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**CONNEXIN EXPRESSION AND BREAST CANCER  
METASTASIS TO BONE**

A Thesis in

Anatomy

by

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## ABSTRACT

Each year, breast cancer causes over 500,000 deaths worldwide. If caught at early stages, metastasis can often be localized, confined, and treated. However, if time for progression of metastasis has occurred, it spreads to bone and surrounding tissues, and quickly metastasizes throughout the body. Breast cancer, in particular is among the most common to preferentially metastasize to bone, with about 73% of those affected developing bone metastasis as a result of breast cancer. However, at this point we do not fully understand why breast cancer cells preferentially metastasize to bone, and through what exact mechanism this occurs.

Studies have been previously conducted utilizing human breast cancer cells extracted from metastatic mammary tumors as a model. These human studies investigated the connexin and cadherin expression profiles, as well as the metastatic characteristics of migration and adhesion. Studies have also demonstrated that it is possible to inject human cells into a mouse model if the model is in an immunocompromised state. However, the utilization of transgenic models, such as Cx43 deficient mice, presents a problem to this scenario because these models are not from an immunodeficient background. Therefore, it is important to develop an *in vivo* murine model of breast cancer metastasis to bone. However, in order to employ a murine model, it is critical to know the connexin and cadherin expression profiles in mice. The results of the murine studies are very different from those found in human models and, in many ways, actually present opposite results. It is important to consider this when working with mice as a model for breast cancer.

At the initiation of this experiment, it was hypothesized that breast cancer cell lines express different connexin and cadherin expression profiles relative to normal breast epithelial cells. To test this hypothesis the connexin and cadherin expression profiles of murine breast cancer cells (4T1.2 cells) and normal breast epithelial cells (NMuMg cells) were characterized through Western blot and RT-PCR analyses. The results of these experiments supported the hypothesis that murine breast cancer cells (4T1.2) possess a different connexin expression profile than normal murine breast epithelial cells (NMuMG). Regarding connexin 43, though both 4T1.2 and NMuMG cells show slight expression of this gene in comparison to the positive control (MLO-Y4), the 4T1.2 cell line expressed it by almost a 50% greater amount than the NMuMG cell line. Regarding connexin 32, NMuMG cells moderately expressed Cx32 in comparison to the positive control (Mouse Liver cells), while the 4T1.2 cell line does not express this protein.

Regarding, connexin 26, 4T1.2 cells display a greater expression than the NMuMG cell line, but neither cell line displays statistically significant expression of Cx26. The results of these experiments revealed that murine breast cancer cells displayed a different cadherin expression profile relative to normal breast epithelial cells. Regarding, E-Cadherin, the 4T1.2 cell line had an abundance of this protein, but little was detected in the NMuMG cell line. In fact, the 4T1.2 cell line expressed E-Cadherin 2.5 times more than the NMuMG cell line. Regarding, N-Cadherin, the 4T1.2 cell line exhibited great expression of N-Cadherin, while NMuMG showed only trace amounts of expression. Regarding OB-Cadherin, neither the 4T1.2 cell line nor the NMuMG cell line showed expression of this cadherin.

It was also hypothesized that murine breast cancer cells would display greater levels of migration and adhesion than normal murine breast epithelial cells. To test this hypothesis, the migration and adhesion in both of these cell types were quantified. The results of these experiments showed murine breast cancer cells displayed increased levels of adhesion in comparison to normal murine epithelial cells. Regarding adhesion, the NMuMG cell line displayed statistically significant higher levels of adhesion and over three times more adhesion than the 4T1.2 cell line. The results from the migration assays revealed that murine breast cancer cells exhibit greater levels of migration than normal mouse epithelial cells. In conclusion, murine breast cancer cells display decreased levels of adhesion and significantly increased levels of migration relative to normal murine breast epithelial cells. These findings support previous human and murine studies on the metastatic properties of adhesion and migration.

Over the last fifteen years, survival rates among breast cancer patients have steadily increased due to major gains in our understanding of this disease. However, future advances will require a better understanding of the genetics and biology of human breast cancer, which will assist with development of new preventative and therapeutic strategies. Scientific findings must be tested in a setting in which potential clinical responsiveness can be assessed. This requires better *in vitro* and *in vivo* models as well as an understanding of the similarities and differences in expression and metastatic behavior between animal models and human models. This study focused on understanding the characteristics of an established murine breast cancer cell line and a normal murine epithelial cell line through which a highly effective *in vivo* model can be developed.

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## Chapter 1

### INTRODUCTION

#### **1.1 The Clinical Problem**

In 2008, the National Cancer Institute predicted that in the United States alone there were over 211,000 new cases of breast cancer and over 500,000 deaths worldwide as a result. Therefore, one in every nine women will develop breast cancer in their lifetime, and the chances are much greater for those who are genetically susceptible. When breast cancer is confined to the breast, long-term survival rates are high, but when cells metastasize, cure rates drop significantly (Welch *et al.*, 2003). Among breast cancer patients, quality of life is substantially worse for those with metastatic disease as opposed to patients with local carcinoma (Welch *et al.*, 2003).

Breast, prostate, and myeloma tumors are among the most unique of various cancers because of their preferential metastasis to bone. Often, the frequency of metastasis to bone is greater than metastasis to other destinations within the body, such as lung and liver metastases. Around 73% of women develop bone metastasis as a result of breast cancer, while only around 33% develop lung and/or liver metastases. A patient can usually survive a relatively long time with bone lesions, but their quality of life is drastically affected by pain, fractures, spinal cord compression, and metabolic complications. Hypercalcaemia is a common complication as a result of excessive bone resorption, caused by increased numbers of active osteoclasts. This condition can lead to breast cancer patients suffering severe pain and bone fractures (Lelekakis *et al.*, 1999).

Besides the complications surrounding bone metastasis, it also inflicts a momentous economic cost, making up over 2/3 of the cost of breast cancer treatment or around \$3 billion per year (Welch *et al.*, 2003). Increased knowledge of the mechanisms involved in breast cancer has led to several improvements in treatment of skeletal metastasis with drugs, such as bisphosphonates. These drugs are designed to reduce loss of bone, but do not replace bone that is already lost (Welch *et al.*, 2003). As more information is gained about the mechanisms involved in breast cancer, it is hoped that more precise methods of treatment can be developed.

## **1.2 Breast Cancer Metastasis**

Metastasis is defined as the spread of tumor cells to establish a discontinuous secondary tumor mass (Welch *et al.*, 2003). Tumor cells can connect to other tissues by direct extension or transport by means of blood vessels, lymphatics, or epithelial cavities. Tumors that metastasize to bone are believed to spread predominantly through the bloodstream.

In breast cancer patients, large numbers of tumor cells ( $\sim 10^7$  cells/day) enter the bloodstream daily, but only around 0.1% of these cells establish secondary lesions. In order for tumor cells to develop a successful metastatic colony, they must possess very specific survival and proliferative advantages over normal cells, as well as extra properties that allow these cells to spread and colonize secondary sites. Metastasis begins when tumor cells migrate away from the primary tumor and enter the circulatory system. To do this, tumor cells must penetrate the basement membrane and endothelial barrier, as

well as escape innate immune surveillance and sheer mechanical forces associated with powerful blood flow. Tumor cells will then arrest at the secondary site because they are larger than the diameter of the capillary or because of tumor cell - endothelial recognition. After they have stopped moving, the tumor cells will then divide or pass out of the vessel by penetrating the intimal layer through the use of proteolytic and motility mechanisms. For tumor cells to further proliferate, they must respond to local growth factors and be resistant to local growth inhibitors (Welch *et al.*, 2003).

### **1.3 Breast Cancer Metastasis to Bone**

The predisposition of cancer cells to colonize bone is a reflection of cancer cell interaction with the bone microenvironment (Cher *et al.*, 2006). Osseous metastases occur predominantly at the end of long bones, vertebrae, and ribs, near their trabecular metaphyses. The metaphyseal region is characterized by a rich blood supply, large amounts of red bone marrow, and a thick meshwork of trabecular bone. Between the rods of the trabeculae is bone marrow that is in close proximity to the vascular sinusoids, separated by a trilamellar structure. This structure is composed of endothelium, supportive adventitial cells, and a basement membrane (Welch *et al.*, 2003). When metastatic breast tumor cells arrive at bone metaphyses they first interact with the endothelial cells of the sinusoids, which line the vascular system. The slow movement of blood flow through the sinusoids, as opposed to capillaries and venules, may allow time for cell-cell interaction to occur. This suggests that specific recognition properties are involved in tumor cell specificity to bone (Cher *et al.*, 2006).

Once breast carcinoma has entered metaphyseal bone, these tumor cells usually find the environment very agreeable. Bone microenvironment is rich in many growth factors. These growth factors are vital in the normal functions of bone marrow, which include sustaining stem cells and hematopoiesis. Also the continuous remodeling of the bony matrix through the joint efforts of osteoblasts and osteoclasts provides an ideal opportunity for further carcinoma growth. When tumor cells enter the trabecular-marrow space, they disrupt the balance between osteoblasts and osteoclasts, usually resulting in a shift towards net bone degradation (osteoclasts) (Cher *et al.*, 2006).

Though blood flow is important in the metastatic process, additional factors must be critical in determining metastatic affinity for a particular tissue (Donahue *et al.*, 2003). In fact, it is likely that complicated homotypic (tumor cell to tumor cell) and heterotypic (tumor cell to target cell) cellular interactions contribute to intravasation and extravasation. Cell-cell adhesion and cell-to-cell communication via gap junctions are two intercellular interactions that may contribute to metastatic potential (Donahue *et al.*, 2003).

#### **1.4 Gap Junctional Intercellular Communication**

Gap junctions are specialized intercellular connections that function as membrane-spanning channels, allowing the passage of small molecules (<1kD) from one cell to another. The structure of a gap junction is composed of two hexamers or connexons, which are each comprised of six subunits, or connexins (Cx). In mammalian species, there are at least twenty-two different connexins that have been identified as

members of the connexin gene family. Connexins are named specifically according to their molecular weight and they usually weigh between 26 and 60 kDa. Connexins may also combine into homomeric and heteromeric gap junctions, causing them to demonstrate varying functional properties, including: voltage gating, chemical gating, size selectivity, and charge selectivity (Donahue *et al.*, 2003)

Specifically in bone tissue, Cx43, Cx45, and Cx46 are expressed, with Cx43 being the predominant connexin (Donahue *et al.*, 2003). In breast tissue, Cx43 also serves as the principal connexin expressed, but Cx26 and Cx32 may also be expressed under particular circumstances such as pregnancy (Cx26) and lactation (Cx32) (Locke *et al.*, 2000).

In 1966, Loewenstein and Kanno first examined the relationship between gap junctional intercellular communication (GJIC) in normal cells and those in cancerous cells. Their results showed that normal liver cells communicated freely with each other through permeable junctional membranes, while cancerous liver cells showed no communication at all (Loewenstein and Kanno, 1966). Since this discovery, many other studies have linked GJIC and tumorigenesis. For example, GJIC is reduced or absent in many neoplastic cell lines and primary tumors and reparation of tumor cell GJIC slows tumor growth (Eghbali *et al.*, 1991). Also, it has been shown that loss of GJIC corresponds with malignant phenotype progression in neoplastic mammary tissue and these changes in GJIC may be related to changes in cell-cell adhesion (Locke, 1998). This suggests a role for cell adhesion molecules in altered GJIC associated with tumorigenesis.

## **1.5 Cadherins**

Along with defects in gap junctional intercellular communication, cell-cell adhesion is also shown to be decreased in neoplastic cells (Behrens, 1999). Other molecules, including: integrins, selectins, CD44, cadherins, and laminins have been shown to contribute to altered cell-cell adhesion in neoplastic cells. Though it is believed that each of these molecules play a role, E-cadherin, or Epithelial cadherin (Cadherin 1), is most closely associated with altered cell-cell adhesion in neoplastic cells (Behrens, 1999). E-cadherin is a cell-cell adhesion protein that fulfills a prominent role in epithelial differentiation. Data found through various model systems suggests that E-cadherin is powerfully involved in invasion and metastasis and also as a tumor suppressor of breast cancer (Oka *et al.*, 1993). Also, a loss in E-cadherin-mediated adhesion has been shown to play a vital role in the conversion of epithelial tumors from a benign to invasive state (Hazan *et al.*, 2000). Clinically, a partial or complete loss of E-cadherin expression has been found to correspond with poor prognoses in breast cancer patients or induction of invasiveness and metastatic potential (Berx and Van Roy, 2001). Also, down regulation of E-cadherin expression has been found in a number of carcinomas (Behrens, 1999). E-cadherin has been linked with gap junctions in several studies, suggesting that E-cadherin expression and function contribute to GJIC (Oka *et al.*, 1993). With the discovery of mutations in the E-cadherin gene family in breast and gastric carcinomas it has been classified as a tumor suppressor (Behrens, 1999).

Since it has been shown that a loss of E-cadherin function is strongly correlated with invasiveness and metastatic potential, and E-cadherin expression contributes to

GJIC, it is thought that GJIC is involved in invasiveness and metastatic potential. GJIC has also been demonstrated between metastatic tumor cells and vascular endothelium, and is directly related to metastatic potential (El-Sabban, 1994).

