Genetic Contributions of the Tumor Microenvironment in Breast Cancer Metastasis

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Abstract

Metastasis is a multistage process involving many complex genetic events which facilitate the spread and growth of cancer cells at a secondary site. *In vivo* models of metastasis are invaluable to our understanding of the interactions between cancer cells and a variety of cell types at the metastatic microenvironment. 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 five 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. We tracked circulating tumor cells over time using a Xenogen *In Vivo* Imaging System (IVIS) and evaluated metastases based on the site of dissemination, morphologic changes, and the genetic profile of the tumor cells at various organ sites (subcutis, mammary fat pad, lung, ovary, adrenal gland, and tibia).

We found that the route of injection did affect the dissemination of PymT tumor cells.

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In addition, the MMTV-PymT tumor cells had different morphologic and genetic profiles depending on their site of growth ($p<0.01$). Specifically, E-cadherin, FGFR1, EGF, and MMP9 expression were greater than 3 fold different in PymT metastases when compared to the cells in the mammary gland control.

We demonstrated that the tumor microenvironment has a site-specific effect on the gene expression profile of the PymT cell line. Interestingly, we have also shown that the injection route may significantly affect the metastatic potential of tumor cells, and therefore the pathology of secondary lesions. This study has significant implications in trying to understand the mechanism by which tumor cells disseminate, and also the genetic interactions between tumor cells and stroma in various organs of metastasis.

Introduction

Breast cancer is the second leading cause of cancer related deaths in women, and accounts for approximately 40,000 deaths annually (American Cancer Society). Although research has improved the detection and treatment of breast cancer, metastasis still remains the most lethal complication of this disease. Metastasis is defined as the process by which cancer cells detach from the primary site of origin to disseminate and grow at a distant site. There are many complex processes involved in metastasis which hinder our ability to fully understand this disease. Cancer cells must acquire the ability to invade through the tissue basement membrane at the primary site, intravasate into a blood or lymphatic vessel, survive and extravasate out of the vessel, and invade and grow in a foreign tissue microenvironment. There are many molecular and biochemical mechanisms which have been discovered to facilitate this multi-stage process, although many are still unknown.

Animal models are an invaluable tool to the understanding of cancer metastasis (Cardiff 224-30). 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 (Vernon, Bakewell, and Chodosh 199-213). 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. Our first objective was to elucidate how different *in vivo* injection models of metastasis can affect the secondary site of growth. Our first hypothesis is that the injection route of cancer cells can affect the metastatic potential of the cells to seed in a variety of secondary microenvironments. In order to test this hypothesis we have utilized five common injection models to study various stages of the multi-stage process of metastasis (Figure 1.) Mammary fat pad injections or subcutaneous injections allow for the modeling of cells leaving the primary site of origin. Alternatively, cardiac or venous injections allow for the modeling of cells which have entered the circulation and can then extravasate out of a vessel to grow at a secondary site. Finally, injection of cells into the tibia allow for the understanding of how breast cancer cells can alter a secondary site of metastasis, with bone being the most common site of breast cancer metastasis.

In addition to determining where breast cancer cells metastasize, our second objective is to study the morphologic and genetic changes that can occur in cancer cells when they reach various tissue microenvironments. Our second hypothesis is that breast cancer cells will undergo different morphologic and genetic changes depending on the site of dissemination, which may allow them to survive and grow at that site. This is the first study to evaluate a commonly used

breast cancer cell line using various routes of injection in order to understand both the pattern of metastasis, as well as the genetic and morphologic changes that occur in that cell line in various microenvironments. Implications from this study can further our understanding of both the molecular mechanisms related to metastasis, as well as the genetic interactions between breast cancer cells and stroma in various metastatic microenvironments.

Materials and Methods

Highly aggressive Mouse Mammary Tumor virus- Polyoma Middle T antigen (MMTV-PymT) cells were selected for this study in order to reliably and reproducibly model metastasis (Borowsky et al. 47-59). Advantages of this model include the rapid formation of metastasis to the lung in virtually 100% of transgenic animals within 20 weeks of age (Borowsky et al. 47-59); as well as an accurate model of multi-stage progression similar to human disease (Qiu et al. 5973-81). Additionally, the isolation of these cells from a primary mouse breast tumor allows for injection into strain matched, immunocompetent animals to allow for the involvement of the immune system in metastasis (Lin et al. 2113-26).

