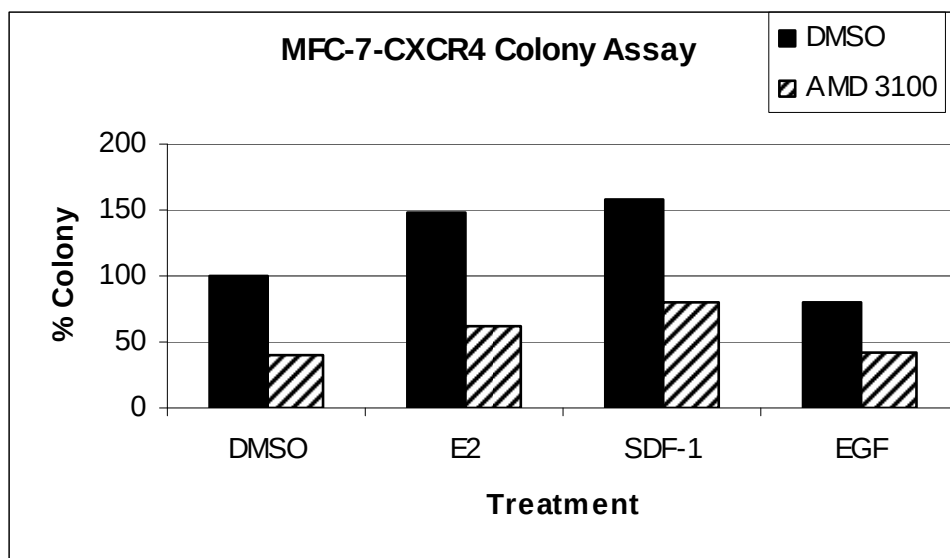
2.C Determine the requirement of ER phosphorylation by CXCR4 for mediation of gene expression.

These experiments have not yet been conducted. We plan to use phospho-mutants of the sites specified by the experiments above in conjunction with luciferase reporter assays to conclude if these sites are required and/or sufficient for ER activation. Mutants will be used alone and in combination.

Task 3. Demonstrate CXCR4/SDF-1 axis mediated gene expression is necessary for hormone independence in breast cancer.

3.A Demonstrate that hormone independence can be disrupted by blocking the CXCR4/SDF-1 axis *in vitro*.

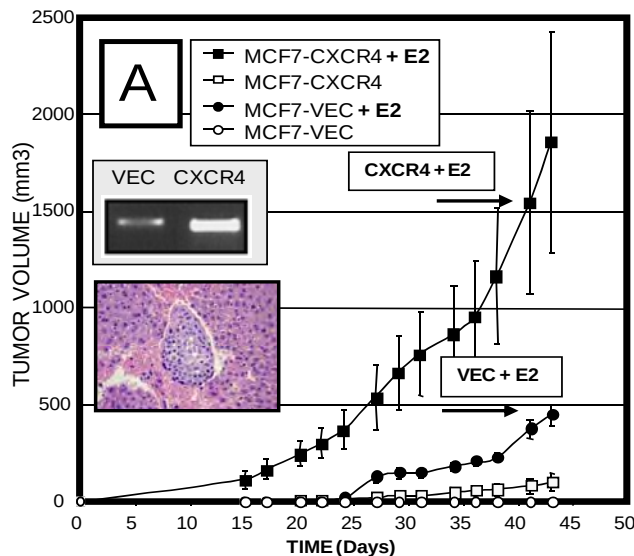
These experiments are currently in progress. Several assays have been performed using MCF-7-VEC, MCF-7-CXCR4, and MDA-MB-231 cells. We show a stimulation of colony formation with the addition of SDF-1 even in the absence of estrogen which can be knocked down with the addition of the CXCR4 specific inhibitor, AMD3100. AMD3100 also appears to inhibit colony formation in the presence of estrogen indicating a link between ER and CXCR4.



3.B Determine the role of CXCR4 expression on progression of breast cancer to invasive and metastatic phenotype dependent on ER phosphorylation *in vitro*.

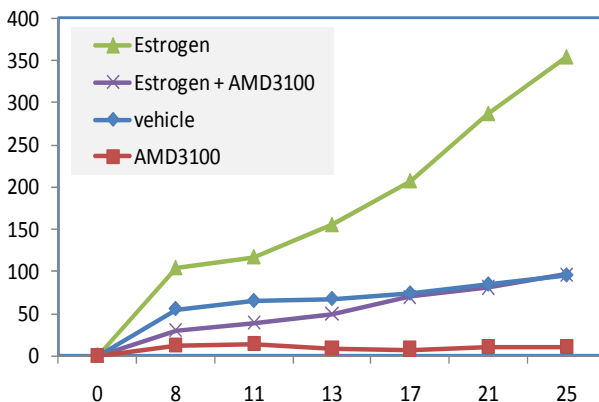
These experiments have not yet been initiated. Transwell experiments will be utilized using MCF-7-VEC, MCF-7-CXCR4, MDA-MB-361 and MDA-MB-231 cell lines treated with and without SDF-1 and/or estrogen.