More recently, N-Cadherin has also been shown to be an important member of the cadherin family that plays a role in tumor metastasis. In a study completed on a mouse breast cancer cell line (MCF-7) by Hazan *et al.* in 2000, researchers concluded that N-cadherin expression is found in highly invasive tumor cell lines that lacked expression of E-cadherin (Hazan *et al.*, 2000). Other results from this study revealed that N-cadherin supports motility, invasion, and metastasis, even in the absence of E-cadherin (Hazan *et al.*, 2000). It is believed that cell lines expressing N-cadherin may possess a superior ability to penetrate matrix protein barriers, and their marked increase in adherence to endothelium may enhance their capacity to enter and exit the vasculature (Hazan *et al.*, 2000). These two properties of N-cadherin-expressing cells may reveal why they seem to have enhanced metastatic properties. Future studies will reveal more specific details about the role N-cadherin plays in metastasis.

Another cadherin that is believed to play a role in breast cancer's preferential metastasis to bone is OB-cadherin, or osteoblast-cadherin (Cadherin 11) (Okazaki *et al.*, 1994). OB-cadherin is expressed in osteoblastic cell lines, precursor cell lines to osteoblasts, and has also been identified at low levels in lung, testis, and brain tissue (Okazaki *et al.*, 1994). As a result of OB-cadherin expression in osteoblastic cells, it is believed to play an important role in bone formation (Okazaki *et al.*, 1994). Elevated expression of OB-cadherin has also been identified in aggressive human breast cancers



(Tamura *et al.*, 2008). It has been suggested that OB-cadherin promotes tumor homing, migration to bone, and osteoclastogenesis by a mediation of breast cancer cell interaction with bone marrow osteoblastic cells (Tamura *et al.*, 2008). Therefore, it is believed that OB-cadherin enhances bone metastases (Tamura *et al.*, 2008). These results suggest that various forms of GJIC, such as expression of specific cadherins, may contribute to the metastatic potential of cancer cells.

It is believed that heterotypic GJIC between tumor cells and endothelial cells may contribute to the metastatic potential of malignant cells (Donahue *et al.*, 2003). It has recently been demonstrated that breast cancer cells communicate with osteoblastic cells via gap junctions and that connexins are differentially expressed by highly metastatic and less metastatic breast cancer cells (Donahue *et al.*, 2003). At this point there has been a great deal of research focused on the expression of connexins in the process of tumorigenesis, but very few studies have focused on the role of connexin expression in metastasis.

## **1.6 Adhesion & Migration**

The process of metastasis involves a complex series of steps, through which cancer cells leave the primary tumor site, migrate to other parts of the body through the bloodstream or lymphatic system and develop sites of secondary tumor development (Khanna & Hunter, 2005). Many previous studies have stated that the processes of cell-cell adhesion and cell migration play an important role in this process.

Cell adhesion is defined as the binding of a cell to a specific substrate, extracellular matrix, or another cell through the use of cell adhesion molecules, such as cadherins, selectins, or integrins (Mauro *et al.*, 2001). In normal organs, including the mammalian breast, cells have the ability to recognize their neighbor cells through cell-adhesion molecules (Sommers *et al.*, 1991). This recognition of neighboring cells allows the cells to grow, divide, attach to each other in an organized manner, and to receive signals to stop growing (Sommers *et al.*, 1991). In cancerous cell lines, studies have shown that cells have lost this ability to communicate with one another and therefore they begin to grow in a disorganized manner (Moss *et al.*, 1999). Many previous studies have investigated the process of cell-cell adhesion in human and murine breast cancer models. Human studies have found that there is a marked increase in the adhesive strength observed in non-metastatic cell lines and a significant decrease in the adhesive strength in metastatic cell lines (Moss *et al.*, 1999). This decrease in adhesive strength in metastatic cell lines seems to be a direct result of the increased migration of tumor cells from the primary tumor mass (Lin *et al.*, 2000). Murine studies have also shown a pronounced disturbance of intercellular adhesion in metastatic cell lines (Behrens, J., 1993). It has been suggested that this loss of adhesion is accomplished by the loss of expression of the epithelial cell adhesion molecule, E-cadherin and other possible factors (Behrens, J., 1993).

Concurrent with a decrease in cell adhesion is a substantial increase in cell migration among breast cancer cells, away from the primary tumor site (Lin *et al.*, 2000). The process of cell migration is an integral part of the process of metastasis and is

defined as an orchestrated movement of cells in a particular direction and to a specific location (Long *et al.*, 2010). Many previous human studies have investigated the process of cell migration in human cancer models. These studies have shown increased levels of cell migration among human breast cancers (Long *et al.*, 2010) (Lester *et al.*, 2005) and murine breast cancers (Strizzi *et al.*, 2005).

### **1.7 Breast Cancer Studies in Human Models**

Many studies have been conducted utilizing human breast cancer cells extracted from metastatic mammary tumors as a model. Human models seemingly present an ideal environment for carrying out various experiments on breast cancer but have left scientists with numerous failed attempts with both injection into a human model and injection of human breast cancer cells into a mouse model.

Many studies have been conducted characterizing the connexin and cadherin expression profiles as well as the metastatic characteristics of adhesion and migration in various metastatic human cell lines. In 2000, Donahue *et al.* first utilized a human fetal osteoblastic cell line (hFOB) and found that hFOB cells expressed abundant levels of connexin 43 protein, lesser levels of connexin 45 protein, and connexin 26 and connexin 32 were undetected (Donahue *et al.*, 2000). In 2001, Saunders *et al.* utilized a metastatic human breast carcinoma cell line (MDA-MB-435) transfected with breast metastasis suppressor 1 (BRMS1) cDNA (Saunders *et al.*, 2001). In this study it was found that gap junctional cell communication (GJIC) was restored in the human breast carcinoma cell line when transfected with breast metastasis suppressor 1 (BRMS1) and there was a

change in the connexin expression profile (Saunders *et al.*, 2001). With the transfection of BRMS1 into the breast carcinoma cell line, Cx43 expression is increased, while Cx32 expression is decreased (Saunders *et al.*, 2001). This study provided supporting evidence that connexin expression profile and gap junctional communication contribute to breast cancer cell's metastatic potential (Saunders *et al.*, 2001).

In 2004, Kapoor *et al* utilized MDA-MB-435 (435) carcinoma cells, a human osteoblastic cell line (hFOB1.19), and transfection with breast metastasis suppressor 1 (BRMS1) to investigate breast cancer's preferential metastasis to bone (Kapoor, *et al.*, 2004). The results of this study suggest that there are differences in the relative homotypic and heterotypic GJIC of cell lines capable of metastasis and cell lines in which metastasis has been suppressed (Kapoor, *et al.*, 2004).

In 2004, Mastro *et al* utilized human breast cancer cells, MDA-MB-435 (435), MDA-MB-231, and a human osteoblast line (hFOB 1.19) to investigate the interaction of metastatic breast cancer cells with osteoblasts (Mastro *et al.*, 2004). As a result, breast cancer cells demonstrated an increase in the prevalence of apoptotic osteoblasts, showing that osteoblasts are the target of breast cancer cell-induced apoptosis (Mastro *et al.*, 2004).

In 2006, Li *et al* utilized immortalized human fetal osteoblastic cell line (hFOB 1.19) with transfected connexin-43 antisense cDNA (hFOB/Cx43(-)) (Li *et al.*, 2006). In this hFOB/Cx43(-) cell line, lower levels of Cx43 protein were detected, as well as a fifty percent decrease in GJIC relative to the control (Li *et al.*, 2006). This suggests that other connexins, such as connexin-45 may contribute to cell-to-cell communication in hFOB

cells (Li *et al.*, 2006). This study also presented the possibility that connexin-43 expression is independent of cell-to-cell communication, bone cell differentiation, and other influences, such as alkaline phosphatase activity (Li *et al.*, 2006).

In 2008, Li *et al* utilized an aggressive bone metastasis breast cancer cell line, MDA-MET (MET) that was transfected with human Cx43 cDNA (MET/Cx43(+)) (Li *et al.*, 2008). The MET/Cx43(+) cell line demonstrated increased levels of Cx43 and Cx26 protein, while Cx32, Cx37, Cx40, and Cx45 were not detected (Li *et al.*, 2008). This study also tested the cadherin expression profile and found no detection of E-cadherin or N-cadherin, but OB-cadherin protein levels were decreased 43% relative to the control (Li *et al.*, 2008). As a result, this study proposed that expression of Cx43 and GJIC contribute to breast cancer cell adhesion and migration through a mechanism utilizing OB-cadherin and therefore regulating breast cancer cell metastatic potential (Li *et al.*, 2008).

In 2008, Li *et al* published their research on how Cx43 expression reduces metastasis to lungs (Li *et al.*, 2008). In this study a human metastatic breast cancer cell line was utilized (MDA-MB-435), transfected with Cx43 cDNA, and four clones (c1, c6, c8, & c14) were derived from the 435 transfectants (Li *et al.*, 2008). It was found that expression of Cx43 in the 435 cell lines caused a decreased expression of Cx32, decreased growth of 435 cells, decreased expression of N-cadherin, and increased sensitivity to apoptosis (Li *et al.*, 2008). It was also found that there were fewer lung metastases in 435/Cx43(+) cells compared to the control (Li *et al.*, 2008). Therefore, Cx43 expression in breast cancer was shown to decrease metastatic potential through a

mechanism related to N-cadherin expression and apoptosis (Li *et al.*, 2008). Taken together these results suggest that human breast cancer cellular models display decreased levels of Cx43 protein, increased levels of Cx32 protein, decreased levels of Cx26 protein, and decreased levels of E-cadherin and N-cadherin. N-cadherin has also been shown to contribute to the mechanism that decreases metastatic potential and OB-cadherin has been shown to contribute to regulation of breast cancer cell metastatic potential.

### **1.8 Mouse Model as an Alternative to Human Model**

In contrast to the large number of studies that have been carried out utilizing human models for breast cancer, there are relatively few studies that have been carried out investigating connexin and cadherin expression in mouse models.

Many studies have demonstrated that it is possible to inject human cells into a mouse model if the model is immunocompromised. However, the use of transgenic models, such as Cx43 deficient mice, presents a problem to this scenario because these models are not from an immunodeficient background. Therefore, it is important to develop an *in vivo* murine model of breast cancer metastasis to bone. An ideal model would allow tumor cells to disseminate from an orthotopic site to bone, allow proliferation, and induce osteolysis. In order to employ a murine model, it is first critical to know the connexin and cadherin expression profiles of the murine cell lines of interest.

After determining the connexin and cadherin expression profiles in mice, future studies may involve development of mouse models of both wild-type mice and connexin-

specific knock-out mice. Various GFP-expressing (green-fluorescence protein) breast cancer cell lines may then be intracardially injected into these mouse models (Naumov *et al.*, 1999). After time for development of breast cancer cell lines, femurs may then be harvested, dissected, and subjected to fluorescent microscopy. This experimental method will allow comparison of breast cancer metastasis to bone in mice with specific connexins present and mice with specific connexins knocked-out.

## Chapter 2

# OBJECTIVES & HYPOTHESES

### 2.1 Specific Aims and Hypotheses

The specific mechanism behind the preferential metastasis of breast cancer cells to bone is poorly understood. The process of breast cancer cell metastasis to bone requires both transendothelial and transosteoblastic migration. Breast cancer cells extravasate through transendothelial migration, they communicate with osteoblastic bone lining cells, and undergo transosteoblastic migration, prior to interacting with the bone extracellular matrix. Previous studies suggest that Cx43 has been shown to be expressed in breast tissue and bone tissue and GJIC has been shown to be reduced or absent in neoplastic cell lines (Loewenstein and Kanno, 1966).

**Sub-hypothesis 1:** Murine breast cancer cell lines express a different connexin expression profile than normal breast epithelial cells.

**Specific Aim 1:** Characterize the connexin expression profile of 4T1.2 cells relative to normal breast epithelial cells.

**Sub-hypothesis 2:** Breast cancer cell lines express a different cadherin expression profile than normal breast epithelial cells.

**Specific Aim 2:** Characterize the cadherin expression profile of 4T1.2 cells relative to normal breast epithelial cells.



**Sub-hypothesis 3:** The 4T1.2 murine breast cancer cells will display greater levels of migration and adhesion than normal murine breast epithelial cells.

**Specific Aim 3:** Quantify migration and adhesion in both normal murine breast epithelial cells and 4T1.2 murine breast cancer cells.

## Chapter 3

# MATERIALS AND METHODS

### 3.1 CELL CULTURE

#### 3.1.1 Cellular Model (4T1.2 - Mouse Breast Cancer Cell Line)

To develop a murine model of breast cancer and accomplish each individual aim, a new murine breast cancer cell, the 4T1.2 cell line was utilized. The 4T1.2 cell line was courtesy of the lab of Andrea Mastro, Ph.D. at The Pennsylvania State University, University Park. The 4T1 cell line was derived from a spontaneously arising BALB/c mammary tumor and functioned as a syngeneic breast cancer model. 4T1.2 cells have been previously found to metastasize to bone when introduced orthotopically, beginning with a rapid growth at the primary site and metastasizing to lungs, liver, bone, and brain over a period of three to six months. The 4T1.2 cell line represented a highly physiological mouse model, which closely resembles breast cancer in humans. The metastatic properties of this cell line have been extensively characterized and have proven to be reproducible in mouse models. Therefore, this cell model proved to be the most appropriate for research purposes and future studies.

