

Award Number:
W81XWH-08-1-0198

TITLE:
Ets2 contributions of the tumor microenvironment in breast cancer
metastasis

PRINCIPAL INVESTIGATOR:
Jillian L. Werbeck, B.S.

CONTRACTING ORGANIZATION:
Ohio State University Research Foundation
Columbus, OH 43210

REPORT DATE:
March 2009

TYPE OF REPORT:
Annual Summary

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

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 31-03-2009		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 MAR 2008 - 28 FEB 2009	
4. TITLE AND SUBTITLE Ets2 contributions of the tumor microenvironment in breast cancer metastasis				5a. CONTRACT NUMBER W81XWH-08-1-0198	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jillian L. Werbeck Email:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Ohio State University Research Foundation Columbus, OH 43210				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 goal of this project is to identify the genetic role of the tumor microenvironment in breast cancer metastasis. In year one, we have characterized our metastasis model, MMTV-PyMT (Aim #1). In addition, through this characterization we discovered that the injection site of breast cancer cells affects the pattern and gene expression of metastasis. We are currently preparing this work for publication. We have also completed a pilot study to evaluate the role of fibroblast specific deletion of Ets2 in MMTV-PyMT metastasis for Aim #2. Preliminary data suggests that deletion of Ets2 reduces the growth of lung metastasis in the MMTV-PyMT model. Based on this finding we will be completing Aims #2 and #3 in the following two years as originally predicted. In addition to these research accomplishments, the PI has also made advancements in her predoctoral training program. Specifically, she has completed her candidacy exam, and prepared 2 first author manuscripts and 6 posters or presentations during this first year (among other accomplishments). In conclusion, we have been able to accomplish the aims and goals originally set forth by this proposal on schedule and we appreciate the opportunity to make significant progress in the next two years.					
15. SUBJECT TERMS Breast cancer, metastasis, tumor microenvironment, fibroblasts, Ras/Ets2, MMTV-PyMT model, OSUCCC					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 31	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	6
Key Research Accomplishments.....	19
Reportable Outcomes.....	20
Conclusion.....	22
References.....	23
Appendices.....	26

I. INTRODUCTION:

A. Background

A1: Breast cancer metastasis:

According to the American Cancer Society, the chance being diagnosed with invasive breast cancer is about 1 in 8 and the chance of dying from breast cancer is about 1 in 33. The recent trend toward a decrease in breast cancer mortality rate is largely due to increased diagnosis of early stage disease, while therapeutic options for advanced stage breast carcinomas are still fairly limited. Currently, metastasis is the most lethal complication of breast cancer and therapies to prevent the spread of breast cancer cells do not exist (1). Little is known about the genetic contributions of metastasis and the role of the microenvironment at the metastatic site. Therefore, in order to find therapeutic drugs to prevent metastasis it is critical to understand the genetic cross talk between different compartments of the metastatic microenvironment which support their growth.

Metastasis proceeds through a multi-step process whereby epithelial cells acquire a number of genetic hits to allow them to spread to distant organ sites. Transformed cells are able to migrate through the basement membrane of the mammary gland and invade through vessels to enter the blood circulation. However, most damage to the patient is caused by their ability to invade and grow at a distant organ sites. There are many molecular and biochemical mechanisms which have been discovered to facilitate this multi-stage process, although many are still unknown.

A2: Animal models of metastasis:

Animal models are an invaluable tool to the understanding of cancer metastasis (2). These models allow for cells to travel *in vivo*, to accurately recapitulate the spread of disease in humans. In an ideal animal model of breast cancer metastasis, breast cancer initiation would occur in the mammary gland, invade out of the primary site and sequentially follow a number of stages of progression to seed and grow at a distant site. Unfortunately, this ideal model does not exist (3). Therefore, it is necessary to use injection models which can recapitulate single or multiple stages throughout this process in order to study the progression of this disease. In addition, it is becoming increasingly evident that the diverse phenotypic changes that occur throughout these complex stages of metastasis cannot be caused by discrete alterations in epithelial cells alone. Several lines of evidence now suggest that concomitant changes also occur in the stromal cells surrounding the epithelial cancers (4).

A3: The role of the microenvironment in metastasis:

The stroma is a supportive platform for epithelial cells which is composed of fibroblasts, endothelial cells, smooth muscle cells, adipocytes, hematopoietic cells, and a macromolecular network of proteoglycans and glycoproteins collectively termed the extracellular matrix (ECM). Factors required for progression, growth and invasion and metastasis are all regulated by stromal interactions, in particular by fibroblasts. Recent studies have shown that tumor-associated fibroblasts secrete growth-promoting factors as well as factors that enhance invasion and metastasis such as MMPs, VEGF, and TGF- β (5). *This strongly indicates that fibroblasts provide oncogenic signals in a paracrine fashion to both promote metastasis from the primary site as well as to support the growth of tumor cells at the sites of metastasis.*

A4: The *ras/ets-2* pathway in breast cancer metastasis:

Mutations in *ras* genes in breast cancer are relatively infrequent, with 2-5% of tumors found to harbor these mutations (6). Despite this finding, activation of *ras* signaling pathways is very important during breast cancer tumorigenesis and likely play a significant role in progression of this disease (7). *Ras* pathways likely affect invasive and metastatic properties of epithelial cells in two ways. First is through activation and/or interaction with the rho/rac subfamily of GTPases. There is increasing evidence that the rho family genes alter cell structure resulting in increased cell motility (8). The second is by affecting the expression of specific sets of genes involved in cell metastasis. Studies by several groups, including our own, led to the identification of cis-elements in *ras*-responsive genes that contain binding sites for *ets*-family members (9,10). The *ets*-family encodes for sequence specific DNA binding proteins that are transcriptional activators and repressors of numerous target genes (11).

Ets-2 is one member of this family of transcription factors that is commonly implicated in breast cancer progression. First, introduction of an *ets-2* dominant negative gene (first characterized by our lab) into the human breast cancer cell line BT20 resulted in a reduction in tumor cell growth and invasiveness as measured

by *in vitro* assays (12). Second, work from Robert Oshima's lab demonstrated that mice in which one allele of *ets-2* was deleted showed a significant reduction in the size and grade of mammary tumors initiated in the aggressive polyoma middle T-antigen breast cancer model when compared to littermates with wild type *ets-2* (13). Moreover, studies have also shown the relationship between *ets-2* and metastasis. Over expression of Ets-2 in MCF-7 breast cancer cells was shown to increase the expression of uPA and MMP-3, two factors implicated basement membrane degradation and migration (14). Furthermore, transfection of Ets2 into a non-tumorigenic cell line, (MCF-12A) lead to enhanced invasiveness and growth factor independent proliferation (15). *Finally, and most importantly, work from our lab and our collaborators have also shown that an Ets-2 deficiency in mammary epithelial cells alone does not affect tumorigenesis. Therefore, this demonstrates that Ets2 supports mammary tumors exclusively through a stromal mechanism (16).*

B. Objectives and Hypotheses

The goal of this work is to study the genetic contributions of the microenvironment in breast cancer metastasis. Specifically, we have three aims:

- 1) To determine the role of the injection model in gene expression and metastasis.
- 2) To measure the effect of Ets2-null fibroblasts on tumor growth at in the lung microenvironment.
- 3) To identify genetic changes downstream of *ras/ets2* in both Ets2-null fibroblasts and breast cancer cells at the metastatic site.

Our overall hypothesis is that *ets-2* activation in fibroblasts leads to the expression of downstream target genes which promote tumor growth at sites of metastasis.

II. BODY

A: Aim 1: To determine the role of the injection model in gene expression and metastasis

A1: Research Design:

The objective of this study is to first understand how different in vivo models of metastasis can affect the site of secondary growth and second, to identify the genetic changes that occur in a single population of cancer cells at various metastatic microenvironments.

For this study, we used a cell line derived from the commonly used and highly metastatic MMTV-PymT murine breast cancer model. In order to accurately model metastasis as a multi-step process we injected the PymT breast cancer cells via 5 different routes of inoculation. Subcutaneous, orthotopic (mammary fat pad), intravascular (tail vein and intracardiac), and intratibial injections allow us to assess the metastatic pattern of this cell line along different stages in the metastatic cascade.

MMTV-PymT cells were cultured using standard laboratory protocols for aseptic cell culture. In order to monitor metastasis in vivo cells were transfected with a firefly luciferase gene construct (YFP-luciferase-pcDNA3.1). Cells were characterized post-transfection and determined to maintain proliferation and tumorigenic properties identical to the parental line (data not shown). Cells were harvested at approximately 70% confluence, 95-100% viability, and verified mycoplasma infection free before injection. For all injections, cells were resuspended in sterile DPBS and kept on ice until inoculation. FVB/N mice (Jackson Laboratories) were injected at 6-8 weeks of age and housed in accordance with the approved guidelines set forth by The Ohio State University Institution for Animal Care and Usage Committee (IACUC).

Number of cells and technique for injection at each site were determined based on both previous literature and original design as approved by The Ohio State University IACUC. For all injections, animals were anesthetized using 3% isoflurane gas and maintained at 2% isoflurane on a rodent heating pad. The injection site was sterilized using 70% ethyl alcohol. For both orthotopic (mammary fat pad) and heterotopic (subcutaneous) injections, 5 million MMTV-PymT cells were injected in 50 μ l of PBS at either site respectively using a 25G needle (N=10 animals each), (17). In order to directly inject cells into the circulatory system cells were injected into either the left cardiac ventricle (N=20) or the lateral tail vein (N=11). For intracardiac injections, mice were placed in a position of dorsal recumbency under anesthesia. A Vevo 660 small animal ultrasound (VisualSonics) was used to visualize the left cardiac ventricle while 100,000 MMTV-PymT cells were simultaneously injected in 100 μ l into the heart using a 27G needle (18). Injection of cells into the arterial circulation was confirmed through ultrasound visualization of the cells in the left ventricular chamber of the heart, as well as a pulsing of blood in the needle upon injection. Alternatively, venous inoculation was performed by injecting 2 million MMTV-PymT cells in 200 μ l of PBS into the dilated lateral tail vein using a 27G needle (19). Finally, intratibial injections (N=10) were performed using a 26G needle by drilling through the patellar ligament and the tibial crest and injecting 100,000 MMTV-PymT cells in 20 μ l volume into the medullary canal (20).

