AD_____

Award Number: W81XWH-08-1-0656

TITLE: Effect of Stromal Adipokines on Breast Cancer Development

PRINCIPAL INVESTIGATOR: Richard A. Woo, Ph.D.

CONTRACTING ORGANIZATION: Southern Illinois University
Springfield, Illinois 62794-9677

REPORT DATE: September 2009

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT:

- ✓ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
30-09-2009	Annual	1 SEP 2008 - 30 AUG 2009

4. TITLE AND SUBTITLE
Effect of Stromal Adipokines on Breast Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0656

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Richard A. Woo, Ph.D.
Email: rwoo@siumed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Southern Illinois University
P.O. Box 19677
Springfield, Illinois 62794-9677

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and 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 Obesity increases the risk of breast cancer in post-menopausal women. The degree of risk increases proportionally with an increase in adiposity. There is mounting evidence that stromal cells in the tumor microenvironment make pivotal contributions to tumor progression. Stromal adipocytes have been shown to exert their influence on breast cancer cells via two secreted adipocytokines, leptin and adiponectin. Leptin stimulates proliferation and invasiveness of breast cancer cell lines. Conversely, adiponectin is down-regulated in obese individuals, and there is an inverse relationship between adiponectin levels and risk of breast cancer. Therefore, in breast cancer development, it is important to know if there are significant interactions or tumorigenic effects of excess adipose tissue on mammary epithelium. The question of how adipocytes exert their influence on pre-malignant mammary epithelial cells has not been resolved. Adiponectin was found to suppress the effects of oncogenic ras or Myc in p53-/- MMECs. These cells slowed their rate of growth in response to adiponectin, and was better able to maintain genomic stability. This lead to reduced transformation potential in a soft agar assay. Conversely, the effects of leptin was very striking. The presence of p53 was able to suppress the oncogenic effects of ras and Myc. However, when leptin was present, oncogene-expressing WT-MMECs behaved just like p53-/- MMECs. These cells had a greatly enhanced rate of growth and became highly aneuploid. Furthermore, p53 was no longer able to function as a tumor suppressor in the presence of leptin, allowing oncogenic transformation.

15. SUBJECT TERMS
Adipocytes, Stroma, Oncogenes, Tumor Suppressor Genes, Genomic Instability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Progress Report:

Grant Title: Effect of Stromal Adipokines on Breast Cancer Development

PI: Richard A. Woo, Ph.D.

Introduction:

Obesity increases the risk of breast cancer in post-menopausal women. The degree of risk increases proportionally with an increase in adiposity. There is mounting evidence that stromal cells in the tumor microenvironment make pivotal contributions to tumor progression. Stromal adipocytes have been shown to exert their influence on breast cancer cells via two secreted adipocytokines, leptin and adiponectin. Leptin stimulates proliferation and invasiveness of breast cancer cell lines. Conversely, adiponectin is down-regulated in obese individuals, and there is an inverse relationship between adiponectin levels and risk of breast cancer. Therefore, in breast cancer development, it is important to know if there are significant interactions or tumorigenic effects of excess adipose tissue on mammary epithelium. The question of how adipocytes exert their influence on pre-malignant mammary epithelial cells has not been resolved. It is apparent that concomitant changes at the organismic level (e.g. degree of adiposity and/or menopausal status) and the tissue microenvironment level (leptin or adiponectin secretion) can vary the effects that adipocytes have on epithelial cells. Conceivably, the epithelial cells may reciprocate and signal back to the adipocytes, or even initiate the signaling.

Hypothesis: It is hypothesized that reciprocal signaling between stromal adipocytes and pre-malignant mammary epithelial cells stimulate epithelial proliferation, which can be a major contributor to the initiating steps required for neoplastic transformation.

Progress report in accordance to the statement of work (SOW)

Task 1. Obtain a breeding pair of $p53^{+/-}$ mice, and establish a breeding colony to obtain wildtype and $p53^{-/-}$ mice of female gender. Generate mouse mammary epithelial cells (MMEC) for all subsequent tasks. **(100% completed)**

Research accomplishment:

Establishing a breeding colony of p53 transgenic mice, as well as the generation of $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse mammary epithelial cells (MMECs).

A single breeding pair of $p53^{+/-}$ mice was obtained (Jax Mice). Following a short quarantine period, the mice were mated, a 20-21 day gestation period ensued, and the first generation of progeny mice was born. After the progeny were weaned (3 weeks after birth), tail tips were snipped for DNA isolation and genotyping. The presence of the p53 and/or nullizygous allele was determined by PCR. When progeny mice were sexually mature (6-8 weeks of age), the breeding was repeated to obtain enough female mice of each genotype for experimentation as well as colony maintenance.