Once the 4T1.2 cells arrived from University Park they were supplemented in media and serum. Specifically, they were cultured in  $\alpha$ -MEM (Invitrogen Catalog #12561-056), which was supplemented with 10% Fetal Bovine Serum and antibiotics (gentamycin & minocycline). The 4T1.2 cell population was expanded for use and several vials were frozen down in liquid nitrogen (-80 degrees C) for future use.

### 3.1.2 Cellular Model (Control - Mouse Epithelium Cell Line)

To serve as a control to the mouse breast cancer cell lines, a normal mouse epithelial cell line (NMuMG) was acquired. This cell line was initiated from the mammary gland tissue of an adult NAMRU mouse and one of a series of epithelial cell lines derived from normal glandular tissue of mice (Sizemore *et al.*, 1982). NMuMg cells express different integral surface proteoglycans and are used for studies of cell adhesion, growth factor-binding molecules, and analysis of growth factor effects on cell proliferation (Sizemore *et al.*, 1982).

### 3.1.3 Other Cell Lines

**Table 3.1.3. Cell Lines Utilized in Experiments.** This table summarizes the various cell lines utilized throughout the experiments which investigated the connexin profile, the cadherin profile, as well as adhesion and migration studies.

<b><u>Cell Line</u></b>	<b><u>Description</u></b>
<b>MLO-Y4</b>	MLO-Y4 was established from transgenic mice and are murine long-bone osteocytes. Compared to osteoblasts, this cell line expresses very large amounts of connexin 43. In these experiments the MLO-Y4 cell line was used as a positive control for Cx43 expression.
<b>MET</b>	The MET cell line is also known as MDA-MET and is characterized as an aggressive human bone metastasis breast cancer cell line. The MET cell line was used in western blot experiments as a negative control for Cx43 and Cx32.
<b>4T1.2</b>	The 4T1.2 cell line is a syngeneic breast cancer model derived from a spontaneously arising BALB/c mammary tumor. 4T1.2 cells have been found to metastasize to bone when introduced orthotopically. The 4T1.2 cell line represents a mouse model that closely resembles breast cancer in humans.
<b>NMuMG</b>	The NMuMG cell line represents a normal murine breast epithelial cell, which initiated from the mammary gland tissue of an adult NAMRU mouse. The NMuMG cell line was utilized as a control.
<b>MC3T3</b>	The MC3T3 cell line represents murine calvarial osteoblasts and is used as a substrate for adhesion studies.
<b>NIH-3T3</b>	The NIH-3T3 cell line represents a mouse embryonic fibroblast cell line and are used as a substrate for adhesion studies.
<b>435</b>	The MDA-MB-435 cell line is a human tumor cell line that has been widely used as a model of human metastatic breast cancer, but recent evidence has shown that it more closely resembles that of a melanoma cell line.
<b>435/BRMS1</b>	The MDA-MB-435/BRMS1(+) cell line is a human breast carcinoma cell line that has been transfected with breast metastasis suppressor 1 (BRMS1), known to cause a change in the connexin expression profile.
<b>Mouse Liver</b>	The mouse liver cell line represents murine hepatocytes and was utilized in these experiments as a positive control for Cx32.

## **3.2 WESTERN BLOT ANALYSIS**

### 3.2.1 Protein Isolation (for Western Blot Analysis)

Before carrying out the Western Blot Analyses, protein was isolated from each cell line to be tested. First, a working buffer solution was prepared, composed of 10 mL 0.1% Triton X100 in dH<sub>2</sub>O (10 mL dH<sub>2</sub>O + 10 ul. Triton X100), 100 ul. Cocktail Set I (Calbiochem #539131), and 100 ul. Tris. EDTA 100 X (SIGMA #T 9285). Media was then aspirated out of 100 mm. dishes of cultured cell lines, cells were washed one time with PBS, and then 1 mL of working buffer was added to each dish to lyse the cells. The cell lysate was then collected using a rubber policeman and transferred to a 1.5 mL. tube. Cells were then broken down by thawing and melting cells three times at -80 degrees C with vortex (or by sonicating the cells at 200 speed, for 10 seconds). Cells were then centrifuged for 30 min at 10,000 rpm, in the cold room, supernatant was removed, and the pellet was resuspended with 500 ul of working buffer. The concentration of each sample was then calculated and 40 ug/tube was measured for use in the Western Blot procedures.

### 3.2.2 Western Blot Procedure

The presence of Cx26, Cx32, and Cx43 protein as well as the various cadherins was evaluated by western blot analysis. To carry out this procedure, various commercially available monoclonal and polyclonal antibodies were utilized. It was a process of trial and error to find which antibodies would reveal the best results.

***Antibodies:***

Finding the correct antibodies to test the cell lines of interest was a long process of trial and error. For each connexin and cadherin, the completed westerns are shown below with details as to the various commercial antibodies utilized.

## Connexin Western Blots

**Table 3.2.2.1. Cx43 Western Blots.** This table summarizes all western blot procedures completed testing expression of Connexin 43. The great number of various antibodies tested verifies that discovering which commercial antibody was most appropriate for each connexin was a true process of trial and error.

<b>Date</b>	<b>Connexin</b>	<b>Company &amp; Catalog Number</b>
2/25/2009	Cx43	Zymed, #13-8300
3/12/2009	Cx43	Zymed, #13-8300
3/18/2009	Cx43	Polyclonal AB – V5 Tag
4/14/2009	Cx43	Zymed, #13-8300, use RIPA lysis buffer to lyse cells
4/22/2009	Cx43	Invitrogen #46-0705, Anti-V5 AB (1:2500)
5/7/2009	Cx43	GenWay, #18-003-42804 – Rabbit Polyclonal AB
5/18/2009	Cx43	GenWay, #18-003-42804 – Rabbit Polyclonal AB
5/26/2009	Cx43	Zymed, #13-8300 – Mouse Monoclonal AB
6/11/2009	Cx43	Replot with Anti-GAPDH (membrane from 5/26/2009)
7/7/2009	Cx43	Cell Signaling, Cat #3512 – Rabbit Polyclonal AB
8/4/2009	Cx43	Zymed #71-0700, Rabbit Polyclonal AB
8/5/2009	Cx43	Cell Signaling, Cat #3512 – Rabbit Polyclonal AB
8/21/2009	Cx43	Cell Signaling, Cat #3512 – Rabbit Polyclonal AB (add 435/BRMS1)
8/28/2009	Cx43	Cell Signaling, Cat #3512 – Rabbit Polyclonal AB (add 435 & 435/BRMS1)
9/2/2009	Cx43	Replot with Anti-GAPDH (membrane from 8/28/2009)
9/11/2009	Cx43	Cell Signaling, Cat #3512 – Rabbit Polyclonal AB – with 435 & 435/BRMS1
11/16/2009	Cx43	Replot with Anti-GAPDH (Cell Signaling AB membrane from 9/11/2009)

**Table 3.2.2.2. Cx32 Western Blots.** This table summarizes all western blot procedures completed testing expression of Connexin 32. There were a great deal less commercially available Cx32 antibodies, but one proved to be successful displaying expression of Cx32. Results were later verified by RT-PCR.

<b>Date</b>	<b>Connexin</b>	<b>Company &amp; Catalog Number</b>
5/18/2009	Cx32	Invitrogen, #71-0600 – Rabbit AB
5/26/2009	Cx32	Terasa (Dr. Li) – Polyclonal Rabbit AB (1:250)
7/7/2009	Cx32	Life Span, #LS-C14540 – Rabbit Polyclonal AB
9/15/2009	Cx32	SIGMA # C-6344

**Table 3.2.2.3. Cx26 Western Blots.** This table summarizes all western blot procedures completed testing expression of Connexin 26. There were a great deal less commercially available Cx26 antibodies and of those tested none were found to display any bands of significance. Connexin 26 results were obtained by RT-PCR as opposed to western blot analysis.

<b>Date</b>	<b>Connexin</b>	<b>Company &amp; Catalog Number</b>
7/7/2009	Cx26	Zymed #33-5800 – Mouse Anti-Cx26 AB
7/7/2009	Cx26	Santa Cruz, #SC-7261-R, Polyclonal Rabbit AB



### **Cadherin Western Blots**

**Table 3.2.2.4. E-Cadherin Western Blots.** This table summarizes all western blots completed testing expression of E-Cadherin. To verify that the band correctly represented E-Cadherin, a Novex Marker was utilized that displayed consistent band migration.

<b>Date</b>	<b>Cadherin</b>	<b>Company &amp; Catalog Number</b>
8/21/2009	E-Cadherin	Rat anti-E-Cadherin (mouse) – Invitrogen #13-1900 (1:1000)
8/28/2009	E-Cadherin	Rat anti-E-Cadherin (mouse) – Invitrogen #13-1900 (1:1000)
9/2/2009	E-Cadherin	Replot with anti-GAPDH (membrane from 8/28/2009)
9/11/2009	E-Cadherin	Rat anti-E-Cadherin (mouse) – Invitrogen #13-1900 (1:1000) -add Novex Marker
11/16/2009	E-Cadherin	Replot with anti-GAPDH (membrane from 9/11/2009)

**Table 3.2.2.5. N-Cadherin Western Blots.** This table summarizes all western blots completed testing expression of N-Cadherin. To verify that the band correctly represented E-Cadherin, a Novex Marker was utilized that displayed consistent band migration.

<b>Date</b>	<b>Cadherin</b>	<b>Company &amp; Catalog Number</b>
8/21/2009	N-Cadherin	Mouse anti-N-Cadherin – Invitrogen #33-3900 (1:1000)
9/2/2009	N-Cadherin	Replot with Anti-GAPDH (membrane from 8/21/2009)
9/11/2009	N-Cadherin	Mouse anti-N-Cadherin – Invitrogen #33-3900 - add Novex Marker
11/16/2009	N-Cadherin	Replot with anti-GAPDH (membrane from 9/11/2009)

**Table 3.2.2.6. OB-Cadherin Western Blots.** This table summarizes all western blots completed testing expression of OB-Cadherin. To verify that the band correctly represented E-Cadherin, a Novex Marker was utilized that displayed consistent band migration.

<b>Date</b>	<b>Cadherin</b>	<b>Company &amp; Catalog Number</b>
8/28/2009	OB-Cadherin	Mouse anti-Cadherin 11 (OB Cadherin) – Invitrogen #32-1700
8/28/2009	OB-Cadherin	Replot with Anti-GAPDH (membrane from 8/28/2009)
9/11/2009	OB-Cadherin	Mouse anti-Cadherin 11 (OB Cadherin) – Invitrogen #32-1700 -add Novex Marker
11/16/2009	OB-Cadherin	Replot with anti-GAPDH (membrane from 9/11/2009)

### **Western Blot: Procedures and Protocols**

#### *Running Western Blot Gel*

To carry out Western Blot analyses, pre-case ready gels from Invitrogen (NuPAGE Novex 4-12% Bis-Tris Gel, 1.5mm, 10 well – Invitrogen Cat #NP0335 BOX) were used. Enough running buffer solution (NuPAGE MOS SDS Running Buffer (20X) – Invitrogen Cat #NP0001) was added to completely cover the gel.

Samples (concentration of 40 ug/tube) were removed from freezer and thawed on ice. A total volume of 40 ul was prepared by adding 10 ul of 4x sample buffer (NuPAGE LDS Sample Buffer (4x) – Invitrogen #NP0007) and 4 ul of 10x sample reducing agent (NuPAGE Sample Reducing Agent (10x) – Invitrogen #NP0004) to the samples. Each sample was then vortexed to mix well.

In first well of gel, 5 ul of 1x protein marker (SeeBlue Plus 2 Prestained Standard (1X) – Invitrogen #LC5925) was added, followed by each subsequent sample (if volume was more than 40 ul – sample was vacuum-dried to a smaller volume before mixing with sample buffer). The gel was then run at 100 V. for around 1.5 hours or less time depending on how quickly the samples traveled to the bottom of the gel.

#### *Western Blot: Transfer Gel to Nitrocellulose Membrane*

After the gel was finished running, it was transferred to a membrane for further analysis. An appropriately-sized piece of nitrocellulose membrane (Pure Nitrocellulose Membrane (0.45 um) 30cm. X 3.5 m. roll – Bio-Rad #162-0115) was placed directly on the gel, and a glass stirring rod was used to get rid of any bubbles that may have appeared between the gel and membrane. Pieces of 3 mm. paper on top of the membrane and wet sponges were added on each side of the membrane and the transfer holder was carefully closed. Transfer holders were then placed into the transfer chamber and around 1 L of blot transfer buffer was added to the transfer chamber (NuPAGE Transfer Buffer (20x) – Invitrogen Cat #NP0006-1). The gel was transferred to the membrane at 20 V/gel in cold room overnight (40 V/2 gels).

#### *Blotting Membranes*

TTBS solution (1 L) was prepared by mixing 900 mL dH<sub>2</sub>O, 100 mL 10X TBS (Bio-Rad Cat #170-6435), and 500 ul. Tween-20 (Bio-Rad #170-6531), and mixing well. The next morning, the membrane was removed from the transfer chamber and blotted

with 1X TTBS in 5-10% milk for 1 hour at room temperature, with shaking. After blotting for one hour, the membrane was washed for 5-10 minutes, three times, with 1X TTBS at room temperature.