Metastasis was tracked using bioluminescent imaging (IVIS, Xenogen) and mice were euthanized using 100% CO₂ at the first clinical sign of disease as approved by The Ohio State University IACUC. Final metastatic lesions were confirmed using gross evaluation at necropsy, radiography, and histopathology. Immunohistochemistry for Ki67 was performed on formalin fixed tumors from each site (N=3) to evaluate differences in proliferation rate of MMTV-PymT cells at each anatomical location.

For genetic evaluation of MMTV-PymT cells, RNA was isolated from representative metastases (N=3) and reverse transcribed into cDNA. RT2 Profiler PCR Arrays were then used to evaluate a set of 88 pathway-focused genes related to cancer pathogenesis. Arrays were performed according to the manufacturer's protocol (SA Biosciences, Frederick, MD). The resulting values were analyzed for pathway-focused clustering of genes with a statistically significant up or down regulation. Additionally, only genes showing a 4 fold or greater change or were considered for final analysis.

Numerical data are expressed as means $\pm$ S.D. Statistical differences were performed using a students T-test with a level of significance at $p < 0.05$ unless stated as otherwise.

A2: Results:

MMTV-PyMT metastasis is injection site dependent. Overall, the route of injection did affect the metastatic potential of MMTV-PyMT cells (Figure 1). We did not observe metastases following orthotopic or heterotopic, or intratibial injections. However, both intravascular routes of injection resulted in metastases with different organs of colonization. Tail vein injection of MMTV-PyMT cells resulted in 9/11 mice with lung metastases and two of these mice also had accompanying ovarian metastases. Following intracardiac injections 7 mice had no metastases, 13 had ovarian metastases, 6 had adrenal gland metastases, and 3 had bone metastases to the skull or humerus, with some mice resulting in multiple metastases.

MMTV-PyMT tumors exhibit multiple phenotypic differences depending on the site of metastasis. To assess the pathologic significance of the MMTV-PyMT lesions, the tumors were examined for histologic evidence of poor prognosis. Morphologic changes in mitotic index, nuclear morphology, and percent of necrosis were all evaluated at the six sites of growth (breast, lung, ovary, tibia, adrenal gland, and subcutis). Overall, MMTV-PyMT lesions were poorly differentiated with a high level of anaplastic and multi-nucleated cells. These characteristics were seen to a higher degree in the ovary and subcutis when compared to other sites (Figure 2). We also evaluated the degree of proliferation of MMTV-PyMT cells at each site through immunohistochemical staining for Ki67. Overall, we found that MMTV-PyMT cells growing in the subcutis had the highest percentage of cells dividing (46%) followed by ovary (41%), breast (38%), adrenal (31%), lung (29%), and bone (16%). This therefore suggests that the tumor microenvironment may affect the rate of proliferation or growth of MMTV-PyMT cells, with the subcutis giving the greatest growth advantage to MMTV-PyMT cells (Figure 3.)

MMTV-PyMT cells undergo differential gene expression depending on the site of growth (N=3). Based on a set of 88 pathway focused genes related to cancer progression we found 36 genes that were statistically up or down regulated in MMTV-PyMT cells in various tissue microenvironments ($p < 0.05$). These changes were then clustered into pathways of importance such as adhesion and metastasis, cell cycle and apoptosis, or angiogenesis (Figure 4.) Hierarchical clustering suggested that the genetic profile of MMTV-PyMT cells in the bone microenvironment was most distinct from the other organ sites. Furthermore, angiogenesis and metastasis genes were greater up regulated in the MMTV-PyMT cells in bone versus a significant down regulation at the other sites.

Additionally, there were five genes that had a greater than four-fold difference in gene expression when compared to the cells in the mammary gland control: E-cadherin, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor-2 (FGFR2), matrix metalloproteinase 9 (MMP9) and endothelial-specific receptor tyrosine kinase (Tek). Interestingly, these differences in gene expression were dependent on the site of MMTV-PyMT growth (Figure 5). MMTV-PyMT cells in the bone had the greatest fold change in gene expression with a 4-fold significant up regulation of EGFR, 27-fold up regulation of fibroblast FGFR2, 44-fold up regulation of MMP9 and 4-fold up regulation of Tek. FGFR2 expression in MMTV-PyMT cells was also significantly up regulated in the lung metastases (9-fold), ovary metastases (4-fold), and adrenal gland metastases (14-fold). Interestingly, the expression of E-cadherin in the ovary metastases were down regulated 6-fold in the ovary when compared to other sites, and this result was confirmed by immunohistochemistry (data not shown). Taken together, these data suggest that the expression of specific genes can be altered in MMTV-PyMT cells depending on the site of growth.

A3: Conclusions:

In vivo models are critical for the elicitation of molecular mechanisms involved in breast cancer pathogenesis. In particular, the MMTV-PyMT model has been key to the identification of important cancer-related molecules such as tyrosine kinases, and the phosphorylation of phosphatidylinositol 3-kinases (21). However, few studies have looked at the relevance of the injection site in progression or metastasis of breast cancer cells. This is the first study to characterize the metastatic, morphologic, and genetic changes in MMTV-PyMT cells following

multiple injection models of metastasis. Furthermore, this study also supports the role of the tumor microenvironment as a critical factor in the pathogenesis of metastatic disease.

The tumor microenvironment is now recognized as an important and prominent factor in tumor progression. Multiple studies have defined the role of different compartments in the microenvironment which facilitate various stages of progression such as proliferation (22), growth (23), angiogenesis (24) and survival of cancer cells (25). Loss of heterozygosity (LOH) studies have also revealed that multiple stromal targets undergo genetic changes to support the growth of breast cancer cells (26). Aside from growth at the primary site, the microenvironment is also known to play a role in the regulation of metastasis by immune cell recruitment, cytokine secretion, structural support, among other actions (27). Interestingly, many studies also suggested that there is tissue-specific tropism for breast cancer cells to metastasize to certain organs (28) based on the pattern of dissemination *in vivo*. However, this study is the first to show that all these factors may be related to not only the metastatic microenvironment, but also the route of inoculation. Specifically, this study suggests that changes in cell morphology and gene expression in the MMTV-PymT cells may depend on the route of dissemination, as well as molecular cues from the microenvironment which provide a tissue specific growth advantage.

There are many morphologic characteristics that are associated with aggressive tumors and therefore poor prognosis (2). These data suggest that MMTV-PymT cells not only exhibit these negative morphologic characteristics, but also that the degree of morphologic variation in these tumors depends on the site of growth or metastasis. Measurements of mitotic index and percent necrosis suggest that MMTV-PymT cells in the subcutis have a more aggressive phenotype versus other sites of growth. Additionally, proliferation rates of MMTV-PymT cells change depending on the site of growth as evidenced through a greater percentage of Ki67 positive MMTV-PymT cells in the subcutis versus other sites. Taken together, these data suggest that the morphology and proliferation of breast cancer cells can change depending on the site of growth. This finding may therefore help in defining the role of morphologic changes in determining the patient prognosis as well as strategies for treatment.

In addition to phenotypic changes, we have identified 5 genes (E-cadherin, EGFR, FGFR2, MMP9 and Tek) which are significantly up or down regulated in MMTV-PymT cells depending on the route of injection and site of metastasis. Furthermore, these genes have all been previously documented to be important in the adhesion, progression and metastasis of breast cancer cells in numerous studies. These data demonstrate that while genes such as FGFR2 may be significantly up regulated at multiple sites of metastasis (ovary, adrenal, bone, lung), other genes such as E-cadherin are significantly regulated at only one site (ovary). This therefore suggests that specific genes may play a critical role in giving a specific survival or growth advantage to MMTV-PymT cells in a site-specific manner.

In conclusion, this study is the first to evaluate the phenotypic and genetic changes in MMTV-PymT cells following multiple routes of inoculation. We do not currently know if the circulation patterns and dissemination of cancer cells throughout the body may have an impact on the pathogenesis or genetic expression of tumor cells at the final site of colonization. However, these results suggest that morphologic and genetic changes do occur in cancer cells depending on the site of growth and the mode of injection. Therefore, breast cancer progression and metastasis is affected by not only the tumor microenvironment, but also the route of metastasis to that microenvironment.

B: Aim 2: To measure the effect of Ets2-null fibroblasts on tumor growth at in the lung microenvironment

B1: Research Design

Previous work from our lab has demonstrated that *ets-2* is activated by a ras/MAPK dependent pathway in a wide range of cells types, including normal and tumor-associated fibroblasts (29-33). However, there is little data concerning the role of this pathway at the metastatic site.