Following genotyping, the desired female mice of each genotype was dissected. Three to five mice of each genotype was required to obtain sufficient mammary tissue. Isolated mammary tissue was minced and enzymatically digested to obtain cell suspension. $P53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ MMECs were successfully isolated and cultured in serum-free media, sub-cultured and grown using standard primary cell culture techniques.

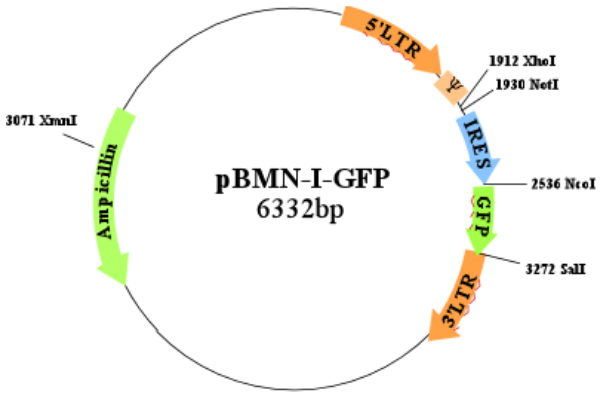
The original timeline of 4 months described in the SOW to accomplish this task was underestimated because the first generation of progeny only had a single heterozygous female and a single heterozygous male. In our 8 progeny litter (assuming 50:50 distribution of males and females, as well as Mendelian distribution of the 3 possible genotypes; 1 : 2 : 1 of wildtype : heterozygote : p53 null), ideally, we would have had two heterozygous females and two heterozygous males. With just one of each, colony expansion was slowed by 2-3 months.

Task 2. To determine the effect of leptin and adiponectin on $p53^{+/+}$ and $p53^{-/-}$ MMEC expressing myc or ras.

(A) Express either GFP-control, myc or oncogenic K-rasV12 or both in MMEC. **(100% completed).**

1. The cDNA clone for K-rasV12 and c-myc was obtained from Addgene. We sub-cloned these two genes separately into the retroviral vector, pBMN-GFP (Figure 1; Orbigen). This is a bicistronic vector that expresses green florescent protein (GFP) from an internal ribosome entry site (IRES). The Ψ promoter controls expression of the inserted gene (either K-rasV12 or c-myc). Successful cloning of rasV12 or c-myc into the vector was confirmed by restriction mapping and PCR sequencing (data not shown). The resulting products were designated pBMN-rasV12 and pBMN-Myc.

Figure 1. pBMN-GFP



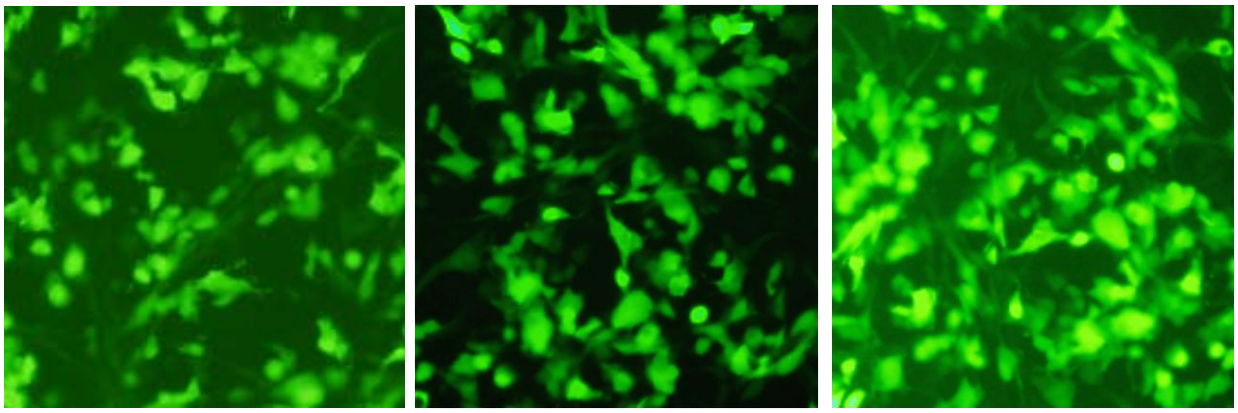
Research accomplishment:

Construction of recombinant DNA, pBMN-rasV12 and pBMN-Myc.