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 50ul of PBS at either site respectively using a 25G needle (N=10 animals each) (Borowsky et al. 47-59). 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 100µl into the heart using a 27G needle (Phadke et al. 809-17). 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 200µl of PBS into the dilated lateral tail vein using a 27G needle (Jessen et al. R157-R169). 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 20µl volume into the medullary canal (Tannehill-Gregg et al. 19-31).

Metastasis was tracked using bioluminescent imaging (IVIS, Xenogen) and mice were euthanized using 100% CO_2 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. RT^2 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 ± S.D. Statistical differences were performed using a students T-test with a level of significance at $p<0.05$ unless stated as otherwise.

Results

MMTV-PymT metastasis is injection site dependent. Overall, the route of injection did affect the metastatic potential of MMTV-PymT cells (figure 2). 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. 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 4.)

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 5.) 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 verses 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. 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.

Discussion

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 (Dilworth 951-56). 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 (Yashiro et al. 307-13), growth (Schor 223-48), angiogenesis (Fainaru et al. 522-29) and survival of cancer cells (Khan et al.). Loss of heterozygosity (LOH) studies have also revealed that multiple stromal targets undergo genetic changes to support the growth of breast cancer cells (Fukino et al. 7231-36). 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 (Joyce and Pollard 239-52). Interestingly, many studies also suggested that there is tissue-specific tropism for breast cancer cells to metastasize to certain organs (Kang 129-38) 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.

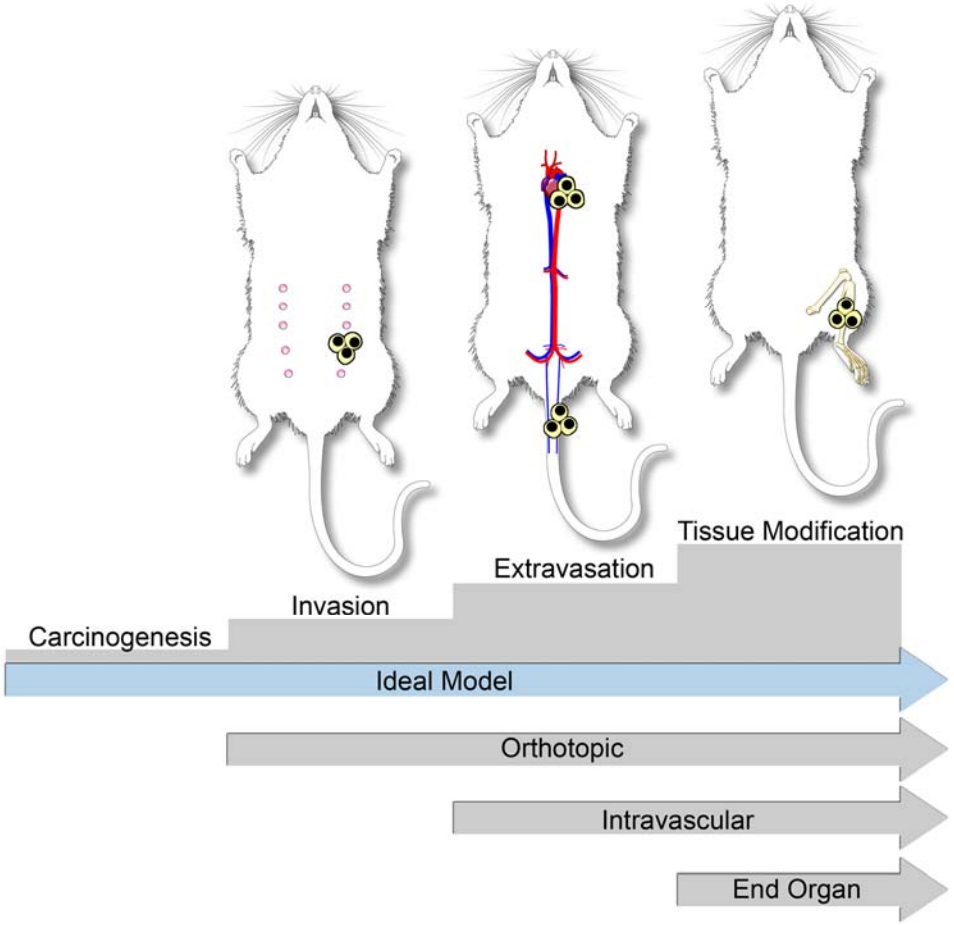
There are many morphologic characteristics that are associated with aggressive tumors and therefore poor prognosis (Cardiff 224-30). These data suggest that MMTV-PymT cells not only exhibit these negative morphologic characteristics, but also that the degree of morphologic variation in these tumors depend 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 verses 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 verses 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.