#### *Treating Membranes with Antibodies*

The first antibody was then added (for appropriate connexin or cadherin being tested) at 1:1000, in 5% milk TTBS, and incubated in a plastic seal-meal bag overnight in cold room. The next morning, the membrane was removed from the seal-meal bag and washed for 5-10 minutes, three times, with 1X TTBS at room temperature. Then membrane was incubated with second antibody at 1:3000 (or 1:4000) in 5% milk TTBS for 1 hour at room temperature, with shaking. After second antibody was incubated for one hour, it was washed three times, for 5-10 minutes, with 1X TTBS at room temperature.

#### *Treat Membrane with ECF*

Next the membrane was treated with ECF and around 2 mL of reagent (ECF Western Blotting Reagent packs – GE Healthcare, Amersham Cat# RPN 5781 (mouse)) was added to a film. The membrane was then placed face down onto the reagent and not disturbed for 1 minute. The membrane was then placed face down (with marker on left hand side) onto the scanning glass of the Typhoon scanner.

### *Quantification of Results using Typhoon Machine*

To obtain images of the western blot membranes the Typhoon scanner (The Pennsylvania State University, Penn State College of Medicine – Floor 4 Lang Lab), was utilized. Appropriate settings were selected, including: 580 BP30 cy 3 TAMRA, AleMFluo546, PMT 375, Laser Blue2(488). Images were then viewed and results and statistical analyses were obtained through use of the “Image Quant 5.2” program.

### **3.2.3 Re-Probing with Anti-GAPDH**

After the completion of running a gel, transferring a gel to a membrane, blotting and treating the membrane with primary and secondary antibodies, and quantifying the results of the membrane, it was important to normalize the membrane with GAPDH antibody. To begin this procedure, first stripping solution (10 mL 20% SDS, 700 ul 2-Mercaptoethanol, 6.19 mL 1 M Tris-HCL (pH 6.7), and 84 mL dH<sub>2</sub>O) was prepared inside a fume hood and the thawed membrane was incubated in 100 mL stripping solution for 30-60 minutes at 50 degrees C, and with shaking (Belly Dancer Machine).

After the membrane was stripped, a standard western blot was then performed with the anti-GAPDH antibody. First, the membrane was washed two times (10 minutes) with 15 mL TTBS, at room temperature. Then the membrane was blotted in Blotto (TTBS in 5% milk) for one hour at room temperature. Following the blotting, the membrane was washed two times (10 minutes) with TTBS, at room temperature. Next, the membrane was incubated with monoclonal anti-GAPDH at 1:1000 in 10 mL Blotto (TTBS in 5% milk) for one hour, at room temperature, then the membrane was rinsed

three times with TTBS. The membrane was then incubated with the second antibody, utilizing the standard mouse AP (monoclonal AB) or rabbit AP (polyclonal AB) and washed three times in TTBS. A standard ECF assay was then performed on the membrane and it was scanned utilizing the Typhoon scanner.

### **3.3 Quantitative Real Time RT-PCR**

#### **3.3.1 Isolation of Total RNA**

RNA was isolated from cells through the use of the RNeasy Mini Kit from Qiagen Inc. (Cat #74104 – 50 tubes). Before carrying out the procedure, RLT and RPE solutions were prepared. For RLT, 1000  $\mu$ L RLT and 10  $\mu$ L  $\beta$ -Mercaptoethanol were added and thoroughly mixed. For RPE, 1 vol. RPE, 4 vol. 96-100% Ethanol were added and thoroughly mixed. Both solutions were stored at room temperature.

To carry out RNA isolation procedure, media was aspirated out of culture dishes, cells were washed with 1X PBS, and 700  $\mu$ L of Buffer RLT (with  $\beta$ -Mercaptoethanol already added) was added. Cell lysate was then collected with a rubber policeman, transferred to a 1.5 mL tube, and vortexed to mix cell lysate (no clumps should be visible). The sample was then homogenized by passing the sample five times through a 20-G needle with a 1 mL syringe and 350  $\mu$ L 70% Ethanol was added to the solution. The sample was then added to a RNeasy mini spin column, sitting in a 2 mL collection tube. Columns were centrifuged for 15 seconds at 10,000 rpm and flow-through was discarded. Next, 700  $\mu$ L of Buffer RW1 was added onto the RNeasy column, centrifuged for 15

seconds at 10,000 rpm, and flow-through was discarded. Then the RNeasy column was transferred to a fresh 2 mL collection tube, 500 ul buffer RPE was added to the RNeasy column, and the column was centrifuged for 15 seconds at 10,000 rpm. Next, 500 ul buffer RPE was added to the RNeasy column and was then centrifuged for 2 minutes at maximum speed to dry the RNeasy column. The RNeasy column was then transferred to a new 1.5 collection tube and 30-50 ul of RNase-free water was placed directly onto the RNeasy column. The column was then centrifuge for 1 min at 10,000 rpm to elute the RNA.

*Measure RNA Concentration using UV Spectrophotometer:*

To measure the concentration of RNA, I utilized a UV Spectrophotometer, courtesy of The Pennsylvania State University, Penn State College of Medicine, Timothy Ritty, Ph.D. Lab. Concentration was measured by adding 2 ul. of sample to 98 ul. of dH<sub>2</sub>O in a 0.5 mL tube, and was mixed thoroughly by pipetting up and down. Distilled H<sub>2</sub>O was used as the blank control and UV light of 260λ to 280λ was utilized.

After UV Spectrophotomer had provided all sample values of absorbency and 260/280 ratio, calculations shown below were performed to obtain the true concentration and appropriate amounts of sample and distilled water (total volume of 100 ul per vial). Necessary quantities of RNA sample vials were prepared and stored in -80 degrees C for future use in RT-PCR procedures.

$$\frac{\text{OD}_{260 \lambda} \# \times 40 \text{ ug/ml}}{1000} \times \text{dilution\# (50)} = \text{ug/ul}$$

**Table 3.3.1. RNA Concentrations for RT-PCR.** The UV spectrophotometer was utilized to obtain the OD and 260/280 values. From these values proper amounts of sample and distilled water were calculated based on the RNA concentration. Each trial of RNA isolation is shown below by date

**9-30-09**

RNA Isolation for RT-PCR			ug/ul		Sample	Distilled Water
Sample	OD	260/280	concentration	ng/ul	200ng/20ul	(ul)
MC3T3	0.264	1.8603	0.528	528	1.893939394	98.10606061
MET	0.1452	1.883	0.2904	290.4	3.443526171	96.55647383
NIH-3T3	0.2058	1.8385	0.4116	411.6	2.429543246	97.57045675
435	0.1367	1.7873	0.2734	273.4	3.657644477	96.34235552
MLOY4	0.0682	1.9525	0.1364	136.4	7.331378299	92.6686217
NMuMG	0.0548	1.9157	0.1096	109.6	9.124087591	90.87591241
4T1.2	0.1397	1.8893	0.2794	279.4	3.579098067	96.42090193

**10/6/2009**

RNA Isolation for RT-PCR			ug/ul		Sample	Distilled Water
Sample	OD	260/280	concentration	ng/ul	200 ng/20ul	(ul)
4T1.2	0.1454	1.8679	0.2908	290.8	3.438789546	96.56121045
NMuMG	0.124	1.9514	0.248	248	4.032258065	95.96774194

**10/27/2009**

RNA Isolation for RT-PCR			ug/ul		Sample	Distilled Water
Sample	OD	260/280	concentration	ng/ul	200 ng/20ul	(ul)
4T1.2	0.1212	2.043	0.2424	242.4	4.125412541	95.87458746
NMuMG	0.0932	2.2164	0.1864	186.4	5.364806867	94.63519313

**1/11/2010**

RNA Isolation for RT-PCR			ug/ul		Sample	Distilled Water
Sample	OD	260/280	concentration	ng/ul	200 ng/20ul	(ul)
4T1.2	0.3109	1.9148	0.6218	621.8	1.608234159	98.39176584
NMuMG	0.3179	1.9116	0.6358	635.8	1.572821642	98.42717836
MET	0.1367	1.9707	0.2734	273.4	3.657644477	96.34235552

**1/15/2010**

RNA Isolation for RT-PCR			ug/ul		Sample	Distilled Water
Sample	OD	260/280	concentration	ng/ul	200 ng/20ul	(ul)
4T1.2	0.2626	2.0239	0.5252	525.2	1.904036558	98.09596344
NMuMG	0.2083	2.0271	0.4166	416.6	2.400384061	97.59961594
MLOY4 (1)	0.0944	2.0616	0.1888	188.8	5.296610169	94.70338983
MLOY4 (2)	0.0621	1.9817	0.1242	124.2	8.051529791	91.94847021



### **3.3.2 Quantitative Real Time RT-PCR**

As a means to examine the steady-state levels of Cx26, Cx32, and Cx43 mRNA and to verify the results found through Western blot analysis, quantitative real time RT-PCR was carried out. This procedure accurately quantified the results with an effective technique, used to assess mRNA.

Before carrying out this procedure, RNA was isolated from cells using a commercially available RNase Easy kit (from QIAGEN, Inc.). In brief, the cells will be lysed, scraped, homogenized, and passed through columns that selectively bind RNA, and total RNA was then eluted from the column. Refer to Chapter 3.3.1 for further detail on this procedure.

#### *DNASE Samples*

To begin RT-PCR procedure, all samples underwent a DNASE procedure. The appropriate components listed below were added to a 0.2 mL PCR tube.

#### ***DNase Mix:***

2 ul of total RNA (20 ng concentration)
2 ul 10X Buffer (ABI #AM8170G)
2 ul DNASE (Ambion PN #2222) in a 1 to 10 dilution
6.67 ul of yeast tRNA (ABI #AM7119) (100 ng/ul)
7.33 DEPC water (Ambion)
20 ul Final Volume of DNase Mix

After adding each of the above components, each sample was placed in the PCR machine and thermocycled using the “DNase program settings” (30 minutes at 37 °C, 5

minutes at 65 °C, & then ramping down to 4 °C). After this process, the samples were immediately removed and placed on wet ice.

### *Reverse Transcription (RT)*

In order to carry out the reverse transcription procedure, I utilized the ABI High Capacity RT kit (PN #4368814). The appropriate components listed below were placed in a 1 mL microvial to make the 2X RT Master Mix. Once all the components were added to the Master Mix, it was mixed gently by pipetting up and down and then placed on ice until use.

#### ***Components of 2X RT Master Mix: (ABI #4390777)***

10X RT Buffer	4.0 ul
25X Dntp Mix (100Mm)	1.6 ul
10 X RT Random Primers	4.0 ul
MultiScribe Reverse Transcriptase	2.0 ul
<u>Nuclease-free H2O</u>	<u>8.4 ul</u>
Total per Reaction	20 ul

#### **Components of 1X cDNA Reverse Transcription Reaction:**

2X RT Master Mix	20 ul
DNased RNA Sample	20 ul

After preparing the 1X cDNA RT reaction mix by combing equal amounts of the 2X RT Master Mix and DNased RNA samples, the samples were placed in the PCR machine to thermocycle using “Reverse Transcription” (RT) settings (10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C, & the remainder of the cycle at 4 °C). After this thermocycle the samples were immediately removed and placed on wet ice. At this

point, the procedure could be continued on to the Real Time RT-PCR procedure or stopped for future completion and storage of the cDNA samples at -20 degrees C.

### *Real Time RT-PCR*

The resulting cDNA mixture from the reverse transcription was utilized in the real time RT-PCR reaction in 5.0 ul amounts. Appropriate amounts of RT-PCR reaction mix were calculated depending on the number of samples and genes being tested. A 384 well plate was used to carry out this procedure.

#### ***Real Time RT-PCR Reaction Mix:***

<b><i>96 Well Plate</i></b>	<b><i>384 Well Plate</i></b>	
25 ul	10 ul	TaqMan 2X Universal Mix (ABI #4324018)
1.0 ul	0.4 ul	Forward Primer (10 uM)
1.0 ul	0.4 ul	Reverse Primer (10 uM)
2.5 ul (or 5.0 ul)	1.0 ul	Target Probe (1 uM)
13 ul	2.2 ul	dH <sub>2</sub> O
5 ul	5 ul	cDNA
2.5 ul	1.0 ul	20X 18S specific gene Control Primers & Probe (separate vials) (ABI #4319413E)
<b>50 uls</b>	<b>20 uls</b>	<b>FINAL VOLUME</b>

In each RT-PCR well, 15 ul of appropriate gene's reaction mix and 5 ul of cDNA was added. After all samples were added to plate, wells were sealed with a clear film, and plate was immediately wrapped with tin foil to protect fluorescence. The plate was then stored in a standard refrigerator until RT-PCR thermocycle (2 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C, 1 minute at 60 °C, 2 minutes at 25 °C) was carried out.

Steady-state mRNA in cells were quantified by real-time RT-PCR using the Perkin-Elmer ABI7700 system at the Penn State Nucleic Acid Core Facility. This technology quantified PCR products in real-time using a custom designed fluorogenic oligonucleotide probe for the 5' nuclease assay with the TaqMan kit (Perkin/Elmer) during PCR amplification. Real-time probe/primers have been previously designed, using Primer Express Software (V1.0 PE Biosystems) by other members of the Donahue lab and were used in my RT-PCR experiments.

After Real Time RT-PCR results were gathered, results were quantified in SDS2.2.2 software by carrying out a relative quantification study. Results were then analyzed and later files were imported into Excel for further analysis.