3.C Test CXCR4/SDF-1 affect on tumor formation, estrogen-independence and metastasis *in vivo*.



CXCR4 expressing breast carcinoma cells exhibit hormone independent tumor formation and increased estrogen sensitivity. Female, 4-6 week old, ovariectomized SCID/beige mice (n=5/group) were injected in the MFP with 5x10⁶ MCF7-vector or MCF7-CXCR4 cells suspended in 50ul of sterile PBS. Tumors were measured every 3 days using digital calipers.

Immunocompromised mouse models were utilized to evaluate tumor growth after injection of MCF-7-CXCR4 cells into the mammary fat pad of ovariectomized female mice with or without estrogen pellets. MCF-7-CXCR4 cells grew faster and larger than control tumors in the presence Metastasis was seen in a few of the CXCR4 mice but results were not conclusive. Livers and lungs were harvested from all mice and will be sent for sectioning to explore for metastasis.



Pharmacologic inhibition of CXCR4 suppresses estrogen stimulated breast cancer tumor growth *in vivo*. 5x10⁶ MCF7-CXCR4 cells were suspended in 50ul of sterile Matrigel (reduced factor) and injected into the MFP of female ovariectomized Nude mice. A pellet of 0.72 mg, 60 day release 17-beta estradiol was implanted subcutaneously. Tumor size was monitored using digital caliper. Animals received twice daily i.p. injections of vehicle or AMD3100 (0.1mg) for 14 days.

Mice were treated with AMD3100 twice daily for 14 days. We were able to decrease the increased growth caused by overexpression of CXCR4 with the use of AMD3100 in MCF7-CXCR4 cells. Tumors were fixed in 10% formalin and have been sent off for sectioning and staining.



Similar results were seen with the use of a CXCR4 specific antibody. Anti-CXCR4 was able to decrease tumor volume in MCF7-CXCR4 cells (+/- estrogen) and MDA-MB-361 cells (+estrogen). The above data indicate that inhibition of CXCR4 is able to decrease tumor volume in vivo.



KEY RESEARCH ACCOMPLISHMENTS

- Determined CXCR4/SDF-1 increase of ER responsive genes.
- Determined CXCR4 relationship with phosphorylated p38.
- Determined SDF-1 phosphorylation of AF1 on ER.
- Determined SDF-1 specific phosphorylation at s118 on ER.
- Demonstrated SDF-1 ability to initiate increased colony formation in a hormone independent manor.
- Demonstrated CXCR4/SDF-1 ability to enhance tumorigenesis and metastasis *in vivo*.

BREAST CANCER TRAINING-RELATED OUTCOMES

- Successfully completed preliminary exam for the Biomedical Science Graduate Program.
- Lecturer, Endocrine Pharmacology class, Department of Pharmacology, Tulane University, New Orleans, LA
- Teaching assistant, Cellular and Molecular Biology Program, Tulane University, New Orleans, LA
- Mentorship, Shannon E. Muir, Pharmacology Masters student, Department of Pharmacology, Tulane University, New Orleans, LA

REPORTABLE OUTCOMES

Presentations:

“CXCR4 Drives Breast Carcinoma to Hormone-Independent and Metastatic Phenotype”. Molecular and Cellular Biology Program Annual Retreat, Primate Center, Tulane University, Covington, LA. October 2006. **Lyndsay Vanhoy Rhodes**

“Chemokines in Breast Cancer”. George A. Pfeiffer Science Symposium, Pfeiffer University, Misenheimer, NC. October 2006. Alumni speaker. **Lyndsay Vanhoy Rhodes**

“CXCR4 Drives Breast Carcinoma to Hormone-Independent and Metastatic Phenotype”. Molecular and Cellular Biology Program Annual Research Days, Tulane University School of Medicine, New Orleans, LA. March 2007. **Lyndsay Vanhoy Rhodes**

Abstracts:

Lyndsay Vanhoy Rhodes, Alberto Salvo, Pablo Fonseca, Syreeta Tilghman, Steven Elliott, and Matthew E. Burow. “CXCR4 Drives Breast Carcinoma to Hormone-Independent and Metastatic Phenotype”, Molecular and Cellular Biology Annual Research Days, Tulane University, Feb. 28 – Mar. 1, 2007, New Orleans, LA.