Figure 1. Injection of MMTV-PymT cells via different anatomical routes accurately recapitulate the multistage process of metastasis.



Metastasis is a multistage process involving invasion of cells out of the primary site, intravasation into a blood or lymph vessel, extravasation out of circulation, and survival and growth at a secondary site. Currently, there are no models which accurately model this process from initiation to metastasis. Therefore, injection models are invaluable to understanding this complex process. The goal of this study is to model metastasis using all the available models of metastasis in order to accurately recapitulate and study this process in multiple stages.

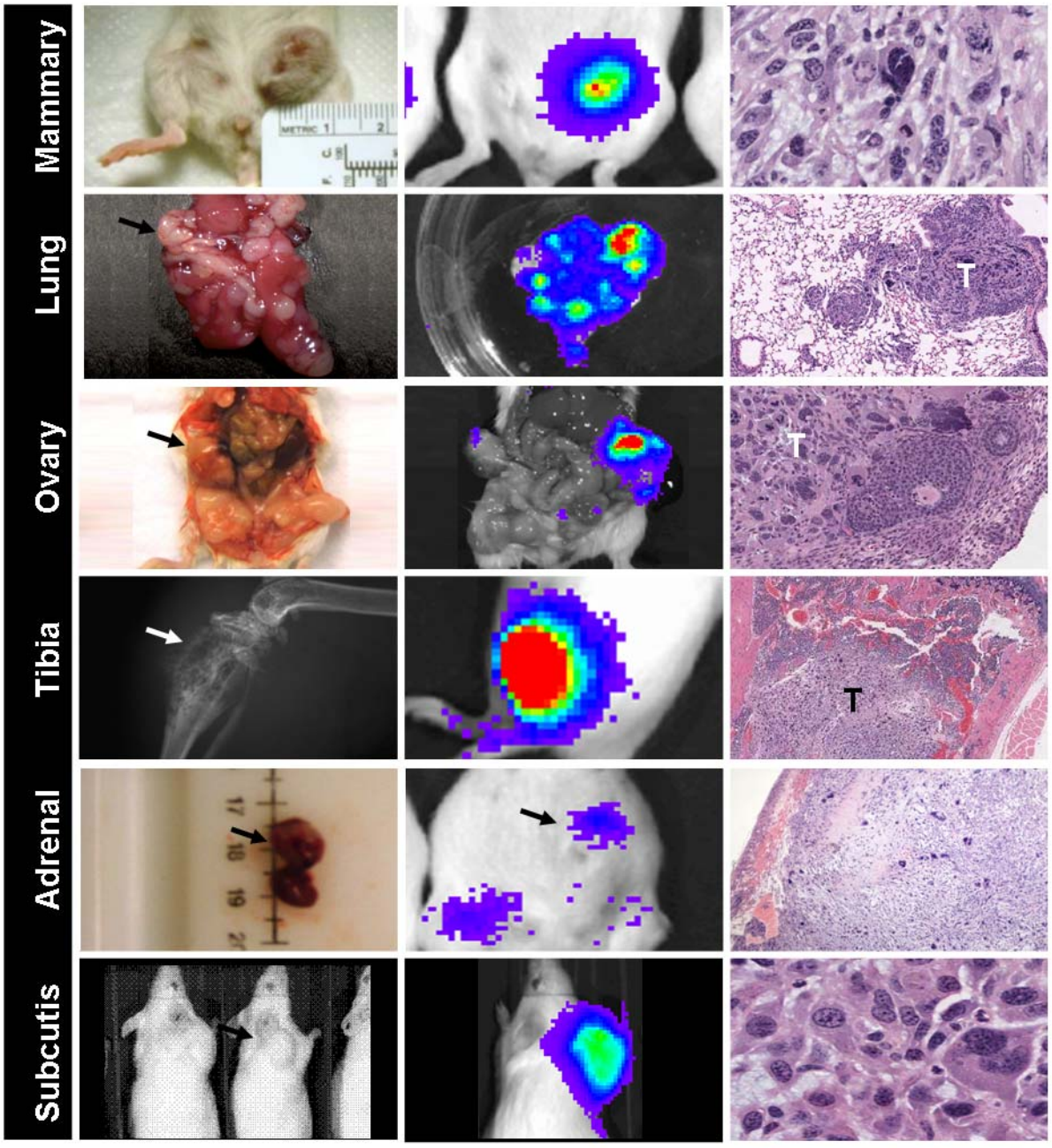
Figure 2. MMTV-PymT metastasis is injection site dependent.