### **3.4 Migration Assay**

In order to carry out the migration assay, the CyQuant NF cell proliferation assay kit (Invitrogen #C35006) was used. To obtain reliable results, it was vital to first grow the appropriate cells types to confluency (usually cells were cultured about 3 days before use for migration assay) in 30 mm culture dishes and then the cells were stored in the incubator until use. Cell culture media must also be supplemented with FBS, serum free media, 0.25% Trypsin-EDTA, 0.05% Trypsin-EDTA, components for lysis buffer (0.05% Triton 100x & Tris EDTA (Sigma #T9285), and cell culture inserts for a 24 well plate (Becton Dickinson #353097 (8 um) & #353096 (3 um)). The procedure of migration assay was dependent on fluorescence and therefore it was important to be

prepared with sheets of tin foil to cover microvials and plates while they contain fluorescent dyes.

To begin the migration assay, cells were collected from a dish by first removing the media, washing the cells once with PBS, followed by trypsinizing the cells with 1 mL of 0.25% Trypsin-EDTA. Cells were then left undisturbed in a 37 °C incubator for around one minute to fully trypsinize. Culture dishes were then removed from incubator and 5-9 mL of culture media (supplemented with FBS) was added to neutralize the Trypsin-EDTA. Cells were then resuspended, collected in a 15 mL tube, and centrifuged at 750-1000 rpm for 5 minutes. The present media was then removed, and 5-9 mL of plain serum-free media was added to re-suspend cells. A hemacytometer was then used to count the cells.

Next the cells were prepared for migration by bringing them to a desired concentration of  $20 \times 10^4$  / ml for 4 mL in plain serum-free media. The cover of a new 24 well plate was then labeled with what will be in each well and cell culture inserts were placed (Becton Dickinson #353097) into appropriate wells. Then 500 ul of appropriate cell suspension was inserted into insert wells, in triplicate and afterwards 600 ul of chemoattractant media (supplemented cell culture media) was added to the bottom of the well. The lid was then placed back on the 24-well plate and the plate was returned to the incubator. The cells were permitted to culture for 20-22 hours in a 37 °C incubator.

After 20-22 hours had passed, a fresh 24 well plate was prepared with 300 ul of 0.05% Trypsin-EDTA to each appropriate well. Remaining suspension was carefully aspirated from each transwell insert, being sure not to touch the membrane in the bottom

of the insert. Transwells were then placed into the new 24 well plate containing the 0.05% Trypsin-EDTA. Cells were incubated in a 37 °C incubator for 30 minutes. While cells were incubating, lysis buffer was prepared by adding the lysis buffer/dye mixture in a ratio of 22 ul Cyquant dye to 5.5 mL lysis buffer (10 mL 0.05% Triton 100X and 100 ul Tris EDTA (Sigma #T9285)). In a mini vial 500 ul of above lysis buffer solution and 2 ul Cyquant dye was placed and the vial was immediately covered with tin foil to protect fluorescence. The mini vial was then vortexed to thoroughly mix the dye solution.

After the 30 minute incubation period ended, plastic scissors or forceps were utilized to rock inserts back and forth in the wells with 0.05% Trypsin-EDTA. This process ensured that all cells had been removed from the inserts. The inserts were then disposed of and 70 ul of dye mixture was added to each well. To make sure that dye was mixed thoroughly, the mixture was repeatedly pipetted up and down. After dye mixture was added and mixed throughout well, the plate was immediately covered with tin foil to protect from light. The cells were then left undisturbed for 20 minutes at room temperature. Fluorescence was then measured using the fluorescence reader at 480/530 nm. Once results have been obtained from the fluorescence reader, data was further statistically analyzed.

### **3.5 Adhesion Assay**

Two different forms of adhesion assays were completed, including: adhesion of cells on a MC3T3 substrate, or mouse osteoblastic cell line, and adhesion of cells on a NIH-3T3 substrate, or a mouse fibroblast cell line. The adherence of the 4T1.2 and

NMuMG cells lines were the two cell lines utilized to test adherence to these differing substrates.

On day one of the adhesion assay, confluency was checked in various cell concentrations. Media was then aspirated out of each dish, cells were washed once with PBS, 1 mL 0.25% Trypsin-EDTA was added to each dish, dishes were placed in incubator for about 1 minute, trypsin was neutralized with 5-9 mL culture medium, the solution was transferred to 15 mL tube, and solution was centrifuged at 750-1000 rpm for 5 minutes. The media was then removed and 5-9 mL of cell culture media was added to re-suspend cells. A hemacytometer was utilized to count the cell concentrations. After having completed cell counts, the appropriate amount of cells were calculated to be added to each well of a 24 well plate in a  $5 \times 10^4$  / well concentration. The lid of a fresh 24 well plate was then labeled with appropriate contents for wells. Then  $5 \times 10^4$  / well concentration was added to appropriate wells and culture media was added to bring to a total volume of 1 mL per well. This 24 well plate was then in a 37 °C incubator and cells were allowed to culture for 3-4 days.

In the same manner on day one, tumor cells were prepared for use by aspirating media out of each dish, washing cells once with PBS, adding 1 mL 0.25% Trypsin-EDTA to each dish, placing dishes in incubator for about 1 minute, neutralizing the trypsin with 5-9 mL culture medium, and transferring solution to 15 mL tube. Then tubes were centrifuged at 750-1000 rpm for 5 minutes. The media present was then removed and 5-9 mL of cell culture media was added to re-suspend cells. A hemacytometer was used to count the cells. After having completed cell counts, the appropriate number of cells were

calculated to be added to fresh 30 mm dish in a concentration of  $0.5 \times 10^6$  / dish. These culture dishes were then placed in a 37° C incubator and cells were allowed to culture for 3-4 days.

On day four, confluency of all cells in 24 well plate and culture dishes was checked before proceeding with the experiment. Then CFDA dye was prepared by first removing DMSO and CFDA dye from freezer and letting them thaw out. It is important that CFDA dye vial remained covered at all times with tin foil to protect fluorescence from light. After these solutions thawed, 90 ul DMSO was added into 1 vial of CFDA SE (Molecular Probe #V-12883), giving a 10 mM concentration. For two cell types, 10 ul CFDA dye was added to 10 mL PBS and the vial was vortexed to mix the dye solution. The cells were then removed from the incubator, the medium was aspirated from each dish and the cells were washed one time with PBS. After aspirating PBS, 5 mL of CFDA dye solution was added to each dish. Immediately the dishes were covered with tin foil and returned to a 37 °C incubator for 15 minutes. After 15 minutes had passed, dishes were removed from incubator, dye solution was aspirated, and 10 mL of appropriate media was added to each dish. These dishes were then recovered with tin foil and incubated for 30 minutes. After 30 minutes, dishes were removed from incubator, medium was aspirated, cells were washed once with PBS, 1 mL 0.25% Trypsin-EDTA was added to each dish, dishes were placed in the incubator for about 1 minute, trypsin was neutralized with 5-9 mL culture medium, and the solution was transferred to a 15 mL tube. Next, the tubes were centrifuged at 750-1000 rpm for 5 minutes and the media was removed, without disturbing the pellet of cells on the bottom of the 15 mL tube. Then 5-



9 mL of cell culture media was added to re-suspend cells. A hemacytometer was then utilized to count the cell concentrations.

After counting cells,  $50 \times 10^4$  cells / well labeled tumor cells were calculated and the appropriate amounts were added onto the cell type in the wells of the 24 well plate (ex. MC3T3, NIH-3T3, hFOB). The plate was then covered with tin foil and placed in a 37 °C incubator for 2 hours. After 2 hours passed, plates were removed from incubator, media was aspirated in each well, each well was washed three times with PBS, and wells were allowed to remain about 50% filled with PBS. Results of adhesion assay were read using a Fluorescence Reader at 485/530 nm. Once we had obtained results from fluorescence reader, data was then further statistically analyzed.

### **3.6 Statistical Analysis of Data**

All raw data in this thesis were statistically evaluated and expressed as mean  $\pm$  standard error (SE) and standard deviations. In the case that a specific experiment contained only two groups of data, a standard t-test was utilized for comparison of data. To compare multiple groups of data (more than two) when standard terms of normality appear to be met, I utilized a one-way analysis of variance test (ANOVA), which was followed by a Tukey post-hoc comparison test (Minitab; Minitab LTD, New York, NY). A value of  $p < 0.05$  was considered statistically significant.

All data were virtually represented using graphs and boxplots. All analyses were carried out using Minitab (Version 15) statistical software system and Microsoft Excel

## Chapter 4

### RESULTS

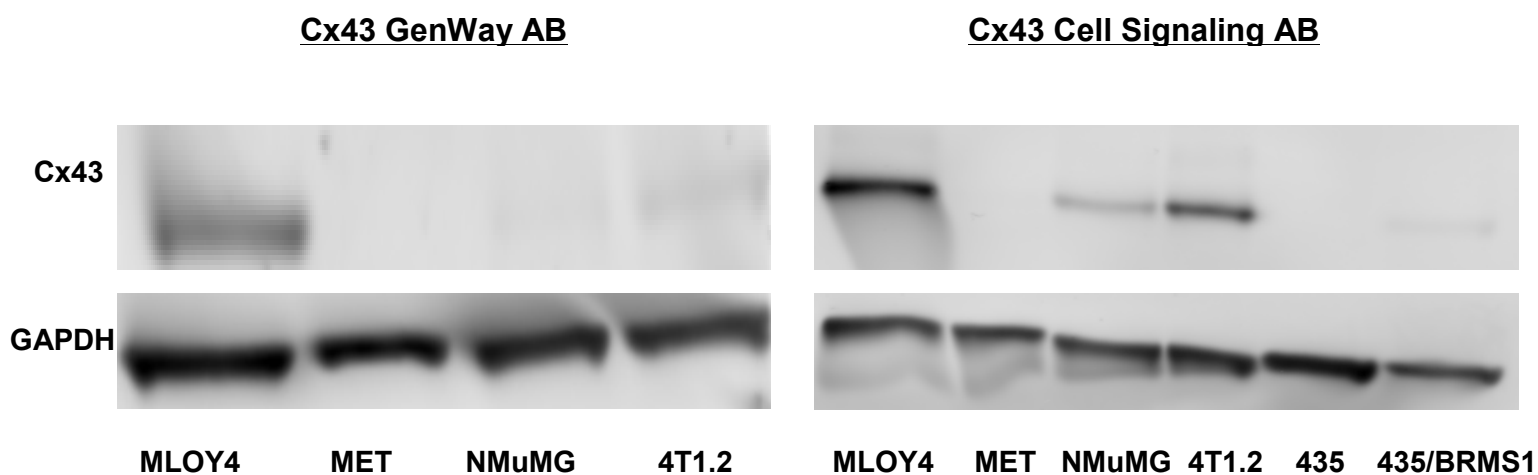
#### **4.1 CONNEXIN EXPRESSION PROFILE**

At the initiation of this experiment, I hypothesized that breast cancer cell lines will express a different connexin expression profile than normal breast epithelial cells and to confirm or deny this hypothesis I characterized the connexin expression profiles of murine breast cancer cells (4T1.2 cells) and normal breast epithelial cells (NMuMg cells). To analyze the expression of Cx43 in these cell lines, I carried out over eighteen western blot analyses testing various antibodies for appropriately displaying clear bands from which I could draw conclusions based on Connexin 43 expression. Two of my best western blots were found by utilizing two repeatedly tested antibodies, including: the Cx43 Gen Way AB (Cat #18-003-42804) and the Cx43 Cell Signaling AB (Cat #3512). Results from both western blots were normalized against GAPDH expression. Results were characterized using ImageQuant Program and Excel and visually expressed in the form of bar graphs.

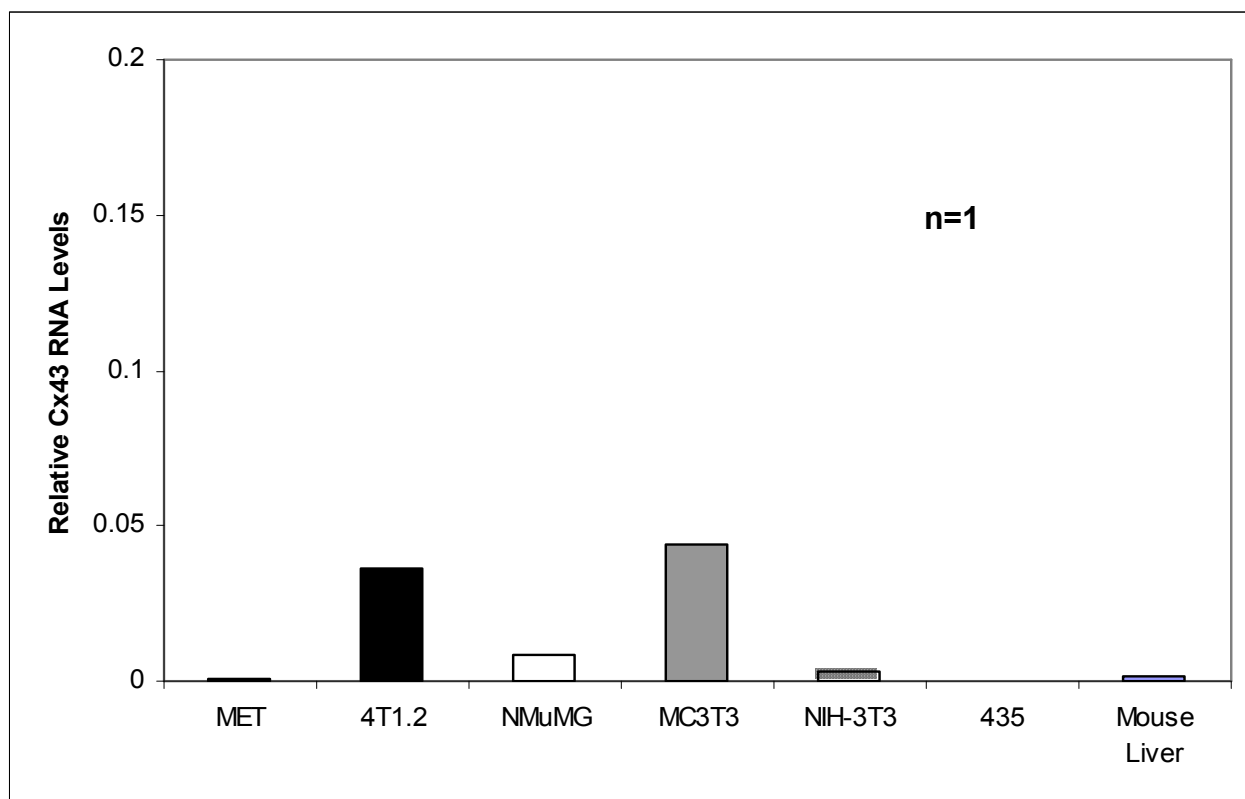
MLO-Y4 was used in both western blot analyses as a known positive control and the MET cell line was utilized as a known negative control that does not express Cx43. Cx43 was found to be moderately expressed in the 4T1.2 mouse breast cancer cell line and markedly less expressed in NMuMG, normal mouse breast epithelial cells. Cx43 was not expressed in the 435 cell line, but was slightly expressed when BRMS1 gene was added to 435 cell line.

