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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 over expression of CXCR4 in breast carcinoma cells leads to a hormone independent phenotype in vivo. It was also determined by our lab that human mesenchymal stem cells in contact with breast cancer cells (MCF7 cell line) could induce proliferation and lead to hormone independent tumors in vivo. Upon analysis of these tumors by real-time PCR, it was found that the MSC containing tumors had increased gene transcription of progesterone receptor as well as SDF-1 indicating ER crosstalk. Future studies are planned to look more closely at the mechanisms involved in this MSC-tumor cell interaction, specifically identifying a role for SDF-1. We propose SDF-1 is the primary factor involved, either being secreted by the MSCs or the MSCs are stimulating its production in the carcinoma cells themselves. Future plans involve using MSCs as source for SDF-1 to test previously outlined objectives.					
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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 AER 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 (SDF-1/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 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. Our previously outlined objectives sought to test one such pathway including phosphatidylinositol 3-kinase (PI3K)/AKT and members of the mitogen-activated protein kinase (MAPK) family (Erk1, JNK, and p38) because both MAPKs and PI3K/AKT have been implicated in development of endocrine therapy resistance in breast carcinoma.

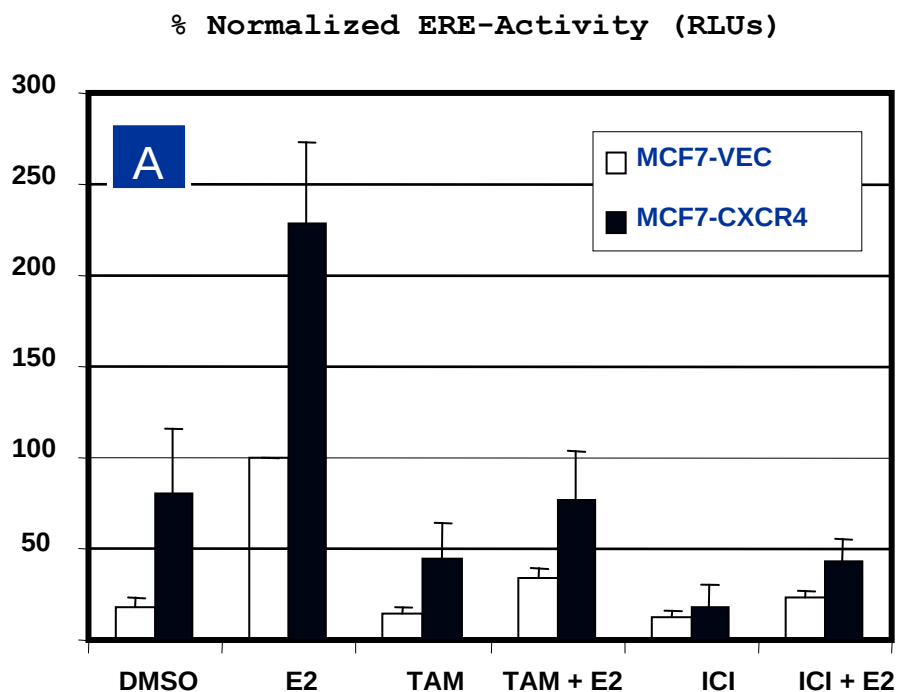
More recently, our lab became interested in the concept of human mesenchymal stem cells and their ability to contribute to the tumor microenvironment. Preliminary studies have shown that MSCs home to sites of breast cancer and integrate into the tumor stroma. We have shown that MSCs enhance primary tumor growth as well as promote hormone-independent tumor growth of the estrogen receptor positive, hormone-dependent breast carcinoma cell line MCF-7 in immunocompromised mice. This is correlated with increase expression of SDF-1, Vascular Endothelial Growth Factor (VEGF), and Progesterone Receptor (PR) in the presence of MSCs as compared to MCF-7 only tumors. Recognizing that SDF-1, VEGF, and PR are all ER mediated genes led us to further investigate the possibility of MSC crosstalk with the ER. We suggest that it is this increase in SDF-1 that acts in a paracrine fashion on the breast cancer cells through its receptor, CXCR4, leading to a shift to hormone independent growth while still remaining estrogen sensitive. The ability of CXCR4 to regulate estrogen receptor signaling and progression to hormone-independence represents an important area of research.