2. The Phoenix™ system (Orbigen) produces replication-incompetent retrovirus that can efficiently transfer genes to mouse cells. pBMN-rasV12 and pBMN-Myc, as well as the

parental retroviral vector, pBMN-GFP were separately transfected into 'Phoenix Eco', ecotropic packaging cells. Transfected cells were selected in puromycin for 3-4 days. Following selection, the packaging cells were analyzed by fluorescence microscopy for the expression of the GFP genes (Figure 2). The resulting cells were designated Eco-GFP, Eco-rasV12 and Eco-Myc.

Figure 2. Phoenix Eco cells stably transfected with either pBMN-GFP parental control, pBMN-rasV12 or pBMN-Myc.



Eco-GFP Eco-rasV12 Eco-Myc

Research accomplishment:

Generating stable retroviral producer cell lines, Eco-rasV12, Eco-Myc and Eco-GFP.

3. Retrovirus from Eco-GFP, Eco-rasV12 and Eco-Myc cells was used to transduce $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ MMECs generated in **Task 1**.

Retroviral supernatant from Eco-GFP, Eco-rasV12 and Eco-Myc cells was purified by ultra-centrifugation. Retroviral pellets were re-suspended in serum-free MMEC growth media, supplemented with polybrene to enhance retroviral infectivity. MMECs of each

genotype ($p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$) was infected with retrovirus coding for either GFP-control, oncogenic rasV12, c-myc, or both rasV12 and Myc. Infected cells were selected with puromycin, and monitored for infectivity by fluorescence microscopy, assaying for the GFP marker present in each of the retroviruses. After 2-3 days virtually all of the MMECs showed GFP fluorescence (Figure 3). The presence of the rasV12 or c-Myc gene was confirmed by PCR

Research accomplishment: MMECs were retrovirally transduced with either GFP-control, oncogenic K-rasV12 or c-myc or both rasV12 and c-myc.

(B) To determine the effect of leptin or adiponectin on growth rate, genomic stability and transformation potential of MMEC expressing myc or rasV12 or both (80% completed).

1. There is an inverse relationship between adiponectin levels and risk of breast cancer. We found that adiponectin (Sigma) had very little effect on the growth rate of non-transduced MMECs, regardless of the genotype. However, the growth rate of oncogene-transduced (rasV12 or myc oncogenes) $p53^{-/-}$ MMECs was greatly suppressed by adiponectin (Figure 4a). Karyotypic profiles of oncogene expressing $p53^{-/-}$ MMECs was highly aneuploid as expected (1). However, treatment of these cells with adiponectin only mildly reduced the degree of aneuploidy (Figure 4b). Furthermore, the number of colonies that formed in a soft-agar in vitro transformation assay was also reduced by the presence of adiponectin in the growth media (Figure 4c), but again the effects were nominal but noteworthy. The effects of adiponectin on oncogene-transduced MMECs that have p53 (wildtype or heterozygous) was minimal.

We would like to further titrate the amount of adiponectin to use to see if the effects on mammary cell genomic stability and oncogenic transformation could be enhanced.

2. It has been reported that leptin stimulates proliferation breast cancer cell lines. Similarly, we found that leptin greatly enhances the growth rate of MMECs, regardless of the genotype. Furthermore, leptin enhance the growth rate of MMECs expressing

rasV12 or Myc (Figure 5a). The absence of p53 in oncogene-expressing cells led to genomic instability (compare wildtype (WT) and p53-/- MMECs, Figure 5b). Most interesting was the observation that leptin nullified the genome protecting effect of p53 in oncogene-expressing WT-MMECs, and these cells showed similar genomic instability as $p53^{-/-}$ MMECs. Furthermore, leptin-treated WT-MMEC lost the ability to suppress oncogenic transformation in the soft-agar assay, and had the same transformation potential as $p53^{-/-}$ MMECs that expressed ras or Myc.

Task 3. To test the effect co-cultured adipocytes (3T3-L1) have on proliferation, genomic stability and in vitro transformation of $p53^{+/+}$ and $p53^{-/-}$ MMEC expressing myc or ras. **(25% completed)**

3T3-L1 cells are pre-adipocytes that can differentiate into adipocytes upon exposure to insulin or insulin-like growth factor (IGF-1). Undifferentiated 3T3-L1 cells expressed a higher level adiponectin than insulin-differentiated 3T3-L1 cells. Conversely, undifferentiated 3T3-L1 cells expressed a lower level of leptin that insulin-differentiated 3T3-L1 cells. These preliminary results should allow us to analyze the different effects of co-cultured undifferentiated 3T3-L1 cells versus differentiated 3T3-L1 cells. Based on previous results adding purified adiponectin or purified leptin (Task 2), there is significant potential in the co-culturing adiponectin-expressing 3T3-L1 cells and leptin-expressing 3T3-L1 cells with the MMECs.