#### 4.1.1. Cx43 Expression

**Figure 4.1.1. Cx43 Protein in 4T1.2 & NMuMG Cells.** Through Western Blot Analysis with Cx43 antibodies from GenWay and Cell Signaling and normalizing with GAPDH, Cx43 expression was evaluated in various relevant cell lines. Cx43 was found to be highly expressed in MLO-Y4, which was used as the positive control and was not expressed in the MET cell line, which was used as the negative control. It was also moderately expressed in the 4T1.2 mouse breast cancer cell line and markedly less expressed in NMuMG, normal mouse breast epithelial cells. Cx43 was not expressed in the 435 cell line, but was slightly expressed when BRMS1 gene was added to 435 cell line. There were over 8 western blots carried out using these two different antibodies. The results shown below are representative of the other gels.



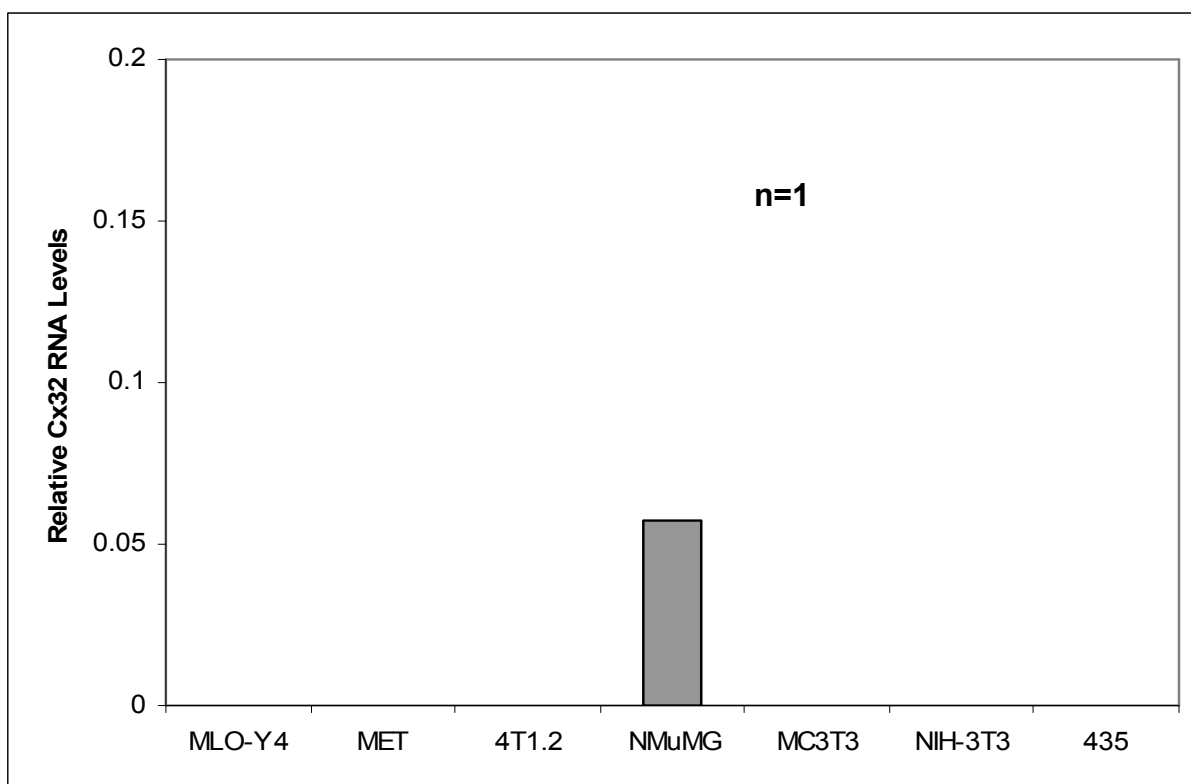
**Figure 4.1.2 Cx43 Expression in 4T1.2 & NMuMG Cells shown in RT-PCR.** This figure summarizes the Cx43 Real Time RT-PCR results. From previous studies, it was known that the MLO-Y4 cell line highly expresses Cx43 and therefore MLO-Y4 was used as a positive control (not shown). All samples were normalized with 18S control probes. Expression of Cx43 is greatest in the MC3T3 cell line, followed by slightly less expression in the 4T1.2 mouse breast cancer cell line. Minimal expression is shown in NMuMG, NIH-3T3, Mouse Liver, MET, and 435 cell lines.



#### 4.1.2. Cx32 Expression

To characterize the connexin expression of connexin 32, there are far less commercial antibodies available for purchase. In the process of trial and error to see which Cx32 antibody best displayed the bands on the membrane, I tested four different antibodies and only found reliable results from the SIGMA (#C-6344) antibody tested on 9/15/09.

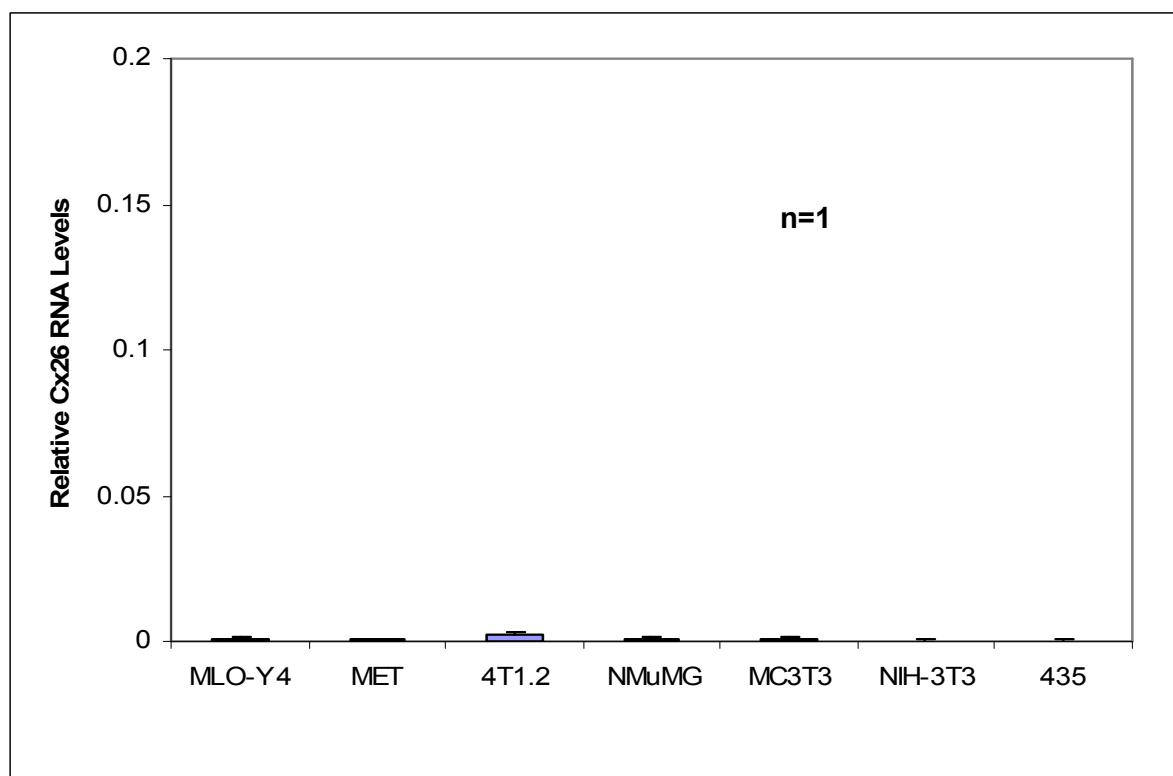
**Figure 4.1.3. Cx32 Protein in 4T1.2 & NMuMG Cells.** This figure summarizes the Cx32 Real Time RT-PCR results. From previous studies, it was known that mouse liver cells highly expresses Cx32 and therefore mouse liver cells were used as a positive control (not shown). All samples were normalized with 18S control probes. Cx32 is moderately expressed in NMuMG, normal mouse epithelial cells. No other relevant cell lines tested display statistically significant expression of Cx32.



### 4.1.3. Cx26 Expression

To characterize the expression of connexin 26 protein, there were fewer commercial antibodies available for purchase. In the process of trial and error to see which Cx26 antibody best displayed the bands on the membrane, I tested two different antibodies and found no reliable results. Conclusions to Cx26 expression are based on the results of testing for Cx26 in RT-PCR (shown in figure 4.5.3)

**Figure 4.1.4. Cx26 Expression in 4T1.2 & NMuMG Cells.** This figure summarizes the Cx26 Real Time RT-PCR results. From previous studies, it was known that mouse liver cells highly expresses Cx26 and therefore mouse liver cells were used as a positive control (not shown). All samples were normalized with 18S control probes. None of the cell lines tested display statistically significant expression of Cx26.



## **4.2 CADHERIN EXPRESSION PROFILE**

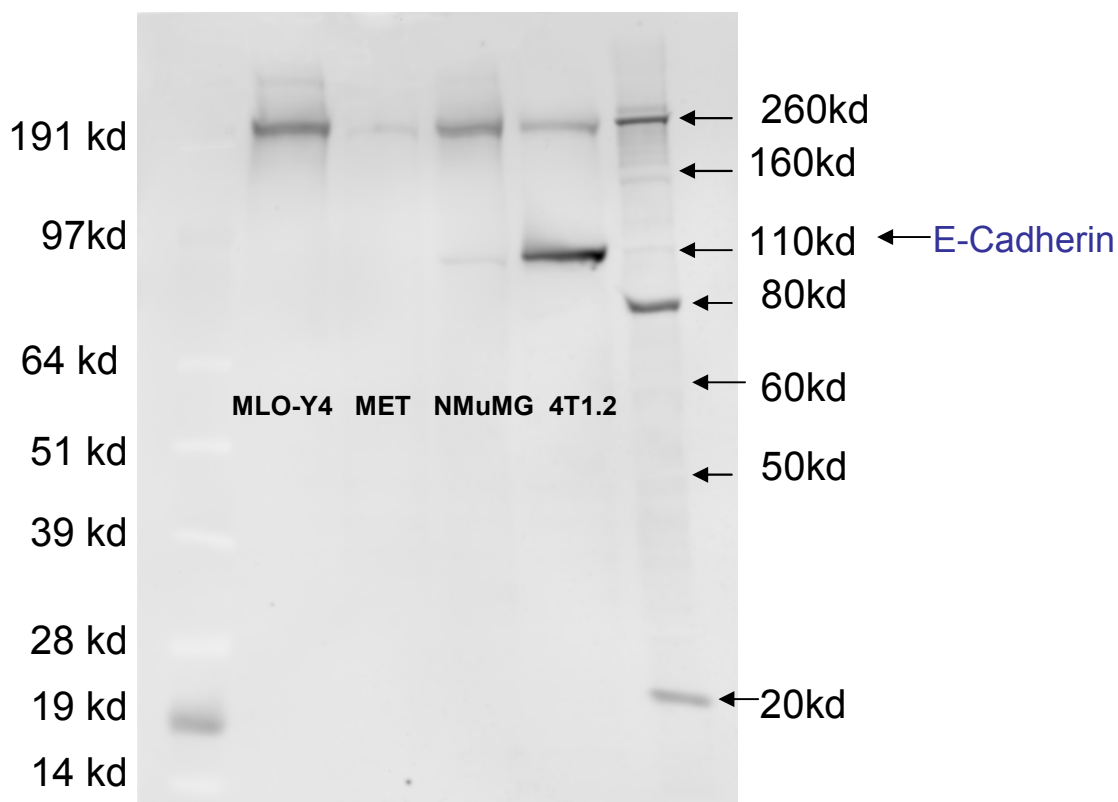
At the initiation of this experiment, I anticipated that breast cancer cell lines will express a different cadherin expression profile than normal breast epithelial cells and to confirm or deny this hypothesis I characterized the cadherin expression profiles of murine breast cancer cells (4T1.2 cells) and normal breast epithelial cells (NMuMg cells). In carrying out my experiments, I focused on three main cadherins that have been shown in many previous studies to possibly play a role in breast cancer metastasis. Specifically I studied, E-Cadherin or Epithelial Cadherin (Cadherin 1), N-Cadherin or Neural Cadherin (Cadherin 2), and OB-Cadherin or Osteoblast Cadherin (Cadherin 11). All Cadherins were tested through Western Blot Analysis and results were normalized against GAPDH expression. Results were characterized using ImageQuant Program and Excel and visually expressed in the form of bar graphs.