	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.

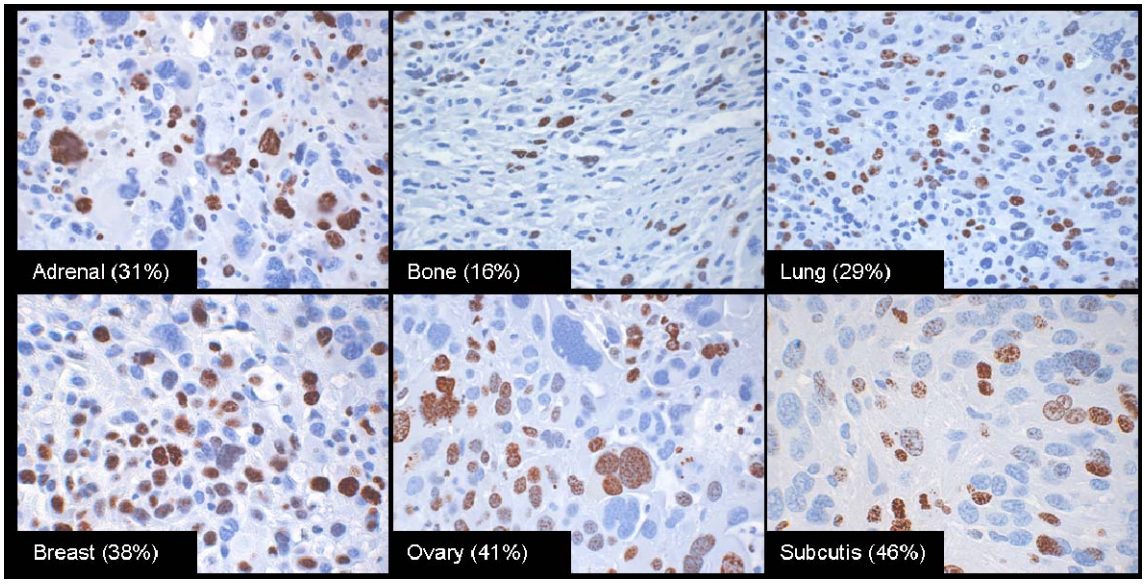
Figure 3. MMTV-PymT cells undergo phenotypic differences at various sites of metastasis.



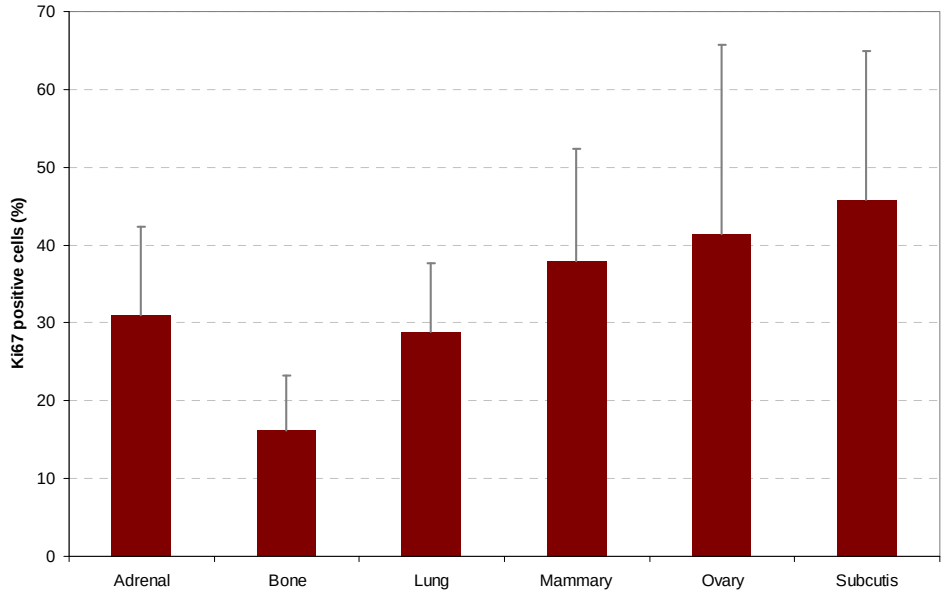
Metastases were detected through gross examination, bioluminescent imaging, and histopathology. Morphologic changes were seen in MMTV-PymT cells depending on the site of growth. Specifically, cells exhibited differences in cell morphology, mitotic index, and percent of necrosis. These findings are significant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

Figure 4. The rate of proliferation of MMTV-PymT cells changes depending on the site of metastasis.