Body

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

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

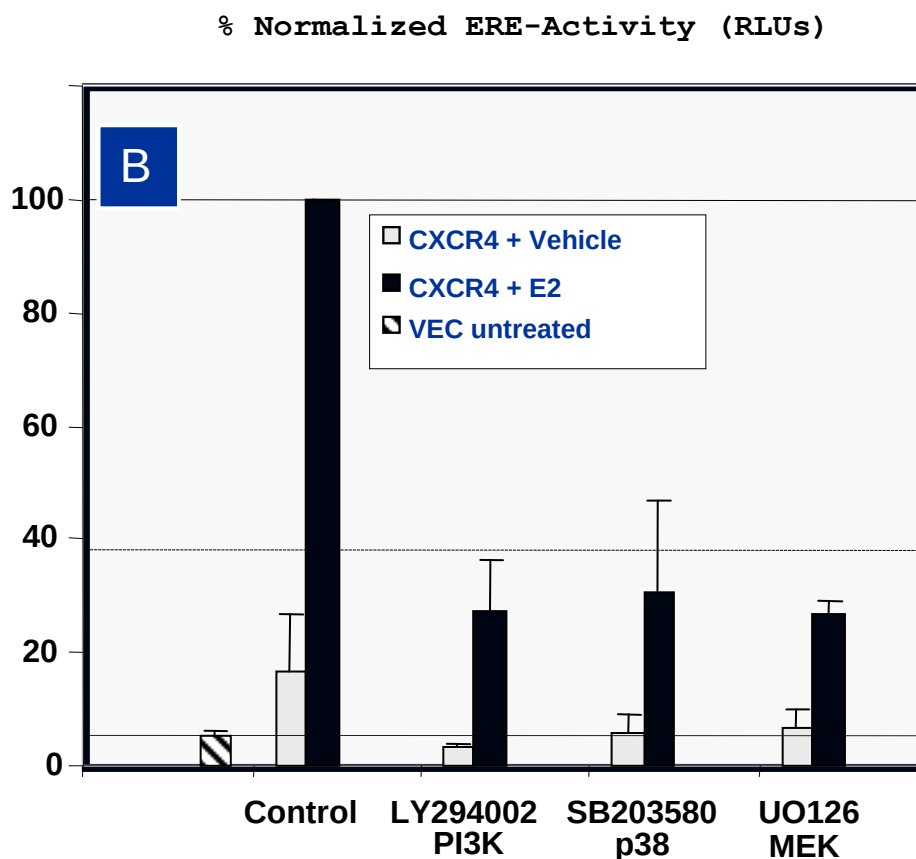
1.A Determine CXCR4 activation leads to increase ER-mediated gene expression.



MCF7-vector or MCF7-CXCR4 overexpressing cell lines were treated with DMSO (control), E2 (100pM), Tamoxifen (100nM), ICI (100nM), or a combination. As seen in the graph above, CXCR4 expression increases ERE activity in all treatment groups when compared to control. These cells do retain sensitivity to E2. ER targeted inhibitors decrease ER response in the CXCR4 cells, but to a lesser extent than control. This indicates endocrine resistance.

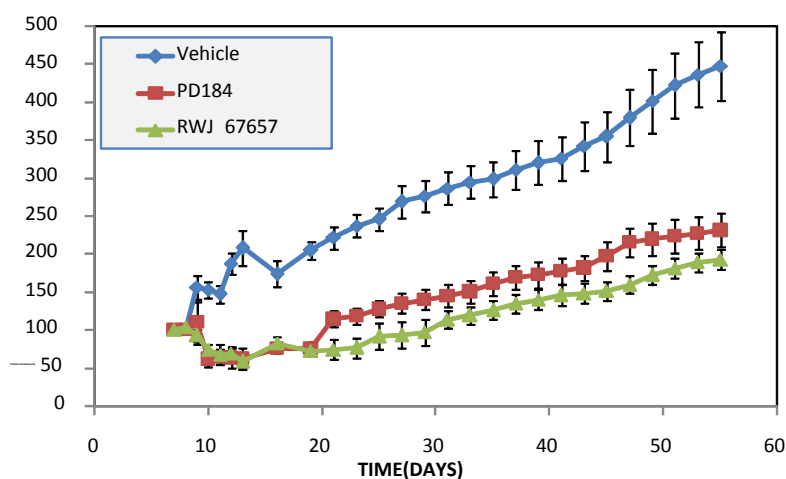
1.B.2 Determine the role of CXCR4 regulation of ER by p38 signaling using p38 luciferase.