Our hypothesis is that Ets2 activation in the lung stroma promotes the growth of breast cancer lung metastases. To test this hypothesis we used a conditional knock-out mouse model generated through our Program Project group. We targeted Ets-2 in embryonic stem cells and successfully produced functional “floxed” sites, resulting in deletion of exons 3-5 (*ets-2* domain) after Cre recombination. Using mice with the

ROSA26-LoxP reporter system we showed that Cre recombinase expression under the promoter for fibroblast-specific protein 1 (FSP1) was specific to drive expression to fibroblasts and not other cell types (as seen in the mammary gland, Figure 6; unpublished results). *Furthermore, we demonstrated that mice with fibroblast specific protein promoter-Cre (fsp-cre) deletion of Ets-2 have a delay in tumor growth when crossed to the MMTV-PyMT oncogenic model (Figure 7; unpublished results).*

After validating this model system, we then used the fsp-cre mice to study the role of Ets-2 in lung metastasis. We injected MMTV-PyMT cells (as described in aim #1) into the lateral tail vein of fsp-cre Ets2^{-/-} mice (experimental) and Ets2^{+/+} mice (control) in order to assess metastatic incidence and tumor burden in the lung (N=5). Metastases were measured using *in vivo* bioluminescence imaging (BLI) and final tumor burden was assessed by gross evaluation, histology and quantitative histomorphometry.

B2: Results

Stromal deletion of Ets2 increases survival time in MMTV-PyMT tumors. In order to determine the effect of Ets2 deletion on tumor onset and mortality, we completed a pilot study with both control, Ets2^{+/+} mice (N=7) and experimental fspcre Ets2^{-/-} mice (N=4). Mice were monitored daily for clinical symptoms of disease, and tumor burden was evaluated through bioluminescent imaging (BLI). Mice were euthanized at the first signs of clinical disease as outlined through our animal use protocol and approved by The Ohio State University IACUC. We found that control mice developed disease approximately 50% sooner (average 5.7 weeks) than experimental mice (average= 10 weeks) as seen in Figure 8. This therefore suggests that fibroblast expression of Ets2 is critical for enhancing the growth and progression of lung metastases.

Stromal deletion of Ets2 decreases tumor burden in MMTV-PyMT tumors. In order to evaluate differences in tumor burden in the lung we injected 5 experimental and 5 control animals with MMTV-PyMT cells into the lateral tail vein. All 10 mice were euthanized simultaneously at 4 weeks (based on pilot study data). After euthanasia, lungs were inflated with 1ml of formalin and fixed and embedded in paraffin for histologic evaluation. Total tumor burden was measured through histomorphometry (ImageProPlus) and represented as a percent measure of tumor area/total lung area (Figure 9). We found that control mice had a statistically significant increase in tumor burden (46.5%) when compared to experimental animals (23.2%). Statistical analysis was performed using a one-sided two-sample t-test ($p < 0.03$).

B3: Conclusions

In conclusion, stromal deletion of Ets2 in lung fibroblasts delayed the incidence of breast cancer lung metastases. Furthermore, metastatic tumor burden was significantly reduced in the lung ($p < 0.03$).

Metastasis is the most lethal complication of breast cancer. Therefore, understanding the genetic contributions of the tumor microenvironment at the metastatic site will be useful in trying to delay or inhibit metastasis. This study is the first to suggest that the deletion of a specific gene (Ets2) in lung fibroblasts may decrease the incidence and growth of lung metastases. Based on these promising results from our pilot study, we are currently injecting a larger number of animals in order to achieve statistical significance. Currently we have injected 10 mice per group; results from this study are pending.

C: Aim 3: To identify genetic changes downstream of ras/ets2 in both Ets2-null fibroblasts and breast cancer cells at the metastatic site.

C1: Research Design

Based on our current observations we have determined that fibroblast deletion of Ets2 significantly reduces tumor burden in our mouse model of metastasis. However, our next objective is to determine the molecular mechanisms which are regulated by Ets2 to enhance the growth of MMTV-PyMT metastases. In order to accomplish this goal we will first determine whether Ets2 deletion has an affect on proliferation, or angiogenesis. This will be determined based on Ki67 or CD31 staining respectively on formalin fixed lung tumors.

Our next goal will be to look at downstream genetic changes in both lung fibroblasts and tumor cells by performing gene expression microarrays. Briefly, the fspcre Ets2^{-/-} mouse will be crossed to a ColI-YFP mouse in order to fluorescently label fibroblasts in this model with yellow fluorescent protein (YFP). Next, we will sterile sort out both cancer cells (transfected with a DsRed positive fluorescent tag) and fibroblast (YFP+) for analysis. Gene microarrays will be conducted on both populations of cells and analyzed to determine changes in gene expression. This study will provide mechanistic insight into the downstream targets of Ras/Ets2 regulation in the tumor microenvironment.

C2: Results & conclusions

In progress/Pending years 2 and 3.

D: Figures

Figure 1. MMTV-PyMT metastasis is injection site dependent.

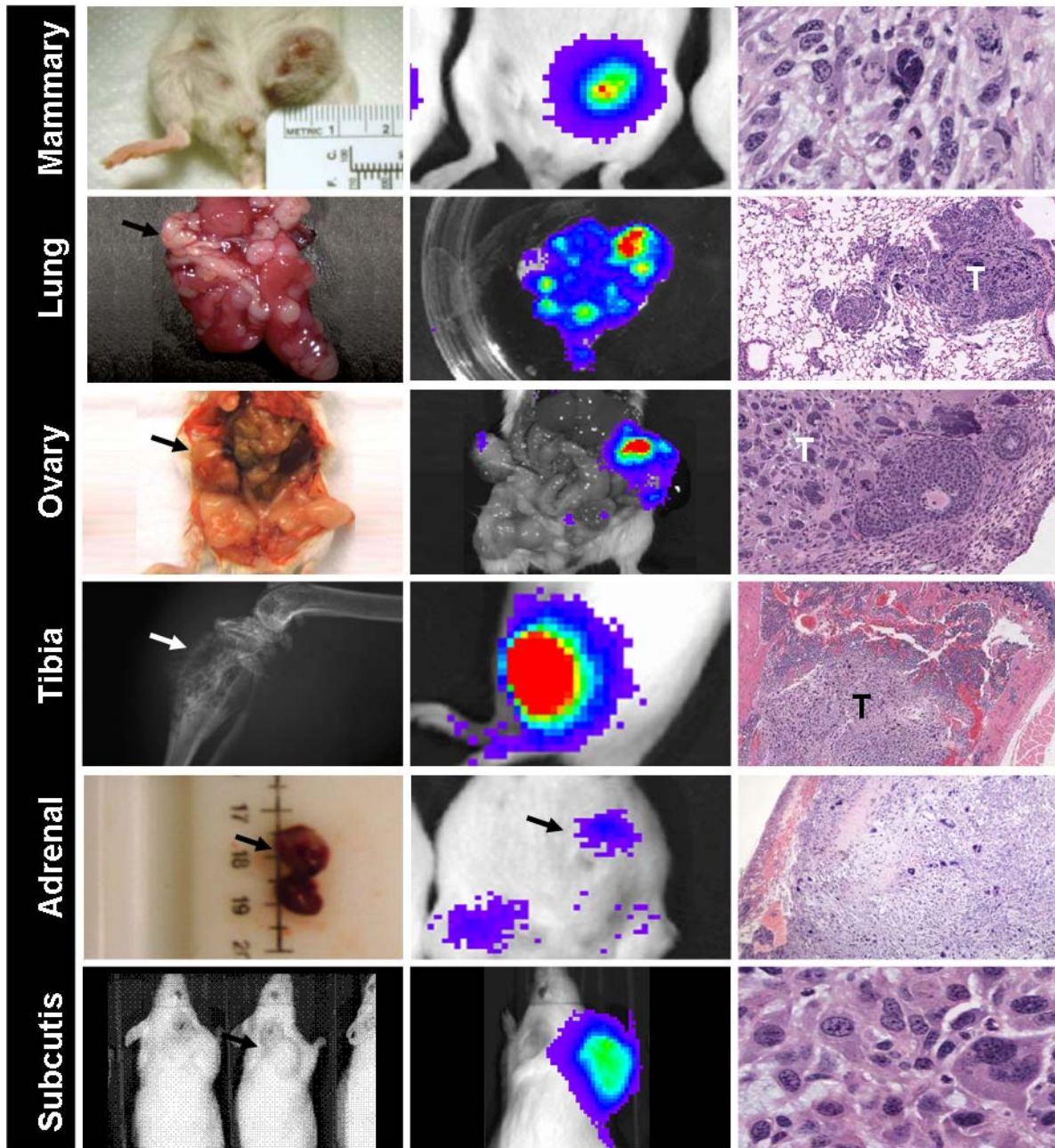
	No Metastasis	Ovary	Lung	Bone	Adrenal gland
Mammary N=10	N=10				
Subcutaneous N=10	N=10				
Tail vein * N=11	N=2	N=2	N=9		
Intracardiac * † N=20	N=7	N=13		N=3	N=6
Intratibial N=10	N=10				