A.

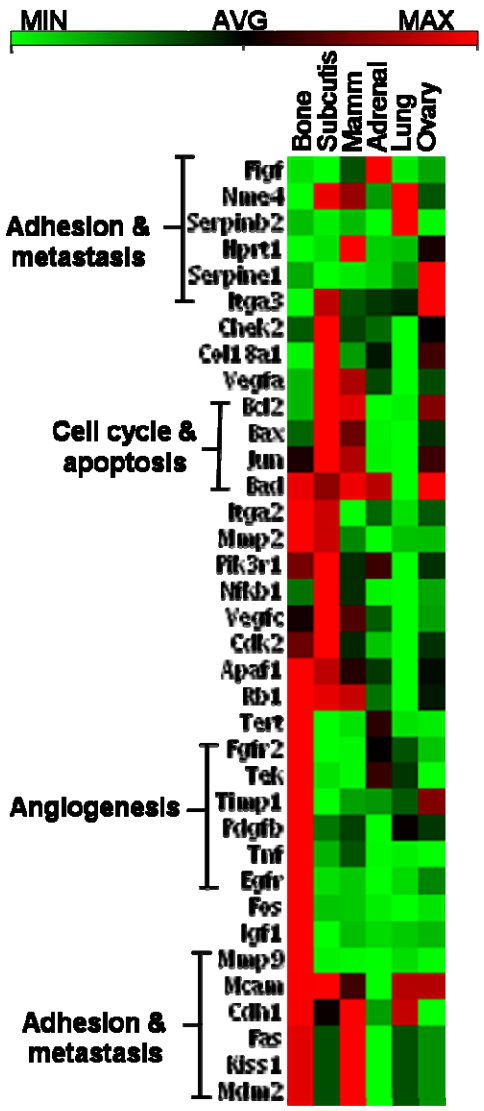


B.



(**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.

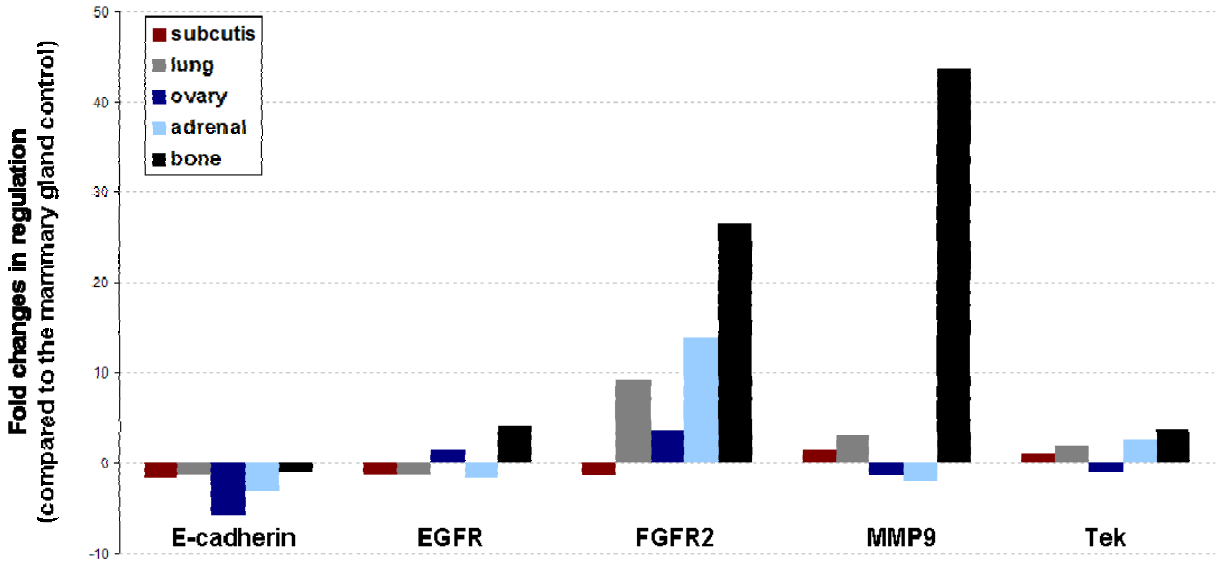
Figure 5. MMTV-PymT cells undergo differential expression of 36 pathway-focused genes depending on the site of growth.



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 (RT^2 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 6. 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).

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