Due to the lack of a p38 luciferase, we conducted the following experiments using ERE-luciferase in combination with p38 chemical inhibitors.



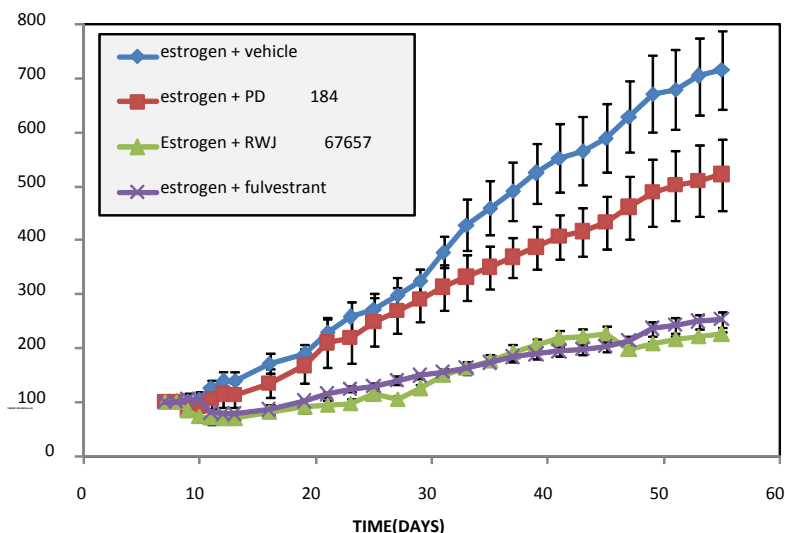
MCF7-CXCR4 cells were treated with specific inhibitors to PI3K, p38, and MEK to determine the role of each on CXCR4 activation of ER. As you can see in the graph above p38 is involved in activation of ER by CXCR4.

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



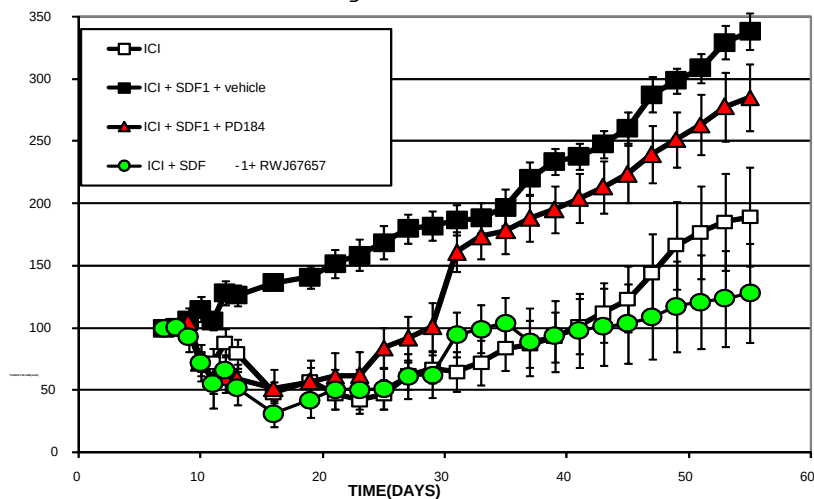
Female ovariectomized Nu/Nu mice (n=5/group) were injected in the MFP with 5×10^6 MCF7-CXCR4 cells suspended in 50ul PBS mixed with 50ul matrigel. After measurable tumor formation animals were randomized into treatment groups and treated twice daily IP with either vehicle, the MEK 11/2-Erk inhibitor PD184135 or the p38 inhibitor RWJ 67657 (20mg/kg/animal).

Using our animal model, we tested the ability of p38 inhibitor (RWJ 67657) to inhibit the ability of CXCR4 to induce hormone-independent growth. As you can see in the above graph, p38 inhibition significantly reduced tumor volume in these animals indicating p38 activation is required in CXCR4 mediate activation of ER.



Female ovariectomized Nu/Nu mice (n=5/group) were injected in the MFP with 5×10^6 MDA-MB-361 cells suspended in 50ul PBS mixed with 50ul matrigel. 17- β estradiol 0.72 mg 60 day release pellet was implanted subcutaneously. After measurable tumor formation animals were randomized into treatment groups and treated once with 5mg i.m. injection of ICI 182,780 or twice daily IP with either vehicle, the MEK 11/2-Erk inhibitor PD184135 or the p38 inhibitor RWJ 67657 (20mg/kg/animal).