*** Mice had 2 metastatic lesions**

† Mice had 3 metastatic lesions

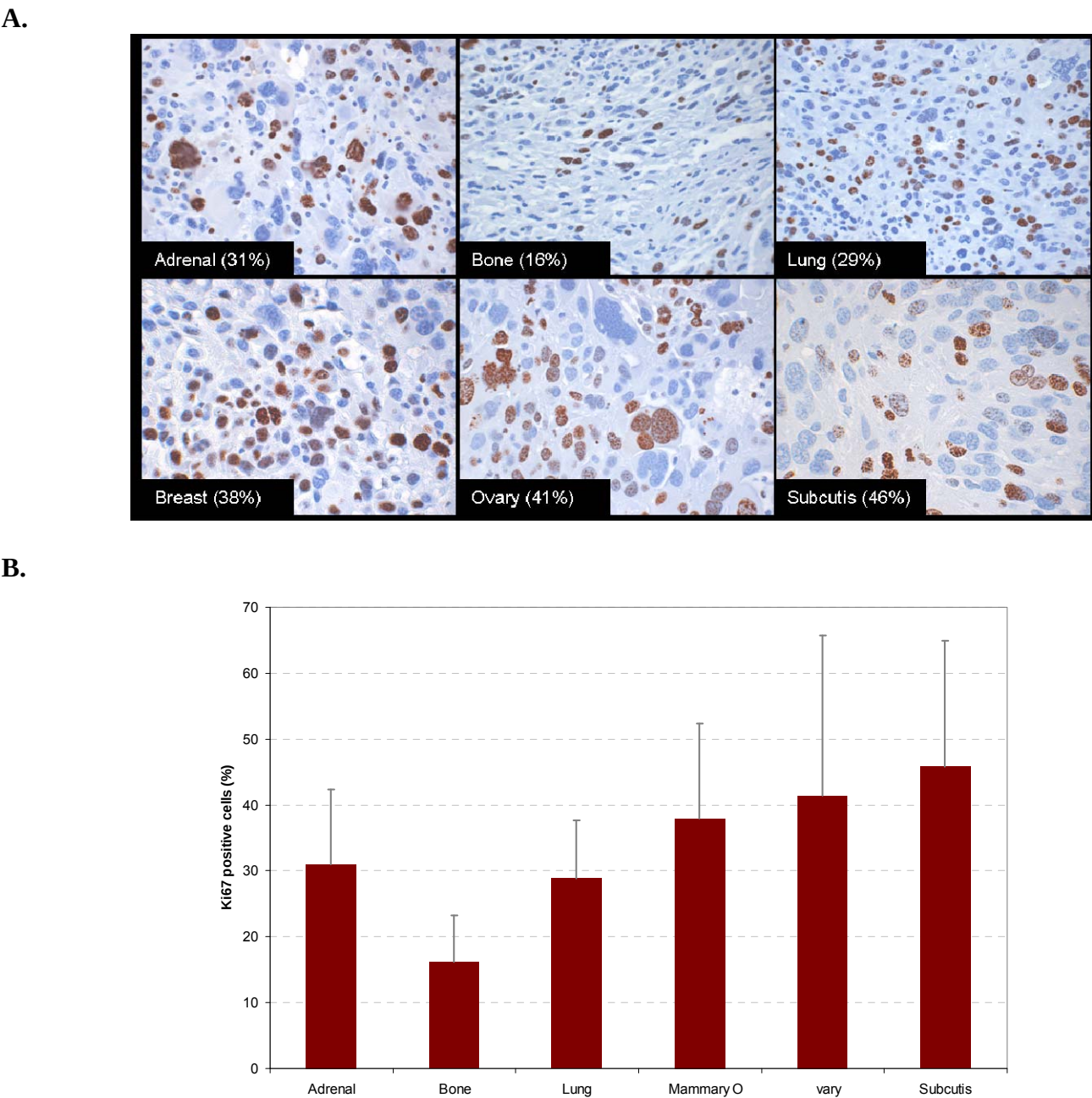
We found that the route of injection did affect the dissemination of MMTV-PyMT tumor cells. We found no metastasis after mammary fat pad injection (N= 0/10), or subcutaneous injection (N= 0/10). Lung metastases were commonly detected after tail vein injection (N= 9/11), along with ovary metastases in a smaller percentage of mice (N= 2/11). Interestingly, alternative intravascular routes of injection (intracardiac) resulted in a different pattern of dissemination: ovary (N= 13/20), adrenal gland (N= 6/20), and bone (N= 3/20). Finally, no metastases were observed following intratibial injections although mild osteolysis was visible via radiographic and histologic evaluation.

Figure 2. MMTV-PymT cells undergo phenotypic differences at various sites of metastasis.



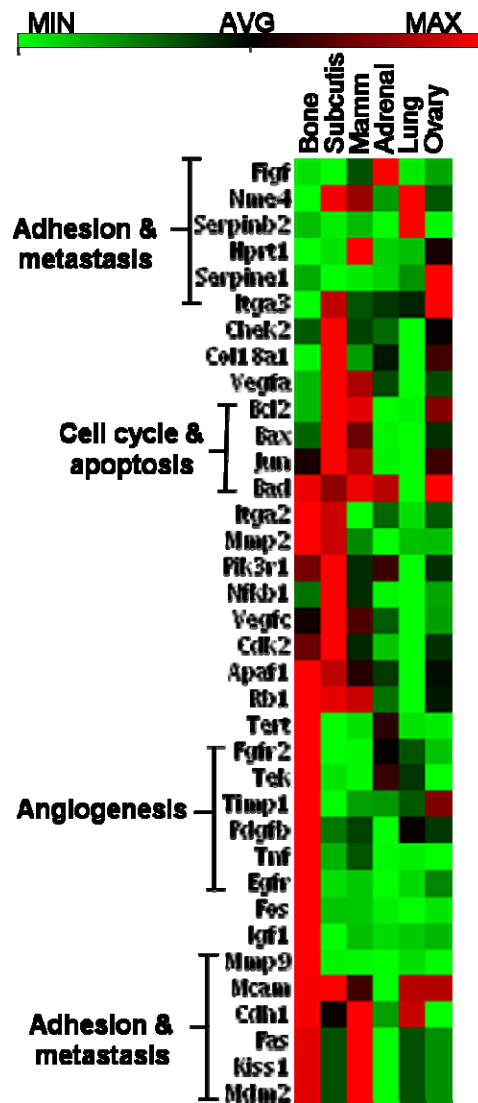
Metastases were detected through gross examination, bioluminescent imaging, and histopathology. Morphologic changes were seen in MMTV-PymT cells depending on the site of growth. Specifically, cells exhibited differences in cell morphology, mitotic index, and percent of necrosis. These findings are significant in determining the aggressiveness of the cells at various sites, and therefore the prognosis for the patient.

Figure 3. The rate of proliferation of MMTV-PymT cells changes depending on the site of metastasis.



(A) Proliferation was measured through immunohistochemistry for Ki67 positive cells. The number of Ki67 positive cells per 100 cells was measured in 3 fields of view, on three mice per group (N=9 measurements per site). (B) Overall, MMTV-PymT cells growing in the subcutis (46%) had the highest percentage of cells dividing followed by ovary (41%), breast (38%), adrenal (31%), lung (29%), and bone (16%). This therefore suggests that the tumor microenvironment may affect the rate of proliferation or growth of MMTV-PymT cells.

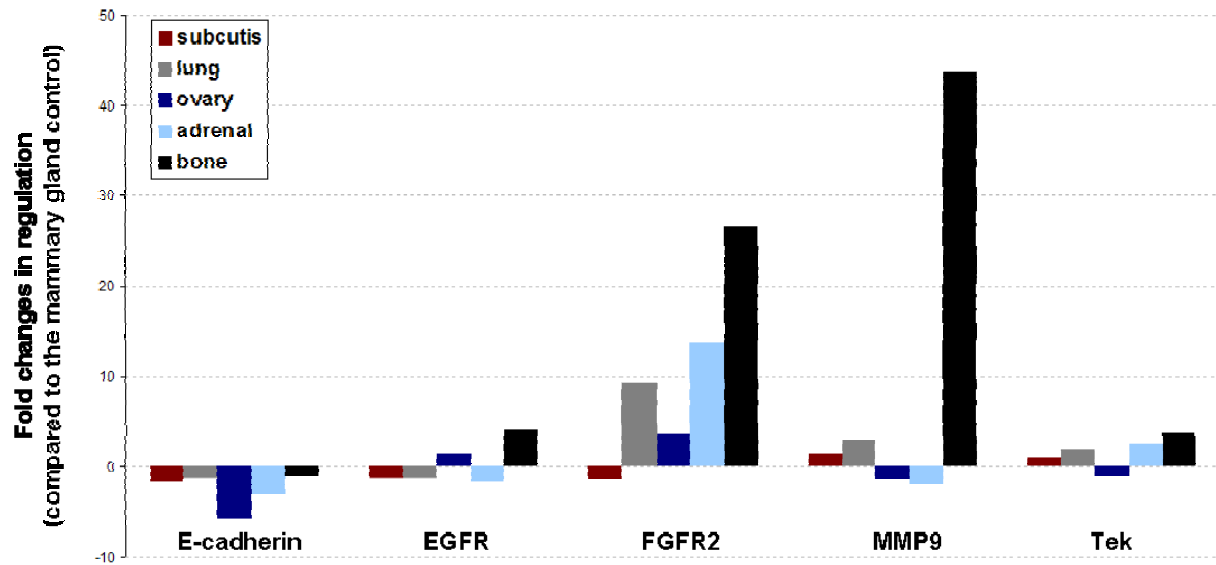
Figure 4. MMTV-PymT cells undergo differential expression of 36 pathway-focused genes depending on the site of growth.



RNA was isolated from MMTV-PymT tumors at six different sites of growth (N=3). Next, Q-RT-PCR was used to examine a set of 88 pathway focused genes related to cancer progression (RT² Profiler, SA Biosciences). Overall, 36 genes were significantly up or down-regulated when compared to the MMTV-PymT cells in the mammary gland control (p<0.05).