Task 4. To investigate if MMEC can 'instruct' co-cultured adipocytes to alter the expression or secretion of leptin or adiponectin. **(25% completed).**

Preliminary results show that ras-expressing MMECs greatly stimulates the expression of co-cultured leptin in 3T3-L1 cells (Figure 6). This occurred regardless of the genotype of the MMECs. Myc-expressing MMECs did not have a similar effect.

Key Research Accomplishments

1. Establishing a breeding colony of p53 transgenic mice, as well as the generation of $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse mammary epithelial cells (MMECs).
2. Construction of recombinant DNA, pBMN-rasV12 and pBMN-Myc.
3. Generating stable retroviral producer cell lines, Eco-rasV12, Eco-Myc and Eco-GFP.
4. Research accomplishment: MMECs were retrovirally transduced with either GFP-control, oncogenic K-rasV12 or c-myc or both rasV12 and c-myc.
5. Adiponectin slows the growth rate of oncogene-expressing, p53-/- MMECs.
6. Adiponectin mildly suppressed the degree of genomic instability of oncogene-expressing.
7. Adiponectin mildly suppressed the oncogenic-transformation potential of oncogene-expressing, p53-/- MMECs.
8. Leptin greatly accelerates the growth rate of oncogene-expressing WT-MMECs.
9. Leptin suppresses the genome-stabilizing effects of p53, leading to gross aneuploidy in oncogene-expressing WT-MMECs.
10. Leptin blocks the tumor-suppressing effects of p53, leading to highly transformed oncogene-expressing WT-MMECs in the soft-agar transformation assay.

Reportable Outcomes

1) R.A. Woo. Stromal adipokines in breast cancer development. *International Journal of Molecular Medicine: Special Supplement Edition*. October, 2009.
2) Preparation of the Manuscript “Stromal leptin co-operates with oncogenic ras to promote genomic instability and oncogenic transformation in mammary epithelial cells”. J.H. Huang, C. Harrison, R.A. Woo. For submission for publication.
3) Recruitment and training of C. Harrison, Research II Technician.
4) Invited speaker at 14th World Congress on Advances in Oncology, 2009. “Stromal adipokines in breast cancer development”

5) Invited speaker at 12th International Symposium on Molecular Medicine, 2009. "Stromal adipokines in breast cancer development"

Conclusions:

Most cancer cells are aneuploid. Oncogenes rasV12 and c-Myc accelerated the growth rate of MMECs leading to genomic instability and aneuploidy. Oncogenes also induced in vitro transformation of MMECs. Adiponectin was found to suppress the effects of oncogenic ras or Myc in p53-/- MMECs. These cells slowed their rate of growth in response to adiponectin, and was better able to maintain genomic stability. This lead to reduced transformation potential in a soft agar assay.

Conversely, the effects of leptin was very striking. The presence of p53 was able to suppress the oncogenic effects of ras and Myc. However, when leptin was present, oncogene-expressing WT-MMECs behaved just like p53-/- MMECs. These cells had a greatly enhanced rate of growth and became highly aneuploid. Furthermore, p53 was no longer able to function as a tumor suppressor, allowing oncogene-induced in vitro transformation in soft agar.

The next steps in this project are to further elucidate the mechanism of how these adipokines, adiponectin and leptin, have their effect on genomic stability, p53 function and oncogenic transformation.

References:

1. **R. A. Woo** and R.Y.C. Poon. Activated oncogenes promote and cooperate with chromosomal instability for neoplastic transformation. *Genes & Dev.* **18:** 1317-1330, 2004.
2. Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., and Weinberg, R.A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth

and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. **121**: 335-348.

3. Lahmann, P.H., Hoffman, K., Allen, N., *et al.,* (2004). Body size and breast cancer risk: Findings from European prospective investigation into cancer and nutrition (EPIC). *International Journal of Cancer*. **111**: 762-771.
4. Harvie, M. and Howell, A. (2006). Energy balance adiposity and breast cancer – energy restriction strategies for breast cancer prevention. *Obesity Reviews*. **7**: 33-47.
5. Somasundar, P., McFadden, D.W., Hileman, S.M., and Vona-Davis, L. (2004). Leptin is a growth factor in cancer. *Journal of Surgical Research.* **116**: 337-349.