We also used an additional breast carcinoma line, MDA-MB-361, which has endogenously high levels of CXCR4 to test the effect of p38. As the graph above shows, p38 inhibition brings tumor volume down the same level as ICI treatment. This indicates to us that CXCR4 actions through the ER mediate downstream effects through p38.



MAPK pathway inhibition blocks SDF1 mediated anti-estrogen resistance. Female ovariectomized Nu/Nu mice (n=5/group) were injected in the MFP with 5×10^6 MCF7-CXCR4 cells suspended in 50ul PBS mixed with 50ul matrigel in the presence or absence of SDF1 (100ng). After measurable tumor formation (day 8) animals were randomized into treatment groups and treated once with 5mg i.m. injection of ICI 182,780 or twice daily IP with either vehicle, the MEK 11/2-Erk inhibitor PD184135 or the p38 inhibitor RWJ 67657 (20mg/kg/animal).

We then looked at the SDF-1 effect on endocrine resistant tumors by injecting MCF7-CXCR4 cells into Nude female ovariectomized mice and treating with ICI. As expected ICI treatment decreased tumor volume in these animals. However, when given SDF-1, the cells were able to overcome ICI treatment, and continue to increase in tumor volume. This indicates that the CXCR4/SDF-1 axis drives an endocrine resistant phenotype. Inhibition of p38 in this study brings tumor volumes back down to ICI levels demonstrating its vital role in this event.

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

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 phosphor-mutants of the ER sites S118 and S167 in conjunction with luciferase reporter assays to conclude if these sites are required and/or sufficient for ER activation by CXCR4. Mutants will be used alone and in combination.

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

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 assays will be utilized using the following cell lines:

MCF7:

Vector	}	
CXCR4		
Vector-vector		
Vector-SDF-1		+/- E2
Vector-CXCR4		+/- SDF-1
SDF-1-CXCR4		+/- E2/SDF-1
MDA-MB-361	}	
MDA-MB-231		

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

Previously we had observed metastasis in a few of the mice injected with MCF7-CXCR4 subcutaneously. We have now developed a Tail Vein mouse model to specifically examine lung metastasis. 1×10^6 cells in 50 μ l, either MCF7-vector or MCF7-CXCR4, were injected into the lateral tail vein of Nu/Nu female ovariectomized mice. Mice were sacrificed between 6 and 8 weeks post injection and lungs were harvested and fixed in formalin. Tissues were then processed, embedded in paraffin and sectioned. H&E sections revealed an increase in tumor cell number of MCF7-CXCR4 cells in the lungs compared to those of control lungs.

Not included in previously outlined tasks, but equally important to the projects success, the following experiments have been conducted or initiated:

S.1 Generation of stable cell lines overexpressing SDF-1, CXCR4, or coexpression of SDF-1/CXCR4 in MCF7 parental line.

MCF7 cells were transfected with pSelect-GFP vector, pSelect-CXCL12 (SDF-1), pSelect-CXCR4, or a combination to result in the following transfected cell lines.

**Vector-vector
Vector-SDF-1**

**Vector-CXCR4
SDF-1-CXCR4**

Cells were kept in selectable marker until individual colonies were large enough to isolate as clones. These clones were then grown up and subjected to PCR for confirmation of stable overexpression of SDF-1/CXCR4.

These stable cell lines will be used in a battery of experiments to help us further understand the role of SDF-1/CXCR4 in breast cancer tumorigenesis, hormone-independence, and metastasis.

We plan the following experiment with the above generated cell lines to readdress task 3.C outlined previously. Female Nu/Nu ovariectomized mice will be injected in the mammary fat pad with 5×10^6 cells/injection site with matrigel. The experimental arms are as follows:

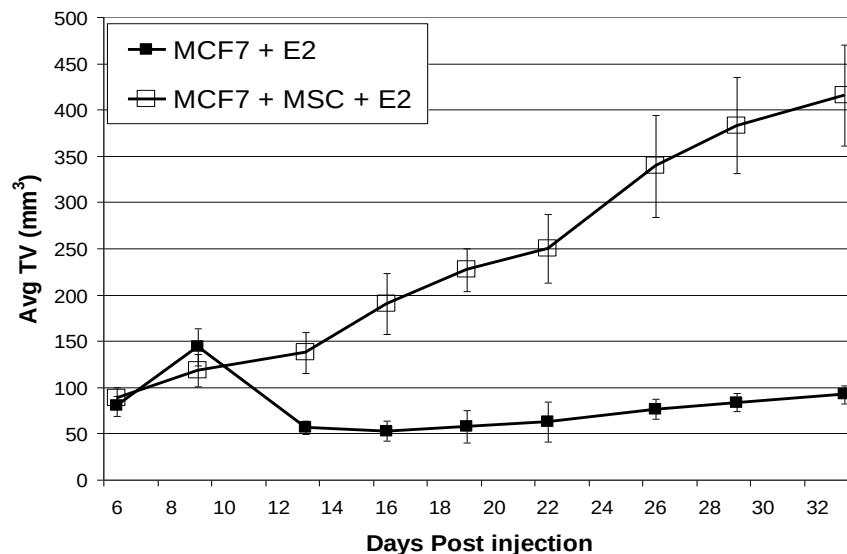
Vector-vector	}	+/- E2
Vector-SDF-1		
Vector-CXCR4		
SDF-1-CXCR4		

We plan to follow this experiment with another in vivo experiment using the same cell lines in combination with anti-estrogens to test the hormone-resistant effects of SDF-1 and CXCR4. We also plan to use CXCR4 and SDF-1 specific inhibitors (proof-of-principle).

The following 4 experimental tasks concern MSC involvement in breast carcinoma tumorigenesis, hormone-independence, and gene expression.

S.2 MSCs increase breast cancer cell sensitivity to estrogen.

When MSCs were coinjected with MCF7 cells tumor size increased more than 4 fold compared to control tumors.

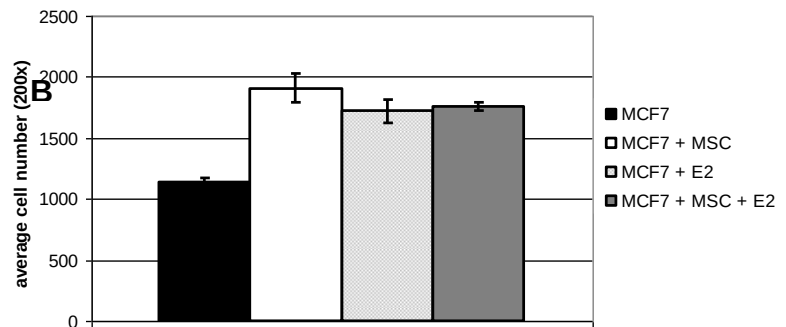
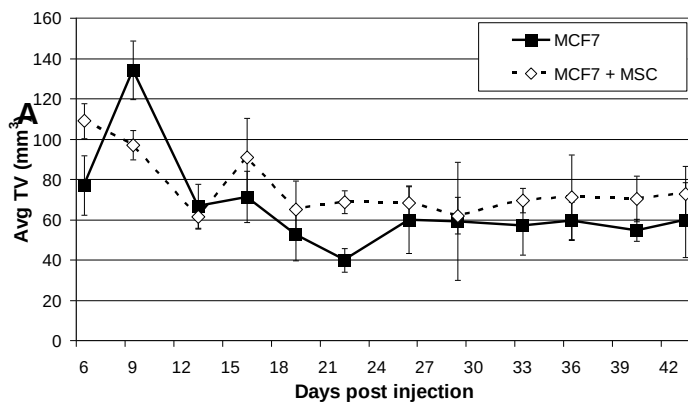


MSC promote tumor growth and estrogen sensitivity of breast tumors in vivo.

Tumor volume in mm³ (mean $\pm$ s.e.m.). 4-6 week old female ovariectomized SCID/beige mice were injected subcutaneously with 1×10^6 MCF7 $\pm$ 1×10^6 MSC in 50 μ l PBS with 100 μ l reduced growth factor matrigel, n = 5 mice per group. All mice were implanted with a 0.72mg 60 day time release estrogen pellet subcutaneously in neck. Tumors were measured every 3 days.

S.3 MSCs increase breast tumor proliferation even in absence of Estrogen.