### **4.2.1 E-Cadherin Expression**

E-Cadherin or Epithelial Cadherin is believed to play a major role in the transition of epithelial tumors from a benign to an invasive state. In various studies it has been shown that the loss of E-Cadherin-mediated adhesion is one of the main factors in contributing to tumor's metastatic character. Specifically for my study, I focused on E-Cadherin expression in the 4T1.2 mouse breast cancer cell line and the control mouse epithelial cell line, NMuMG. Previous studies on the 4T1 cell line, the parent cell line of my 4T1.2 cell line, have concluded that 4T1 cells show strong epithelial markers, which

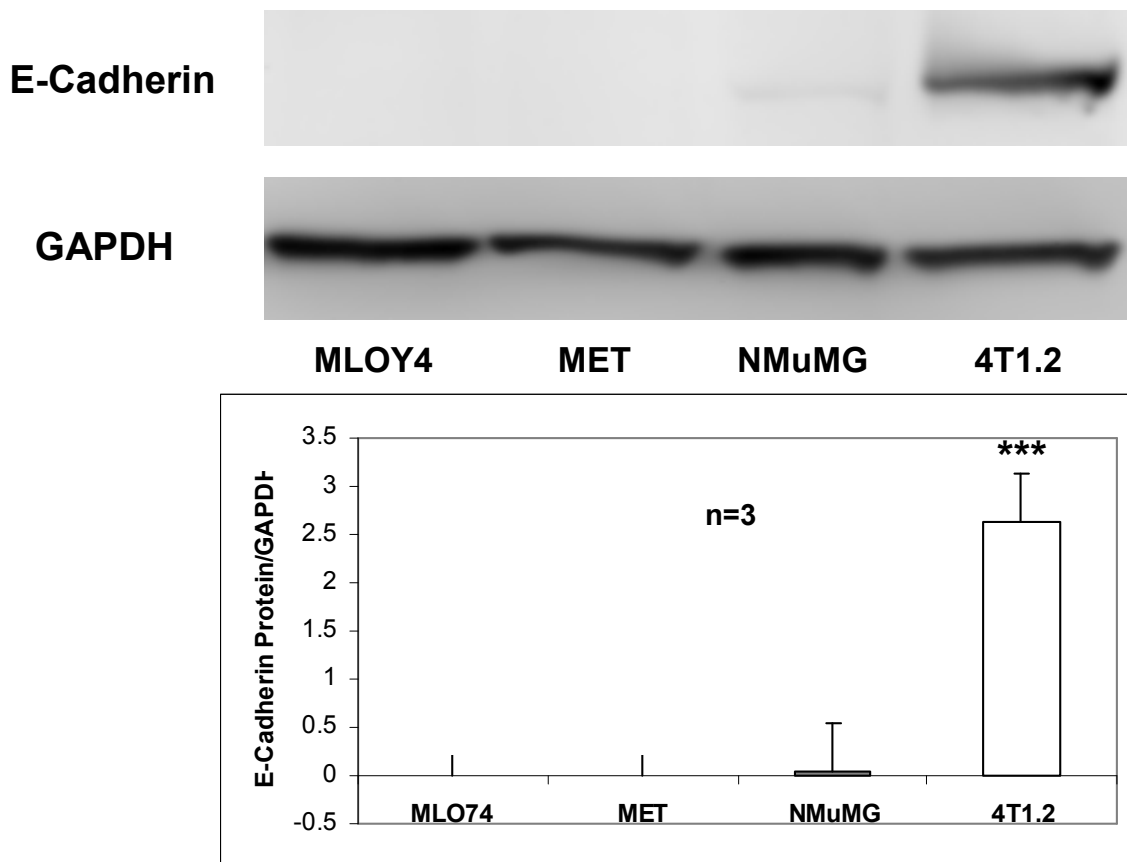
could contribute to breast cancer cells characteristic retention of certain epithelial attributes and their control of migration (Berdinelli *et al*, 2009)

**Figure 4.2.1. E-Cadherin Protein in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first E-Cadherin antibody from Invitrogen (#13-1900). According to Invitrogen and various other companies, bands for E-Cadherin are normally expressed around 110-135 kD and I found a very prominent band around 110 kD on this membrane, which I have identified as E-Cadherin. Therefore, E-Cadherin is expressed strongly in the 4T1.2 cell line and mildly expressed in the NMuMG mouse epithelial cell line. E-Cadherin was not found to be expressed strongly in any of the other cell lines tested, including: MLO-Y4 or MET.





**Figure 4.2.2. E-Cadherin Protein Summary in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first E-Cadherin antibody from Invitrogen (#13-1900). These Western Blot results were normalized against GAPDH and the results are shown below. Therefore, E-Cadherin is expressed very strongly in the 4T1.2 cell line and mildly expressed in the NMuMG mouse epithelial cell line. E-Cadherin was not found to be expressed strongly in any of the other cell lines tested, including: MLO-Y4 or MET.



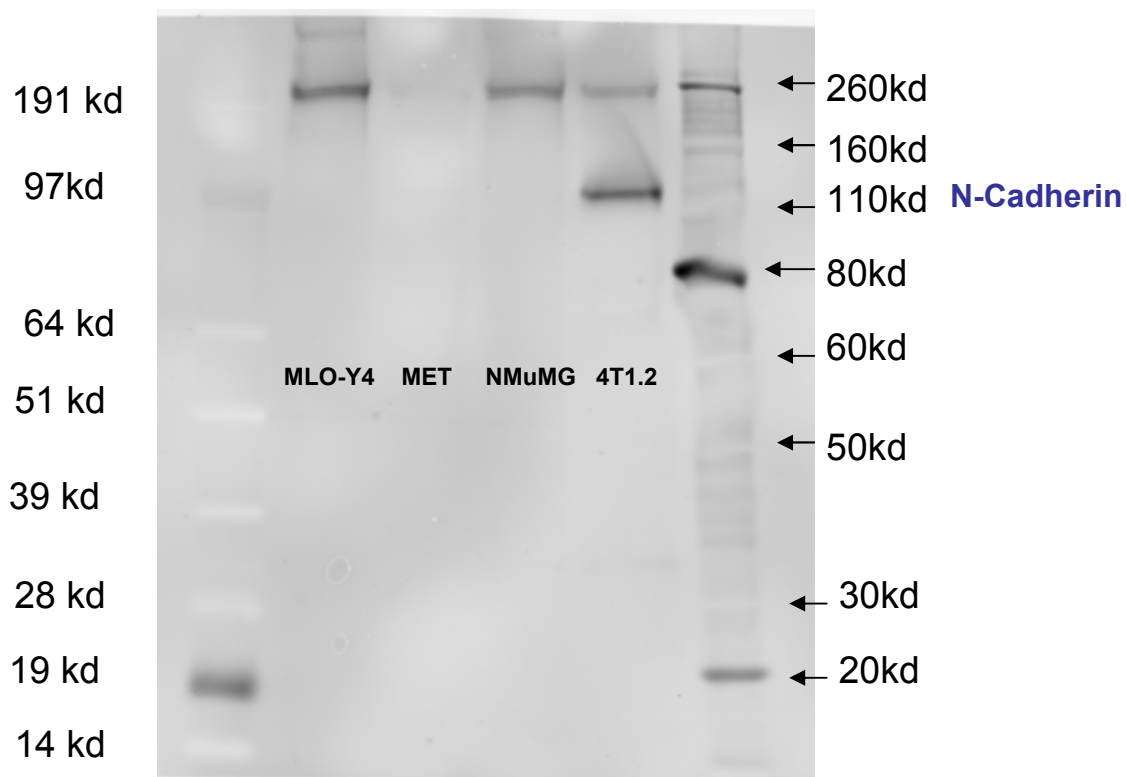
\*\*\*p<0.001

#### 4.2.2 N-Cadherin Expression

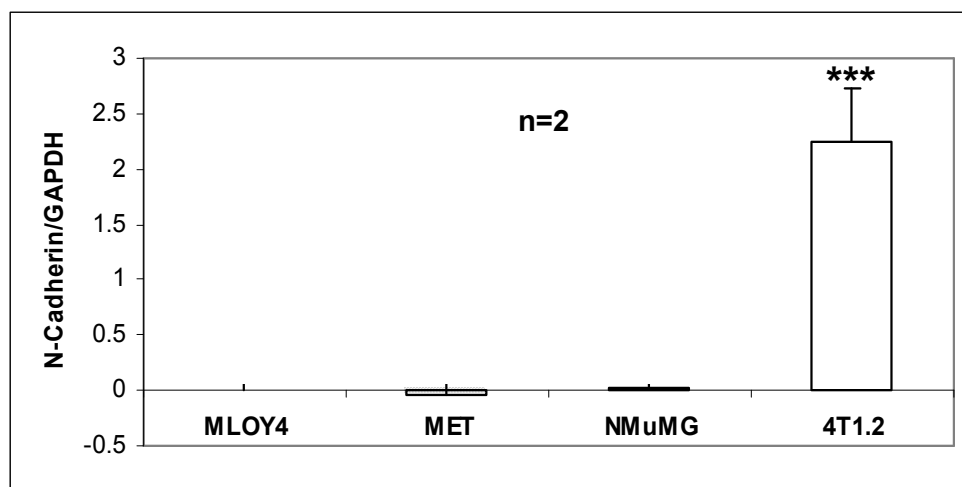
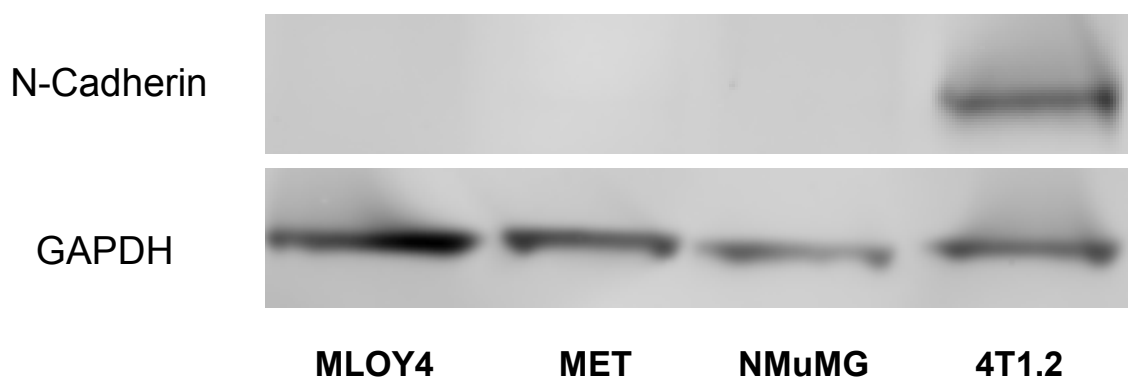
N-Cadherin or Neural Cadherin is believed to play a major role in contributing to the invasive phenotype of cancer cells. In various studies it has been shown that N-Cadherin-expressing cells may possess a greater capacity to enter vasculature and to

penetrate matrix protein barriers, two important assets to any cancer cell. Previous studies on the 4T1 cell line, the parent cell line of my 4T1.2 cell line, have concluded that 4T1 cells show strong neural cadherin markers, which could contribute to breast cancer cells characteristic epithelial to mesenchymal transition and metastasis (Berdinelli *et al*, 2009)

**Figure 4.2.3. N-Cadherin Protein in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first N-Cadherin antibody from Invitrogen (#33-3900). According to Invitrogen and various other companies, bands for N-Cadherin are normally expressed around 110-140 kD and I found a very prominent band around 120 kD on this membrane, which I have identified as N-Cadherin. Therefore, N-Cadherin is expressed strongly in the 4T1.2 cell line and minutely expressed in the NMuMG mouse epithelial cell line. N-Cadherin was not found to be expressed strongly in any of the other cell lines tested, including: MLO-Y4 or MET.



**Figure 4.2.4. N-Cadherin Protein Summary in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first N-Cadherin antibody from Invitrogen (#33-3900). These Western Blot results were normalized against GAPDH and the results are shown below. Therefore, N-Cadherin is expressed very strongly in the 4T1.2 cell line and mildly expressed in the NMuMG mouse epithelial cell line and MET. E-Cadherin was not found to be expressed strongly in MLO-Y4, another cell line that was tested.