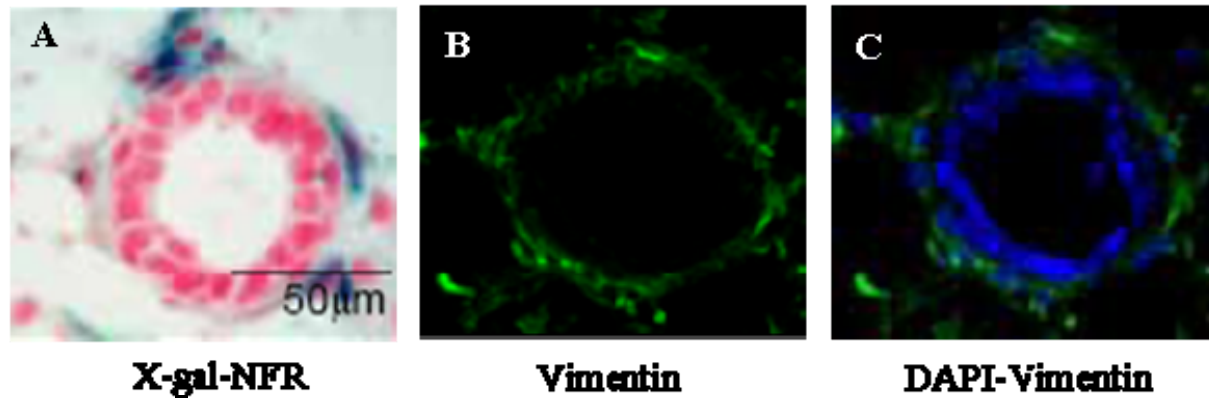
Furthermore, hierarchical clustering of these genes reveals pathways of importance such as adhesion, cell cycle and apoptosis, angiogenesis, and metastasis. Therefore, this suggests that differences in growth and metastasis of MMTV-PymT cells may be related to certain pathways that play a role in the tumor microenvironment.

Figure 5. Fold differences in MMTV-PymT gene expression change depending on the site of growth or metastasis.



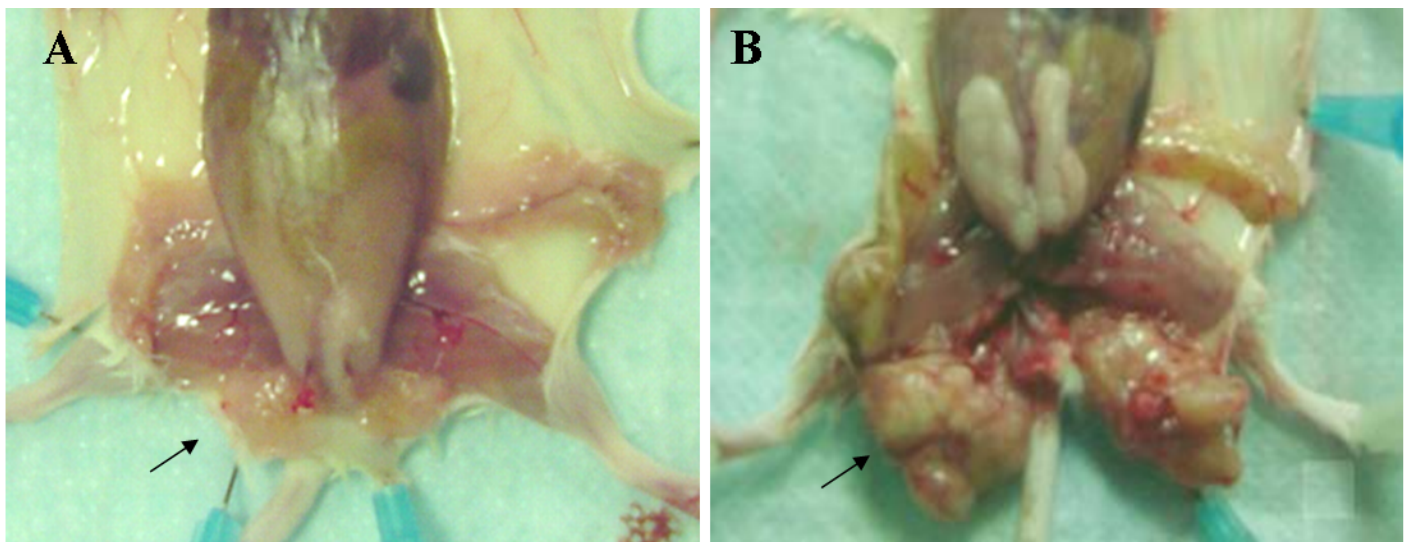
Five genes had a greater than four fold difference in gene expression when compared to the MMTV-PymT cells in the mammary gland control: E-cadherin, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor-2 (FGFR2), matrix metalloproteinase 9 (MMP9) and endothelial-specific receptor tyrosine kinase (Tek). These fold changes in gene expression were dependent on the site of growth or metastasis ($p < 0.05$). MMTV-PymT cells in the bone had the greatest fold change in gene expression with significant up regulation of EGFR (4-fold), FGFR2 (27-fold), MMP9 (44-fold) and Tek (4-fold). Up regulation of FGFR2 was also seen in lung metastases (9-fold), ovary metastases (4-fold) and adrenal metastases (14-fold). Interestingly, E-cadherin was significantly down regulated MMTV-PymT ovary metastases (6-fold).

Figure 6. The Fsp-cre promoter specifically drives expression to mammary fibroblasts.



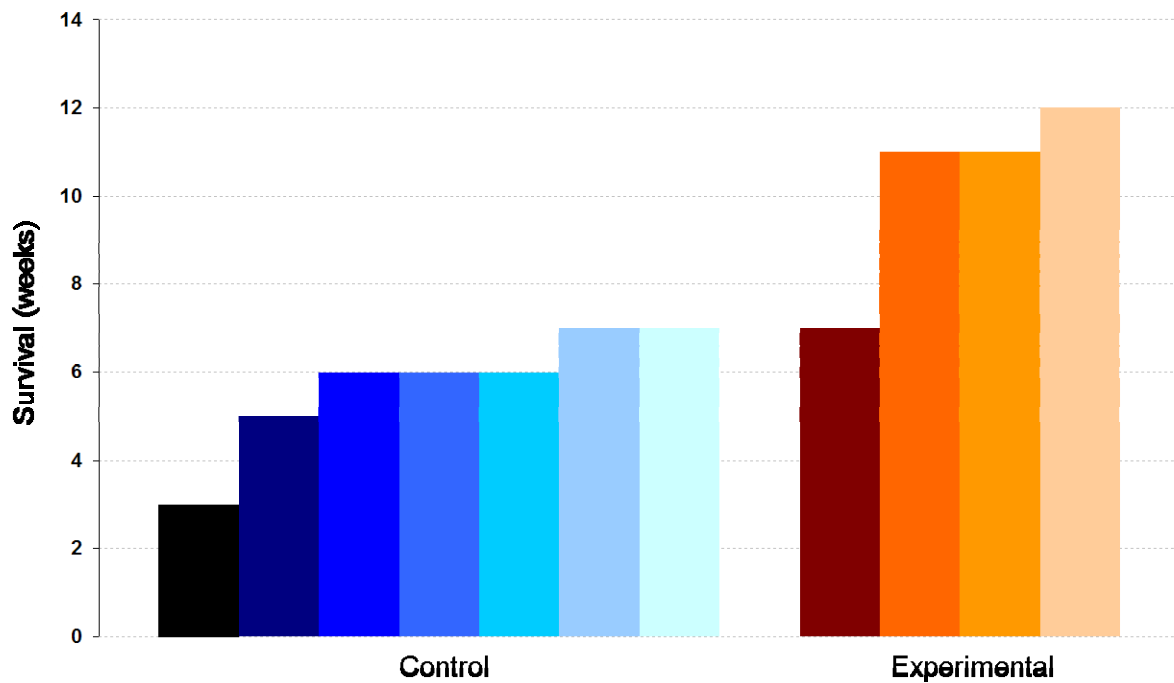
(A) B-galactosidas staining driven by the Fsp-cre promoter correlates with vimentin staining (B) as seen in stromal cells of the mammary duct (C).

Figure 7. Fibroblast deletion of Ets-2 delays tumor progression in the PymT transgenic mouse model.



At 14 days, tumors are significantly smaller in MMTV-PymT-Fsp-cre Ets-2^{-/-} mice (A) when compared to control MMTV-PymT animals (B).

Figure 8. Stromal deletion of Ets2 increases survival time in MMTV-PymT tumors.



In order to determine the effect of Ets2 deletion on tumor onset and morbidity, we completed a pilot study with both control (Ets2^{+/+}) and experimental (fspcre Ets2^{-/-}) mice. MMTV-PymT breast cancer cells were injected into the lateral tail vein of both control (N=7) and experimental (N=4) mice and mice were monitored daily for clinical symptoms of disease. We tracked tumor burden in mice using bioluminescent imaging and mice were euthanized. We found that control mice developed disease approximately 50% sooner (average 5.7 weeks) than experimental mice (average= 10 weeks).

A.



III. KEY RESEARCH AND TRAINING ACCOMPLISHMENTS

A. Research

- In the past year we have completed Aim #1 and I am currently preparing the data for publication
 - Injected 100 mice via 5 different routes of inoculation
 - Weekly bioluminescent imaging
 - Euthanasia and full necropsy of all mice on study
 - Analysis of data: histopathology, immunohistochemistry, RNA extraction of tumors, RT² Profiler PCR Superarrays (SA Biosciences)
- We have also completed a pilot study for Aim #2 and currently have mice on study to complete this aim
 - Completed breeding for the pilot study and the main study
 - Completed injections of pilot study mice and the main study
 - Completed results and analysis of the pilot study: Euthanized animals, formalin fixed and paraffin embedded lungs, completed histomorphometry to look at tumor burden in the lungs
- We will be completing Aim#2 and Aim#3 in the following two years, as outlined by the original statement of work

B. Training

- Weekly obligations: Have attended departmental and cancer related seminars, journal clubs and invited seminar talks at the OSU Comprehensive Cancer Center, as well as weekly lab meetings in the Rosol lab.
- Monthly obligations: Molecular biology and cancer genetics program seminar (MBCG)
- Courses: I have now completed all course work required by the Veterinary Biosciences Graduate Program
- Qualifying exam: I passed both my written and oral candidacy exam on October 20, 2008 in accordance with the guidelines set forth by the Department of Veterinary Biosciences
- Presentations: I presented my work in my yearly Departmental Seminar, as well as monthly in the Rosol lab meetings.
- Conferences: I presented my work at the Advancements in Veterinary Medicine Research Day (OSU), The OSUCCC meeting (annual), The Keystone Symposia for Immunology, Microenvironment and Cancer, I was selected for an oral presentation at the OSUCCC Molecular Biology and Cancer Genetics Bi-Annual Retreat (November 11-12, 2007),
- Future Presentations: Annual AACR meeting (April, 2009), International Tumor Microenvironment and Cancer Meeting (October, 2009), OSU Advancements in Veterinary Medicine Research Day (April, 2009)
- Other professional accomplishments: Elected President of the Veterinary Biosciences Graduate Student Association, Elected as a Delegate for the OSU Council of Graduate Studies. Serving on the OSU Graduate Compensation and Benefits Committee. Served on the Quality of Life Committees for both Veterinary Biosciences and The College of Veterinary Medicine.
- Honors and Awards: Won first place in Molecular and Cellular Biology in the Advances in Veterinary Medicine Research Day. Selected for an oral presentation in the 2009 Hayes Graduate Research Forum.