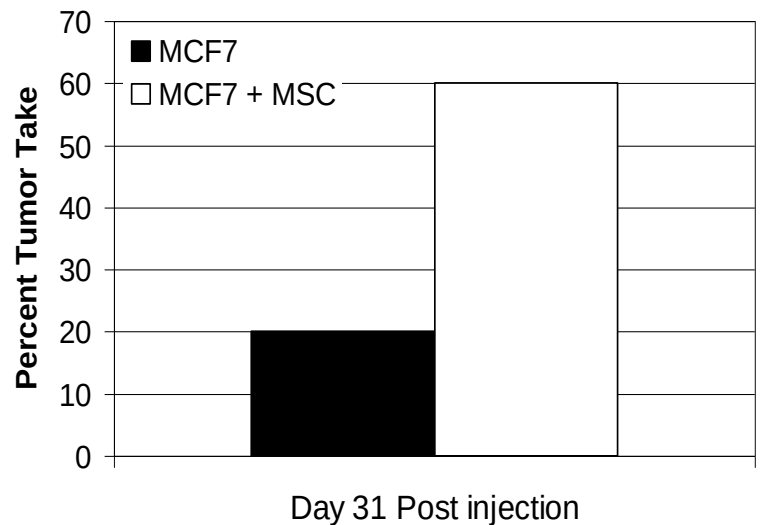
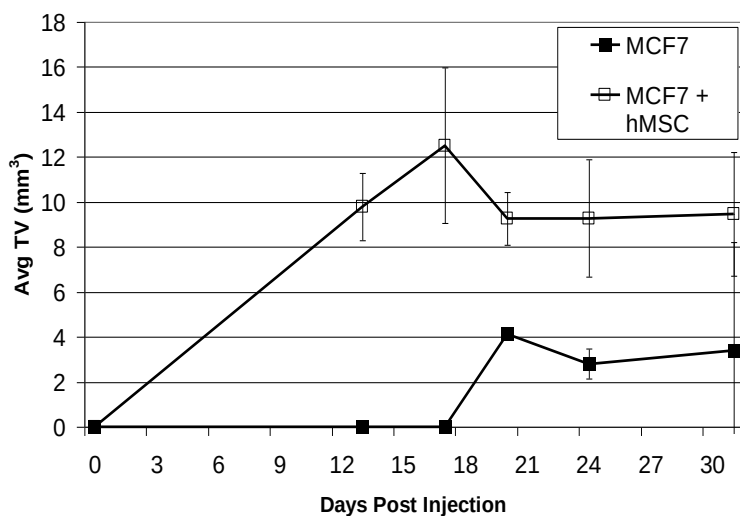
In the absence of estrogen, no significant MSC effect was seen in our tumor data. Upon closer examination of H&E sections of the tumors, it was observed that cellular density of tumors between groups was striking. Therefore, the number of cells in 5 representative fields of view for 3 tumors from each group were counted and averaged. Through this observation, it was found that MSC containing tumors had almost double the cell number as compared to control indicating increased proliferation. No differences are seen in the estrogen groups as these tumors have reached the maximum cell density for the field.



hMSCs promote tumor cell proliferation in the absence of estrogen. A. Tumor volume in mm³ (mean $\pm$ s.e.m.). 4-6 week old female ovariectomized SCID/beige mice were injected subcutaneously with 1×10^6 MCF7 $\pm$ 1×10^6 MSC in 50 μ l PBS with 100 μ l reduced growth factor matrigel, n = 5 mice per group. Tumors were measured every 3 days. **B.** Cell count averages per field of view at 200x. *: p = 0.001. Bars represent average cell number $\pm$ SE.

S.4 MSC promote hormone independent growth of breast carcinoma.

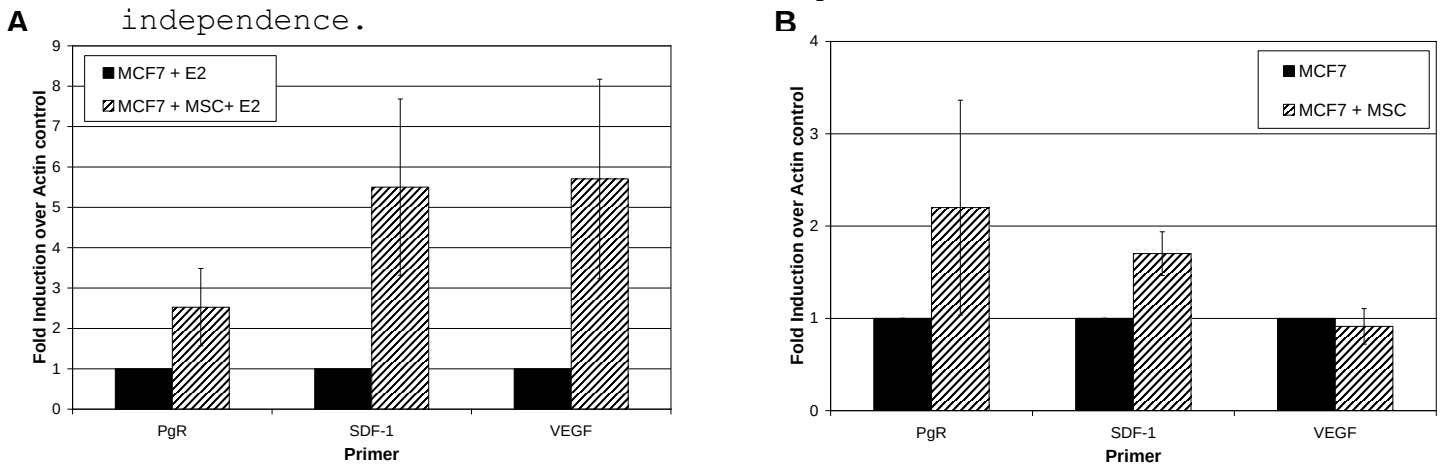
It has been shown previously that MCF7 cells do not form tumors in vivo in the absence of either estrogen or matrigel. However, we found that coinjection of MSCs and MCF7 cells in ovariectomized female SCID/beige mice were able to form measurable tumors in this setting, indicating a role for hormone independence.