CONCLUSION

The hypothesis that overexpressing CXCR4 cancer cells crosstalk with ER to upregulate their own ligand (SDF1) and form a feed-forward autocrine loop which activates CXCR4 signaling constitutively provides a novel mechanism for tumor progression and metastasis. Our research will provide strong evidence that combining SDF1/CXCR4 blockade with current endocrine therapy strategies will be synergistically effective in treatment for endocrine resistant and metastatic breast cancer. As a novel mechanism of hormone independence this could prove to be a unique approach to breast cancer treatment. New treatment options for patients are necessary for improved patient care.

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BIOGRAPHICAL SKETCH

NAME		POSITION TITLE	
Lyndsay Vanhoy Rhodes		Graduate Student of Molecular and Cellular Biology	
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Pfeiffer University, Misenheimer, North Carolina	B.S	2000 - 2004	Biology
Pfeiffer University, Misenheimer, North Carolina	B.A.	2000 - 2004	Psychology
Tulane University, New Orleans, Louisiana	Ph.D. (in progress)	2004 – current	Molecular and Cellular Biology

Research Experience:

2000 – 2004	Undergraduate Honors Independent Research Projects, Pfeiffer University, Misenheimer, NC Antibiotic Susceptibility in Animal Products, Plasmid Isolation, Bioinformatics Program, Development of Plant Tissue Culture Protocol
2002	Teaching & Resident Assistant, Southern Piedmont Educational Consortium (SPEC), Pfeiffer University, Misenheimer, NC
2003	Research Intern, Plant Molecular and Cell Biology Program, University of Florida, Gainesville, FL
2004	Lab Assistant, James Robinson Lab, Tulane University Medical Center, New Orleans, LA
2004-2006	Teaching Assistant, Tulane University, New Orleans, LA
2005-present	Graduate Student Research, Interdisciplinary Program in Molecular and Cellular Biology, Tulane University Medical Center, New Orleans, LA (Thesis Advisor: Matthew Burow)

Honors, Awards, and Other Professional Activities:**Scholarships:**

2000 – 2004	Presidential Scholarship, Pfeiffer University
2000 – 2004	Medical Scholarship, Pfeiffer University
2000 – 2004	Athletic Scholarship, Varsity Swimming, Pfeiffer University

Honors:

2000 – 2004	Dean's List, Pfeiffer University
2000 – 2004	Honor Scholar, Pfeiffer University
2000 – 2004	Scholar Athlete, Pfeiffer University
2003 – Present	Psy Chi, Pfeiffer University Chapter
2004	Who's Who among American Universities and Colleges
2004 – Present	Order of the Sundial, Pfeiffer University
2005 – Present	Phi Delta Sigma, Pfeiffer University

Activities:

2003 – 2004	Vice President, Honors Colloquium, Pfeiffer University Steering Committee Student Representative, Molecular and Cellular Biology Program, Tulane University
2005 – 2006	Molecular and Cellular Biology Graduate Student Association President, Tulane University
2006 – 2007	Biological Sciences Steering Committee Student Representative, Tulane University
2006 – 2007	Biological Sciences Student Board of Representatives, Tulane University

Recent Publications:

Duong, B.N., Elliott, S., Frigo, D.E., Melnik, L.I., **Vanhoy, L.**, Tomchuck, S., Lebeau, H.P., David, O., Beckman, B.S., Alam, J., Bratton, M.R., McLachlan, J.A., Burow, M.E. AKT regulation of ER-beta transcriptional activity in breast cancer. *Cancer Research*. 66(17):8373-8381. (2006).

Recent Presentations:

“CXCR4 Drives Breast Carcinoma to Hormone-Independent and Metastatic Phenotype”. Molecular and Cellular Biology Program Annual Retreat, Primate Center, Tulane University, Covington, LA. October 2006.

“Chemokines in Breast Cancer”. George A. Pfeiffer Science Symposium, Pfeiffer University, Misenheimer, NC. October 2006. (invited Alumni speaker).

“CXCR4 Drives Breast Carcinoma to Hormone-Independent and Metastatic Phenotype”. Molecular and Cellular Biology Program Annual Research Days, Tulane University School of Medicine, New Orleans, LA. March 2007. (Poster session and brief talk).

Research Support:

Current:

DoD Breast Cancer Predoctoral Traineeship Award BC061597 “The SDF1-CXCR4 Axis Functions Through p38-MAPk Signaling to Drive Breast Cancer Progression and Metastasis”. My role: PI. 8/30/2006 – 9/29/2009.