IV. REPORTABLE OUTCOMES

A. Publications

*Adam W. Studebaker, *Gianluca Storci, **Jillian L. Werbeck**, Pasquale Sansone, A. Kate Sasser, Simona Tavolari, Tim Huang, Michael W.Y. Chan, Frank C. Marini, Thomas J. Rosol, Massimiliano Bonafè and Brett M. Hall. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Research*. 68(21): 9087-9095, 2008.

JL Werbeck, NK Thudi, C Premanandan, CK Martin, MC Ostrowski, TJ Rosol. Gene expression in the metastatic microenvironment is injection site-dependent in the MMTV-PyMT breast cancer model. (*manuscript in preparation for submission to Cancer Research*)

J.L. Werbeck, B.M. Hall. Mesenchymal Stem Cells as Fibroblasts in Tumor Stroma.
- Invited book chapter for Humana Press, "Cancer Stem Cells Methods" (*In press*)

Martin, CM, **Werbeck, JL.**, Thudi, NK, Wolfe, T, Gooding, LM. Rosol, TJ. Effect of zoledronic acid therapy in an in vivo model of bone invasive feline oral squamous cell carcinoma. (*manuscript in preparation*)

B.E. Hildreth III, **J.L. Werbeck**, N.K. Thudi, X. Deng, T.J. Rosol, R.E. Toribio. Conditioned media from parathyroid hormone-related protein transfected LCC15-MB cells enhances in vitro bone formation depending on the concentration and duration of exposure. (*in review, Journal of Bone and Mineral Research*)

Ramiro E Toribio, Holly A Brown, Chad M Novince, Lisa M Gooding, **Jillian L Werbeck**, Sherry T Shu, Gwendolen Lorch, John Foley, Laurie K McCauley, Thomas J Rosol. Skeletal dysmorphology and post-natal lethality of mice lacking the mid-region, nuclear localization sequence, and C-terminus of PTHrP. (*in review, PNAS*).

Xiyun Deng, Guangchun He, **Jillian Werbeck**, Andrea Levine, Ramiro Toribio and Thomas J Rosol. Biphasic Responses of Bone to Parathyroid Hormone-related Protein-141 In Vitro. (*in review*)

A. Kate Sasser, Bethany Mundy, **Jillian Werbeck**, Adam W. Studebaker, Michael W.Y. Chan, Tim Huang, Frank C. Marini, Nilsa Ramirez, Brett Hall. Breast fibroblasts promote epithelial to mesenchymal transition (EMT) of breast carcinoma cells through paracrine IL-6. (*manuscript in preparation*)

B. Posters and Presentations

Werbeck, JL, Thudi, NK, Ostrowski, MC, Rosol, TJ. Gene expression in the metastatic microenvironment is injection site-dependent in the MMTV-PyMT breast cancer model.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2008, 2009
- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008
- Poster Presented at AACR Annual Meeting, 2009

Martin, CM, **Werbeck, JL.**, Thudi, NK, Wolfe, T, Gooding, LM. Rosol, TJ. Effect of zoledronic acid therapy in an in vivo model of bone invasive feline oral squamous cell carcinoma.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2008, 2009
- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

JL Werbeck, AK Sasser, AE Axel, CE Linardic, TJ Rosol, BM Hall. Ras induced IL-6 secretion enhances the growth rates of breast cancer cells through autocrine and paracrine mechanisms.

- Poster presented at the Keystone Symposia for Immunology, Microenvironment and Cancer, 2008

B.E. Hildreth III, **J.L. Werbeck**, N.K. Thudi, X. Deng, T.J. Rosol, R.E. Toribio. Conditioned media from parathyroid hormone-related protein transfected LCC15-MB cells enhances in vitro bone formation depending on the concentration and duration of exposure.

- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

Martin S. Vonau, Zachary M. Rossfeld, Brett M. Hall, **Jillian Werbeck**, Joseph J. Pinzone. Dickkopf-1 (DKK1) inhibits differentiation of bone marrow-derived human mesenchymal stem cells (hMSC) and promotes growth of breast cancer cells in the presence of hMSC.

- Poster Presented at the Annual AACR meeting, 2008

Ramiro E Toribio, Holly A Brown, Chad M Novince, Lisa M Gooding, **Jillian L Werbeck**, Sherry T Shu, Gwendolen Lorch, John Foley, Laurie K McCauley, Thomas J Rosol. Skeletal dysmorphology and post-natal lethality of mice lacking the mid-region, nuclear localization sequence, and C-terminus of PTHrP.

- Poster presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

Jillian Werbeck. The role of the tumor microenvironment in breast cancer metastasis.

- Veterinary Biosciences Annual Departmental Seminar, 2008

J.L. Werbeck, Genetic contributions of the tumor microenvironment in breast cancer metastasis.

- Finalist in the 2009 Hayes Graduate Research Forum

- Paper selected for an oral presentation

J.L. Werbeck, F. Li, M. Gutik, M.C. Ostrowski, T.J. Rosol. Ets2 in lung fibroblasts promotes the growth of metastatic breast cancer cells.

- Poster presented at The Ohio State University Advancemetns in Veterinary Medicine Research Day, 2009

V. CONCLUSIONS

This annual review summarizes our progress to investigating the genetic contributions of the microenvironment in breast cancer metastasis. Overall, we have made progress in accordance with our proposed statement of work. We have now completed Aim#1 of this study (as planned) and we are currently preparing our data for publication. Results from this first study suggest that the injection site can affect metastatic potential and gene expression in the MMTV-PyMT breast cancer model. This novel finding has allowed us to not only add to the current understanding of the relevance of the MMTV-PyMT model for metastasis research, but has also allowed us to characterize this model system for future work in Aims #2 and #3.

We have made significant progress in Aim#2 as outlined in our original statement of work. Results from our pilot study justify moving forward with this project as originally outlined. Preliminary results demonstrate that fibroblast specific deletion of *Ets2* decreases tumor burden in the MMTV-PyMT model. We are currently expanding this study to a larger group of animals in order to complete Aims #2 and #3 of this proposal.

In the past year, the PI (Jillian Werbeck) has been first author on 2 manuscripts (one in preparation) and 5 posters or presentations. She also co-authored 6 papers and 4 posters.

In addition to our research accomplishments, the PI (Jillian Werbeck) has also made significant progress in her training program. She completed all required coursework and successfully passed her qualifying exam in October, and is now a PhD candidate in the Veterinary Biosciences program. She presented in my yearly Departmental Seminar, as well as monthly in the Rosol lab meetings. She also had the opportunity to attend meetings such as the Advancements in Veterinary Medicine Research Day (OSU), The OSUCCC meeting (annual), and The Keystone Symposia for Immunology, Microenvironment and Cancer. She was also selected for an oral presentation at the OSUCCC Molecular Biology and Cancer Genetics Bi-Annual Retreat (November 11-12, 2007), and won first place in Molecular and Cellular Biology in the Advances in Veterinary Medicine Research Day, 2008. In the future, she will be presenting her work at the Annual AACR meeting (April, 2009), International Tumor Microenvironment and Cancer Meeting (October, 2009), OSU Advancements in Veterinary Medicine Research Day (April, 2009), and was selected to give an oral presentation at the 2009 OSU Hayes Graduate Research Forum.

Other professional accomplishments include election as President of the Veterinary Biosciences Graduate Student Association, and as a Delegate for the OSU Council of Graduate Studies. In addition she is also serving on the OSU Graduate Compensation and Benefits Committee and the Quality of Life Committees for both Veterinary Biosciences and The College of Veterinary Medicine.

In conclusion the PI has accomplished the research, training and professional obligations outlined by this original proposal and statement of work. She anticipates being on schedule to continue the proposed initiatives over the next two years, and to be able to make significant progress to defining the genetic role of the microenvironment in breast cancer metastasis.