MSCs promote hormone independent breast tumor growth in vivo. Tumor volume in mm³ (mean $\pm$ s.e.m.). 4-6 week old female ovariectomized SCID/beige mice were injected subcutaneously with 1×10^6 MCF7 $\pm$ 1×10^6 MSC in 50 μ l PBS, n = 5 mice per group. Tumors were measured every 3 days. **Insert.** Percent tumor take at day 31 post injection of non-matrigel MCF7 tumors +/- hMSCs.

S.5 MCS induce gene expression of ER mediated genes.

Endpoint tumors from above studies were harvested for use in Real-time PCR analysis. As shown in the graphs below, Progesterone Receptor, SDF-1, and VEGF gene expression was upregulated in tumors containing MSCs. As these are ER mediated genes, these results indicate an ER-MSC crosstalk. Increased levels of SDF-1 led us to speculate MSC as a source of SDF-1 or stimulate an increase in SDF-1 production by the cancer cells. We believe this strengthens our case for the role of SDF-1/CXCR4 in breast tumorigenesis and hormone independence.



hMSCs induce estrogen mediated gene activation. Total RNA was isolated from tumors, reverse transcribed into cDNA and subjected to real-time PCR analysis for quantification. **A.** Real time PCR results from matrigel + estrogen tumor samples. MCF7 + E2 control tumors are set to 1. **B.** Real time PCR results from matrigel tumor samples. MCF7 control tumors are set to 1. In all figures, bars represent mean fold change $\pm$ SE of 3 separate tumors from each experimental group run in duplicate. *: $p = 0.0414$.

Future studies are in the works to determine the mechanism by which SDF-1 is being upregulated in these tumors. We hope to define the source of the SDF-1, be it the MSCs themselves or the breast carcinoma cells. We also have planned to use specific inhibitors against SDF-1/CXCR4 to validate this as the primary method of action by the MSCs. Plans to use anti-estrogens in this system are also underway to validate the MSC-ER crosstalk.

We believe the MSC studies to be a great asset to the SDF-1/CXCR4 project previously outlined as this would be a natural source of increased SDF-1. Biologically relevant systems are always of benefit, and we believe this new direction will benefit the field as well as lead to new avenues of treatment options.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that CXCR4 activation leads to increased ER-mediated gene expression.
- Determined a role for p38 signaling in CXCR4 regulation through ERE-luciferase.
- Determined that p38-MAPK signaling is involved in CXCR4 mediated breast cancer progression in vivo.
- Determined the ability of CXCR4/SDF1 to affect tumor formation, estrogen-independence and metastasis in vivo using a lung metastasis model.
- Generated stable cell lines overexpression SDF-1, CXCR4, or both in the MCF7 parental line.
- Discovered the ability of MSCs to increase breast cancer cell sensitivity to estrogen.
- Determined MSCs increase breast tumor proliferation even in the absence of estrogen.
- Discovered that MSCs promote a hormone independent phenotype in breast carcinoma.
- Found that MSCs induce gene expression of ER mediated genes in breast tumors.

REPORTABLE OUTCOMES**Presentations:**

"Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence". American Association for Cancer Research Pathobiology Workshop, Snowmass, CO. July 2008.

"Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence". DOD Breast Cancer Research Program Era of Hope Meeting, Baltimore, MD. June 2008, Invited Platform Session.

"Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence". American Association for Cancer Research Annual Meeting, San Diego, CA. April 2008.

"Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence". Tulane University Health Sciences Center Research Days, Tulane University, New Orleans, LA. Mar 2008.

"Human Mesenchymal Stem Cells in Breast Cancer". Center for Bioenvironmental Research joint laboratory meeting, Tulane University School of Medicine, New Orleans, LA. February 2008.

Abstracts:

Lyndsay V. Rhodes, Shannon E. Muir, Steven Elliott, Lori M. Guillot, James W. Antoon, Patrice Penfornis, Syreeta L. Tilghman, John A. McLachlan, Brian G. Rowan, Radhika Pochampally, Matthew E. Burow. "Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence". AACR Annual Meeting, April, 2008.

CONCLUSION

With our discovery that MSCs have the ability to increase proliferation and hormone independence of breast carcinoma, which we believe is achieved at least in part through the SDF-1/CXCR4 axis, it is even more imperative that we understand their functions. Now with a biologically relevant source of SDF-1, we can also better study the effects of SDF-1 in our model systems. By defining the pathway, we can better understand how to control the effects of SDF-1 and CXCR4, leading to better treatment options in the future.

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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 (if applicable)	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

Teaching Experience:

2008	Instructor, Endocrine Pharmacology, Department of Pharmacology, Tulane University, New Orleans, LA
2004-2006	Teaching Assistant, General Biology and Cellular and Molecular Biology Program, Tulane University, New Orleans, LA
2002	Teaching & Resident Assistant, Southern Piedmont Educational Consortium (SPEC), Pfeiffer University, Misenheimer, NC

Research Experience:

2005-present	Graduate Student Research, Interdisciplinary Program in Molecular and Cellular Biology, Tulane University Medical Center, New Orleans, LA (Thesis Advisor: Matthew Burow)
2004	Lab Assistant, James Robinson Lab, Tulane University Medical Center, New Orleans, LA
2003	Research Intern, Plant Molecular and Cell Biology Program, University of Florida, Gainesville, FL
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

Honors, Awards, and Other Professional Activities:

Honors:

2005 – Present	Phi Delta Sigma, Pfeiffer University
2004 – Present	Order of the Sundial, Pfeiffer University

Activities:

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

Member:

2008 – Present	American Association for Cancer Research,
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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:

“Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence”. American Association for Cancer Research Pathobiology Workshop, Snowmass, CO. July 2008.

“Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence”. DOD Breast Cancer Research Program Era of Hope Meeting, Baltimore, MD. June 2008, Invited Platform Session.

“Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence”. American Association for Cancer Research Annual Meeting, San Diego, CA. April 2008.

“Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence”. Tulane University Health Sciences Center Research Days, Tulane University, New Orleans, LA. Mar 2008.

“Human Mesenchymal Stem Cells in Breast Cancer”. Center for Bioenvironmental Research joint laboratory meeting, Tulane University School of Medicine, New Orleans, LA. February 2008.

“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.

“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 Alumnus Speaker.

Abstracts:

Lyndsay V. Rhodes, Shannon E. Muir, Steven Elliott, Lori M. Guillot, James W. Antoon, Patrice Penfornis, Syreeta L. Tilghman, John A. McLachlan, Brian G. Rowan, Radhika Pochampally, Matthew E. Burow. “Adult human mesenchymal stem cells enhance breast cancer tumorigenesis and promote hormone independence”. AACR Annual Meeting, April, 2008.

Shannon Muir, Lyndsay Vanhoy-Rhodes, Yun Zhu, Virgilio A. Salvo, Steven Elliott, Lori Guillot, Juan P. Fonseca, John A. McLachlan, Brian Barnett, Barbara S. Beckman, Tyler J. Curiel, Matthew E. Burow. “The chemokine receptor CXCR7 mediates estrogen-stimulated breast cancer tumorigenesis”. AACR Annual Meeting, April, 2008.

Meacham WD, Antoon JW, Burow ME, Smith CD, Slaughter EM, Rhodes LV, Muir SE, Guillot LM, Bratton MR, Beckman BS. “Effect of Novel Sphingosine Kinase Inhibitors on Estrogen Receptor in Endocrine Sensitive and Endocrine Resistant Breast Cancer”. Tulane University Health Sciences Center Research Days, Tulane University, New Orleans, LA. Mar 2008.

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.