VI. REFERENCES

1. Debies, M. T. and D. R. Welch. 2001. Genetic basis of human breast cancer metastasis. *J. Mammary Gland Biol. Neoplasia* 6:441-451.
2. Cardiff, R. D. 2001. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. *Microsc. Res. Tech.* 52:224-230.
3. Vernon, A. E., S. J. Bakewell, and L. A. Chodosh. 2007. Deciphering the molecular basis of breast cancer metastasis with mouse models. *Rev. Endocr. Metab Disord.* 8:199-213.
4. Fukino, K., L. Shen, S. Matsumoto, C. D. Morrison, G. L. Mutter, and C. Eng. 2004. Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. *Cancer Res* 64:7231-7236.
5. Kalluri, R. and M. Zeisberg. 2006. Fibroblasts in cancer. *Nat. Rev. Cancer* 6:392-401.
6. von Lintig, F. C., A. D. Dreilinger, N. M. Varki, A. M. Wallace, D. E. Casteel, and G. R. Boss. 2000. Ras activation in human breast cancer. *Breast Cancer Res Treat.* 62:51-62.
7. Malaney, S. and R. J. Daly. 2001. The ras signaling pathway in mammary tumorigenesis and metastasis. *J. Mammary Gland Biol. Neoplasia.* 6:101-113.
8. Bar-Sagi, D. and A. Hall. 2000. Ras and Rho GTPases: a family reunion. *Cell* 103:227-238.
9. Graves, B. J. and J. M. Petersen. 1998. Specificity within the ets family of transcription factors. *Adv. Cancer Res* 75:1-55.
10. Reddy, M. A., S. J. Langer, M. S. Colman, and M. C. Ostrowski. 1992. An enhancer element responsive to ras and fms signaling pathways is composed of two distinct nuclear factor binding sites. *Mol. Endocrinol.* 6:1051-1060.
11. Wasylyk, C., A. Gutman, R. Nicholson, and B. Wasylyk. 1991. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *EMBO J* 10:1127-1134.
12. Sapi, E., M. B. Flick, S. Rodov, and B. M. Kacinski. 1998. Ets-2 transdominant mutant abolishes anchorage-independent growth and macrophage colony-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. *Cancer Res* 58:1027-1033.
13. Neznanov, N., A. K. Man, H. Yamamoto, C. A. Hauser, R. D. Cardiff, and R. G. Oshima. 1999. A single targeted Ets2 allele restricts development of mammary tumors in transgenic mice. *Cancer Res* 59:4242-4246.
14. Baker, K. M., G. Wei, A. E. Schaffner, and M. C. Ostrowski. 2003. Ets-2 and components of mammalian SWI/SNF form a repressor complex that negatively regulates the BRCA1 promoter. *J Biol. Chem.* 278:17876-17884.
15. Schedin, P. J., K. L. Eckel-Mahan, S. M. McDaniel, J. D. Prescott, K. S. Brodsky, J. J. Tentler, and A. Gutierrez-Hartmann. 2004. ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. *Oncogene* 23:1766-1779.

16. Tynan, J. A., F. Wen, W. J. Muller, and R. G. Oshima. 2005. Ets2-dependent microenvironmental support of mouse mammary tumors. *Oncogene* 24:6870-6876.
17. Borowsky, A. D., R. Namba, L. J. Young, K. W. Hunter, J. G. Hodgson, C. G. Tepper, E. T. McGoldrick, W. J. Muller, R. D. Cardiff, and J. P. Gregg. 2005. Syngeneic mouse mammary carcinoma cell lines: two closely related cell lines with divergent metastatic behavior. *Clin. Exp. Metastasis* 22:47-59.
18. Phadke, P. A., K. S. Vaidya, K. T. Nash, D. R. Hurst, and D. R. Welch. 2008. BRMS1 suppresses breast cancer experimental metastasis to multiple organs by inhibiting several steps of the metastatic process. *Am. J. Pathol.* 172:809-817.
19. Jessen, K. A., S. Y. Liu, C. G. Tepper, J. Karrim, E. T. McGoldrick, A. Rosner, R. J. Munn, L. J. Young, A. D. Borowsky, R. D. Cardiff, and J. P. Gregg. 2004. Molecular analysis of metastasis in a polyomavirus middle T mouse model: the role of osteopontin. *Breast Cancer Res.* 6:R157-R169.
20. Tannehill-Gregg, S. H., A. L. Levine, M. V. Nadella, H. Iguchi, and T. J. Rosol. 2006. The effect of zoledronic acid and osteoprotegerin on growth of human lung cancer in the tibias of nude mice. *Clin. Exp. Metastasis* 23:19-31.
21. Dilworth, S. M. 2002. Polyoma virus middle T antigen and its role in identifying cancer-related molecules. *Nat. Rev. Cancer* 2:951-956.
22. Yashiro, M., K. Ikeda, M. Tendo, T. Ishikawa, and K. Hirakawa. 2005. Effect of organ-specific fibroblasts on proliferation and differentiation of breast cancer cells. *Breast Cancer Res. Treat.* 90:307-313.
23. Schor, S. L. 1994. Cytokine control of cell motility: modulation and mediation by the extracellular matrix. *Prog. Growth Factor Res.* 5:223-248.
24. Fainaru, O., A. Adini, O. Benny, I. Adini, S. Short, L. Bazinet, K. Nakai, E. Pravda, M. D. Hornstein, R. J. D'Amato, and J. Folkman. 2008. Dendritic cells support angiogenesis and promote lesion growth in a murine model of endometriosis. *FASEB J.* 22:522-529.
25. Khan, S., J. R. Aspe, M. G. Asumen, F. Almaguel, O. Odumosu, S. cevedo-Martinez, M. N. De Leon, W. H. R. Langridge, and N. R. Wall. 2009. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential. *Br J Cancer.*
26. Fukino, K., L. Shen, S. Matsumoto, C. D. Morrison, G. L. Mutter, and C. Eng. 2004. Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. *Cancer Res.* 64:7231-7236.
27. Joyce, J. A. and J. W. Pollard. 2009. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9:239-252.
28. Kang, Y. 2006. New tricks against an old foe: molecular dissection of metastasis tissue tropism in breast cancer. *Breast Dis.* 26:129-138.
29. Yang, B. S., C. A. Hauser, G. Henkel, M. S. Colman, C. Van Beveren, K. J. Stacey, D. A. Hume, R. A. Maki, and M. C. Ostrowski. 1996. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol. Cell Biol.* 16:538-547.
30. McCarthy, S. A., D. Chen, B. S. Yang, J. J. Garcia Ramirez, H. Cherwinski, X. R. Chen, M. Klagsbrun, C. A. Hauser, M. C. Ostrowski, and M. McMahon. 1997. Rapid phosphorylation of Ets-2 accompanies

mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. *Mol. Cell Biol.* 17:2401-2412.

31. Yang, B. S., C. A. Hauser, G. Henkel, M. S. Colman, C. Van Beveren, K. J. Stacey, D. A. Hume, R. A. Maki, and M. C. Ostrowski. 1996. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol. Cell Biol.* 16:538-547.
32. Fowles, L. F., M. L. Martin, L. Nelsen, K. J. Stacey, D. Redd, Y. M. Clark, Y. Nagamine, M. McMahon, D. A. Hume, and M. C. Ostrowski. 1998. Persistent activation of mitogen-activated protein kinases p42 and p44 and ets-2 phosphorylation in response to colony-stimulating factor 1/c-fms signaling. *Mol. Cell Biol.* 18:5148-5156.
33. Patton, S. E., M. L. Martin, L. L. Nelsen, X. Fang, G. B. Mills, R. C. Bast, Jr., and M. C. Ostrowski. 1998. Activation of the ras-mitogen-activated protein kinase pathway and phosphorylation of ets-2 at position threonine 72 in human ovarian cancer cell lines. *Cancer Res* 58:2253-2259.

VI. APPENDICES

Abbreviations

NCI	National Cancer Institute
OSUCCC	The Ohio State University Comprehensive Cancer Center
MVIMG	Molecular Virology Immunology and Molecular Genetics program
IBGP	Integrated Biomedical Graduate Program
PATH	Pathology Department
IVIS	<i>In vivo</i> Imaging System
BLI	Bioluminescence imaging
Micro-CT	Micro-computed tomography
RT-PCR	Reverse transcriptase polymer chain reaction
AACR	American Association for Cancer Research
<i>MMTV</i>	Mouse mammary tumor virus
<i>PymT</i>	Polyoma Middle T antigen
Fsp-Cre	Fibroblast specific protein 1 promoter- Cre recombinase
TAF	Tumor associated fibroblast
ECM	Extracellular Matrix
FGF	Fibroblast growth factor
VEGF	Vascular endothelial growth factor
TGF- β	Transforming growth factor- beta
MMP	Matrix metalloproteinase
PECAM	Platelet/endothelial cell adhesion molecule

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Jillian L. Werbeck	Graduate Research Associate

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Binghamton University Binghamton, NY	BS	2001-2005	Cellular and molecular biology
The Ohio State University Columbus, OH	PhD	2005- present	Veterinary Biosciences

ACADEMIC POSITIONS

Academic Positions (present)

- 2005-present Graduate Research Associate, Department of Veterinary Biosciences, The Ohio State University, Columbus, OH
Genetic contributions of the microenvironment in breast cancer metastasis

- 2008-present President, Graduate Student Association, Veterinary Biosciences, The Ohio State University

- 2008-present Delegate, Council of Graduate Students, The Ohio State University
- Graduate compensation and benefits committee

- 2008-present Quality of Life committee member
-Department of Veterinary Biosciences
-College of Veterinary Medicine

Academic Positions (past)

- 1999-2000 Research Assistant, Department of surgery, SUNY Upstate Medical University, Syracuse, NY
Histopathologic analysis of cytokines related to breast pain.

- 2000-2001 Research Assistant, Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse NY
The effect of estrogenic-like polychlorinated biphenyls on breast cancer and leukemia cells.

- 2003 Summer Research Assistant, Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY
The effect of estrogen on cardiac angiotensin converting enzyme, (ACE) mRNA expression.

- 2004 Summer Undergraduate Research Fellowship, Department of Orthopedic Surgery, SUNY Upstate Medical University, Syracuse NY
Characterization of a primary human metastatic breast cancer cell line.

- 2003-2005 Undergraduate Research Associate, Department of Biological Sciences, Binghamton University, Binghamton, NY
Identification of an auto-inducer molecule of dispersion in *Pseudomonas aeruginosa*.

HONORS AND AWARDS

2009	Selected for an oral presentation at the 2009 Hayes Graduate Research Forum
2008	Advancements in Veterinary Medicine travel award for Molecular and Cellular Biology
2007	Selected for oral presentation at the 2007 OSU MBCG meeting
2007	Second Place, Roche Distinguished Graduate Seminar Award
2004	Summer Undergraduate Research Fellowship, SUNY Upstate Medical University
2004	Louis M. Azzara scholarship '87, Binghamton University Graduation Scholarship
2003	Dean's List, Fall
2002	<i>Young woman of promise</i> , The National Organization for Women, Syracuse, NY
2002	<i>Student of the Year</i> , Broome-Tioga workforce association, Binghamton, NY
2001	Third place, Intel 52 nd International High School Science and Engineering Fair First place United States Army
2000	Finalist, Intel 51 st International High School Science and Engineering Fair

FUNDING AND GRANTS

BC073043 (PI: J.L. Werbeck)	03/01/08-02/29/11	100%
CDMRP, Department of Defense Predoctoral Fellowship		\$97,000
Ets2 contributions of the tumor microenvironment in breast cancer metastasis		

PUBLICATIONS

Ramakrishnan R, **Werbeck J**, Khurana KK, Khan SA. Expression of interleukin-6 and tumor necrosis factor alpha and histopathologic findings in painful and nonpainful breast tissue. *Breast Journal*. 9(2):91-7, 2003.

N.K.Thudi, C.K.Martin, M.V.P. Nadella, **J.L.Werbeck** and T.J.Rosol. Osteoblastic lesions and tumor burden are independent of osteolysis in a nude mouse model of canine prostate cancer. *The Prostate*. 68(10): 1116-1125, 2008.

Sherry T. Shu, M.V.P. Nadella, Wessel P. Dirksen, Soledad A. Fernandez, Nanda K. Thudi, **Jillian Werbeck**, Thomas J. Rosol. A novel bioluminescent mouse model and effective therapy for humoral hypercalcemia of malignancy in adult T-cell leukemia/lymphoma. *Cancer Research*. 67:11859-11866, 2007.

*Adam W. Studebaker, *Gianluca Storci, **Jillian L. Werbeck**, Pasquale Sansone, A. Kate Sasser, Simona Tavolari, Tim Huang, Michael W.Y. Chan, Frank C. Marini, Thomas J. Rosol, Massimiliano Bonafè and Brett M. Hall . Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Research*. 68(21): 9087-9095, 2008.

Werbeck, JL, Thudi, NK, Ostrowski, MC, Rosol, TJ. Gene expression in the metastatic microenvironment is injection site-dependent in the MMTV-PyMT breast cancer model. (*manuscript in preparation*)

J.L. Werbeck, B.M. Hall. Mesenchymal Stem Cells as Fibroblasts in Tumor Stroma.
- Invited book chapter for Humana Press, "Cancer Stem Cells Methods" (*In press*)

Martin, CM, **Werbeck, JL.**, Thudi, NK, Wolfe, T, Gooding, LM. Rosol, TJ. Effect of zoledronic acid therapy in an in vivo model of bone invasive feline oral squamous cell carcinoma. (*manuscript in preparation*)

B.E. Hildreth III, **J.L. Werbeck**, N.K. Thudi, X. Deng, T.J. Rosol, R.E. Toribio. Conditioned media from parathyroid hormone-related protein transfected LCC15-MB cells enhances in vitro bone formation depending on the concentration and duration of exposure. (*in review, Journal of Bone and Mineral Research*)

Ramiro E Toribio, Holly A Brown, Chad M Novince, Lisa M Gooding, **Jillian L Werbeck**, Sherry T Shu, Gwendolen Lorch, John Foley, Laurie K McCauley, Thomas J Rosol. Skeletal dysmorphology and post-natal lethality of mice lacking the mid-region, nuclear localization sequence, and C-terminus of PTHrP. (*in review, PNAS*).

Xiyun Deng, Guangchun He, **Jillian Werbeck**, Andrea Levine, Ramiro Toribio and Thomas J Rosol. Biphasic Responses of Bone to Parathyroid Hormone-related Protein-141 In Vitro. (*in review*)

A. Kate Sasser, Bethany Mundy, **Jillian Werbeck**, Adam W. Studebaker, Michael W.Y. Chan, Tim Huang, Frank C. Marini, Nilsa Ramirez, Brett Hall. Breast fibroblasts promote epithelial to mesenchymal transition (EMT) of breast carcinoma cells through paracrine IL-6. (*manuscript in preparation*)

POSTERS AND PRESENTATIONS

Davies D, Marques C, Chan W,* **Werbeck J,*** Jang H, Cuadra G, Duddipudi L. Autoinduction of *Pseudomonas aeruginosa* Biofilm Dispersion.

- Poster presentation at the 2005 General Meeting for the American Society of Microbiology.

J.L.Werbeck, C.K. Martin, N.K. Thudi, M.C. Ostrowski, T.J. Rosol. Ets-2 contributions of the tumor microenvironment in breast cancer metastasis.

-Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2007

-Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2007

-Poster Presented at "Cell Signaling in Cancer", 2007

Jillian Werbeck. Genetic contributions of the tumor microenvironment in breast cancer metastasis

-Veterinary Biosciences Annual Departmental Seminar, 2006

N.K.Thudi, C.K.Martin, M.V.P. Nadella, **J.L.Werbeck** and T.J.Rosol. Osteoblastic lesions and tumor burden are independent of osteolysis in a nude mouse model of canine prostate cancer.

- Poster presentation at the European Calcified Tissue Society meeting, 2006

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2007

- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2007

- Poster Presented at "Cell Signaling in Cancer", 2007

Sherry T. Shu, M.V.P. Nadella, Wessel P. Dirksen, Soledad A. Fernandez, Nanda K. Thudi, **Jillian Werbeck**, Thomas J. Rosol. A novel bioluminescent mouse model and effective therapy for humoral hypercalcemia of malignancy in adult T-cell leukemia/lymphoma.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2007

- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2007

- Poster Presented at the National AACR meeting, 2007

- Poster Presented at the 13th international conference on human retrovirology HTLV and related viruses, 2007

Jillian Werbeck. Estrogen Independent growth mechanisms in hormone responsive breast cancer.

- Veterinary Biosciences Annual Departmental Seminar, 2007

Chelsea Martin, **Jillian Werbeck**, Nandu Thudi, Tobie Wolfe and Thomas Rosol. Tumor-Bone Interaction in Models of Head and Neck Cancer.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2007
- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2007
- Poster Presented at "Cell Signaling in Cancer", 2007

B.E. Hildreth III, **J.L. Werbeck**, X. Deng, T.J. Rosol, R.E. Toribio. In vitro kinetics of parathyroid hormone-related protein (PTHrP) (1-141) produced by transfected LCC15-MB cells.

- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2007

J.L. Werbeck, A.K. Sasser, A.E. Axel, C. Linardic, T.J. Rosol, B.M. Hall. Ras and IL-6 cooperate in an additive fashion to enhance ER α positive breast cancer growth rates.

- Selected for oral presentation, OSUCCC Molecular Biology and Cancer Genetics Bi-Annual Retreat. November 11-12, 2007.

Werbeck, JL, Thudi, NK, Ostrowski, MC, Rosol, TJ. Gene expression in the metastatic microenvironment is injection site-dependent in the MMTV-PyMT breast cancer model.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2008, 2009
- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008
- Poster Presented at AACR Annual Meeting, 2009

Martin, CM, **Werbeck, JL.**, Thudi, NK, Wolfe, T, Gooding, LM. Rosol, TJ. Effect of zoledronic acid therapy in an in vivo model of bone invasive feline oral squamous cell carcinoma.

- Poster Presented at The Ohio State University Comprehensive Cancer Center Meeting, 2008, 2009
- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

JL Werbeck, AK Sasser, AE Axel, CE Linardic, TJ Rosol, BM Hall. Ras induced IL-6 secretion enhances the growth rates of breast cancer cells through autocrine and paracrine mechanisms.

- Poster presented at the Keystone Symposia for Immunology, Microenvironment and Cancer, 2008

B.E. Hildreth III, **J.L. Werbeck**, N.K. Thudi, X. Deng, T.J. Rosol, R.E. Toribio. Conditioned media from parathyroid hormone-related protein transfected LCC15-MB cells enhances in vitro bone formation depending on the concentration and duration of exposure.

- Poster Presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

Martin S. Vonau, Zachary M. Rossfeld, Brett M. Hall, **Jillian Werbeck**, Joseph J. Pinzone. Dickkopf-1 (DKK1) inhibits differentiation of bone marrow-derived human mesenchymal stem cells (hMSC) and promotes growth of breast cancer cells in the presence of hMSC.

- Poster Presented at the Annual AACR meeting, 2008

Ramiro E Toribio, Holly A Brown, Chad M Novince, Lisa M Gooding, **Jillian L Werbeck**, Sherry T Shu, Gwendolen Lorch, John Foley, Laurie K McCauley, Thomas J Rosol. Skeletal dysmorphology and post-natal lethality of mice lacking the mid-region, nuclear localization sequence, and C-terminus of PTHrP.

- Poster presented at The Ohio State University Advancements in Veterinary Medicine Research Day, 2008

Jillian Werbeck. The role of the tumor microenvironment in breast cancer metastasis.

- Veterinary Biosciences Annual Departmental Seminar, 2008

J.L. Werbeck, Genetic contributions of the tumor microenvironment in breast cancer metastasis.

- Finalist in the 2009 Hayes Graduate Research Forum
- Paper selected for an oral presentation

J.L. Werbeck, F. Li, M. Gutik, M.C. Ostrowski, T.J. Rosol. Ets2 in lung fibroblasts promotes the growth of metastatic breast cancer cells.

- Poster presented at The Ohio State University Advancemetns in Veterinary Medicine Research Day, 2009