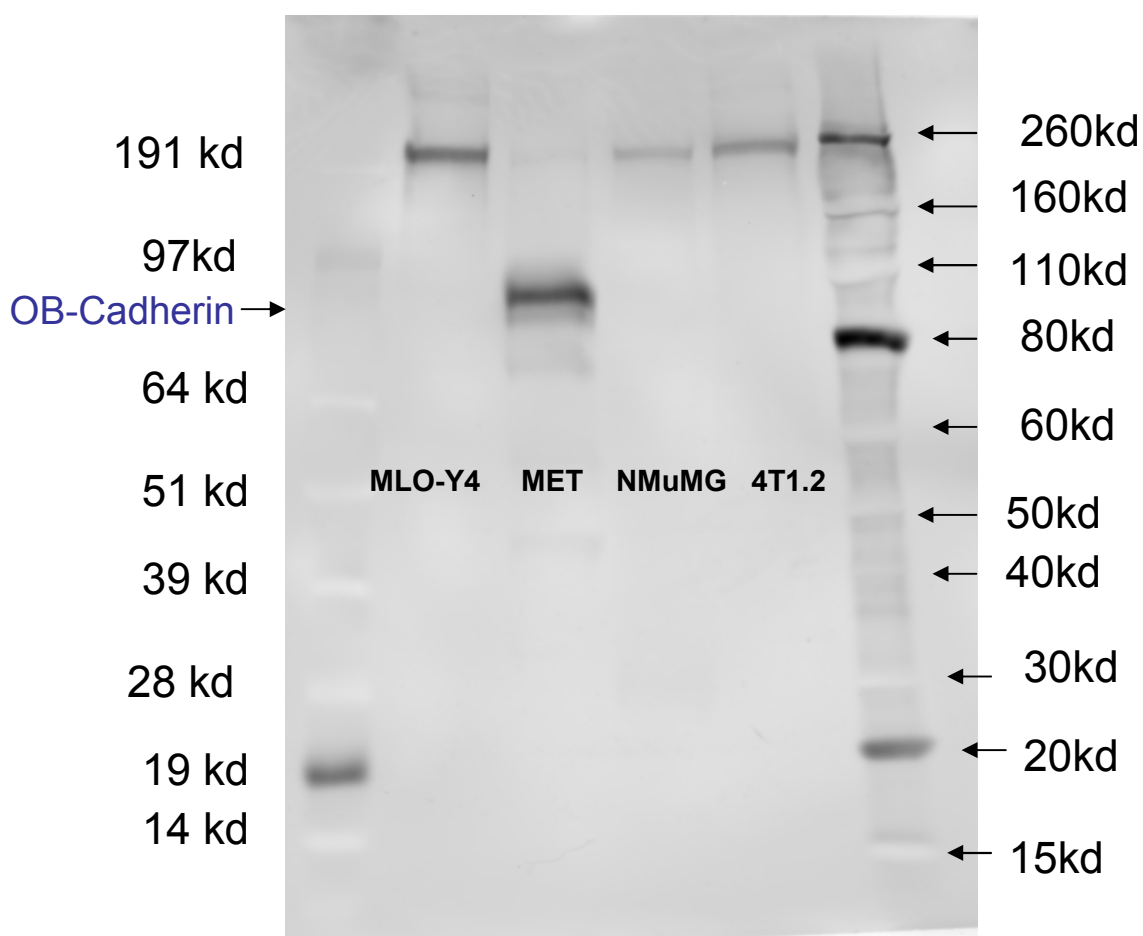
\*\*\* $p < 0.001$

### 4.2.3 OB-Cadherin Expression

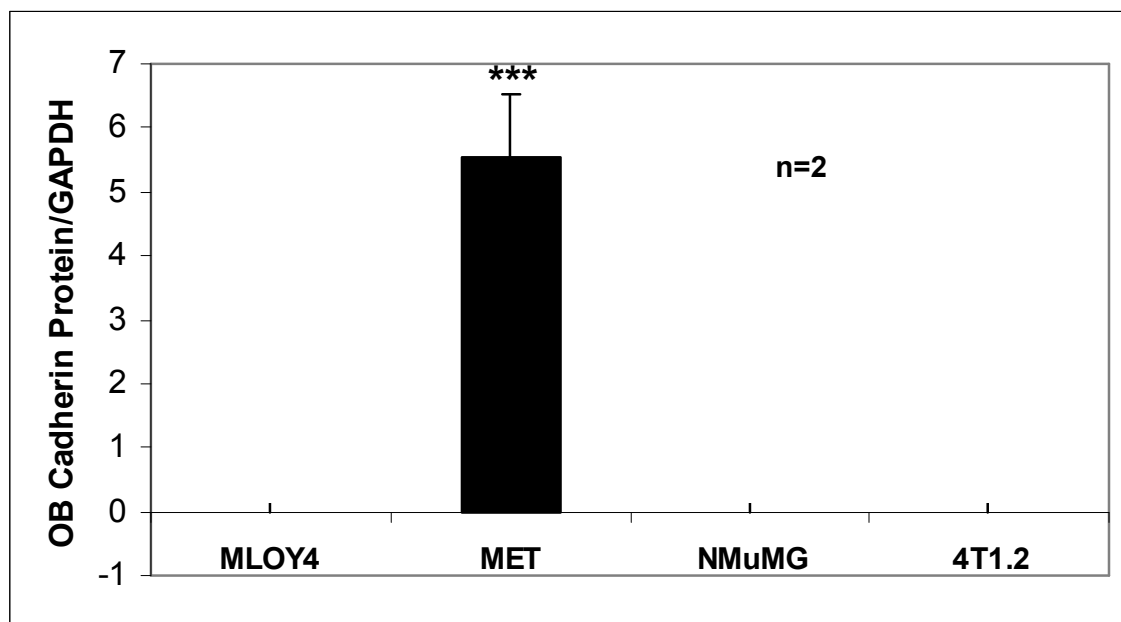
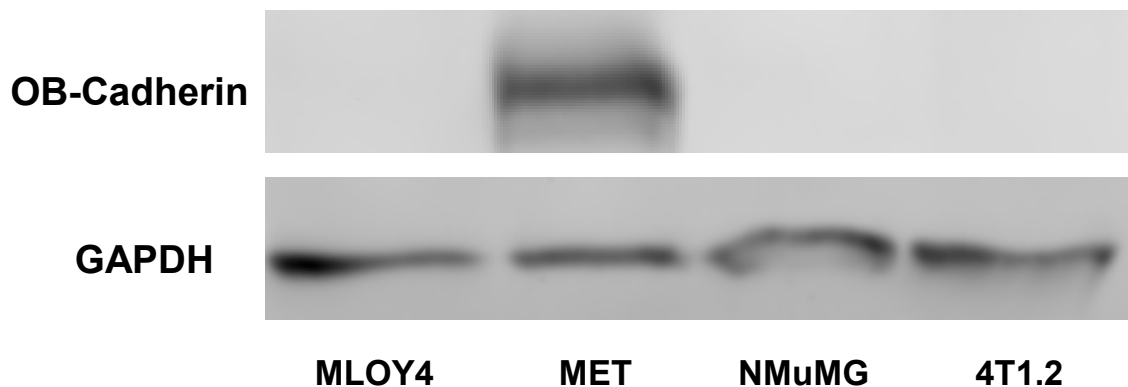
OB-Cadherin or osteoblast cadherin is an integral membrane protein that is believed to arbitrate calcium-dependent cell-cell adhesion interactions. It has also been shown to be expressed in osteoblastic and stromal cells in bone marrow. In various

studies Cadherin 11 (OB-Cadherin) has been shown to be increased in human breast cancer cell lines that have a selective ability to metastasize to bone. The mechanism by which OB-Cadherin contributes to this metastasis to bone is not fully understood and OB-Cadherin has not been greatly tested in various animal cancer models, nor was there evidence of previous studies showing expression in the 4T1 parent cell line.

**Figure 4.2.5. OB-Cadherin Protein in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first OB-Cadherin antibody from Invitrogen (#33-1700). According to Invitrogen and various other companies, bands for OB-Cadherin are normally expressed around 85-110 kD and I found a very prominent band around 90 kD on this membrane, which I have identified as OB-Cadherin. Therefore, OB-Cadherin is only expressed strongly in the MET cell line, minutely expressed in the 4T1.2 cell line and was not found to be expressed in any of the other cell lines tested, including: MLO-Y4 or NMuMG.



**Figure 4.2.6. OB-Cadherin Protein Summary in 4T1.2 & NMuMG Cells.** This figure displays a Western Blot Analysis with a first OB-Cadherin antibody from Invitrogen (#32-1700). These Western Blot results were normalized against GAPDH and the results are shown below. Therefore, OB-Cadherin is expressed very strongly in the MET cell line and minutely expressed in the 4T1.2 mouse breast cancer cell line. OB-Cadherin was not found to be expressed in the other cell lines, including MLO-Y4 or NMuMG.



\*\*\*p<0.001

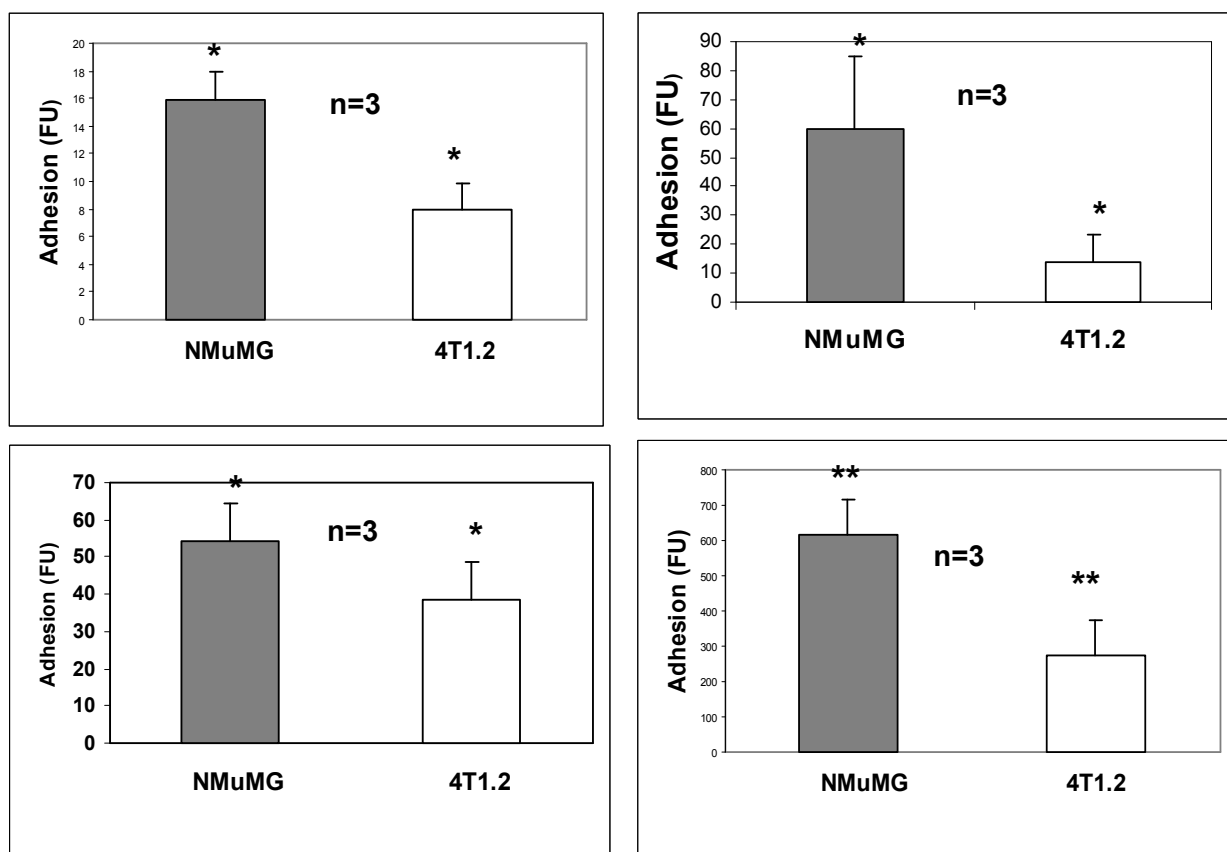
### 4.3 **ADHESION ASSAY**

Before carrying out my adhesion assays and obtaining my results, I anticipated that murine breast cancer cells will display greater levels of adhesion relative to normal murine breast epithelial cells. To verify this hypothesis, I quantified the levels of adhesion in both of these cell types. Adhesion was tested on two cellular substrates, including a MC3T3 cell line and a NIH-3T3 cell line. MC3T3 is a mouse calvaria osteoblastic cell line. In both of these cell lines, the “3T3” designation refers to the abbreviation of a three day transfer and inoculated at the density of  $3 \times 10^5$  cells. To maintain this cell line it is stored in alpha-MEM culture media, supplemented with 10% FBS and 1% P/S. NIH-3T3 is a fibroblastic cell from mouse embryos. To maintain this cell line it is stored in DMEM culture media, supplemented with 10% calf serum and 1% P/S.

#### 4.3.1 **Adhesion of Cells on MC3T3**

Adhesion assays are time-consuming experiments, each one taking 4 days to complete. Four separate adhesion assays on MC3T3 were completed in this experiment, each with an n=6. Figure 4.3.1 displays the results for the four separate adhesion assays, while Figure 4.3.2 displays the combined overall results with n=24. Overall there is a statistically significant difference between the adhesion on MC3T3 shown in the NMuMG cell line and the 4T1.2 cell line, with the NMuMG cell line showing greater than 50% more adhesion on MC3T3 than the 4T1.2 mouse breast cancer cell line.

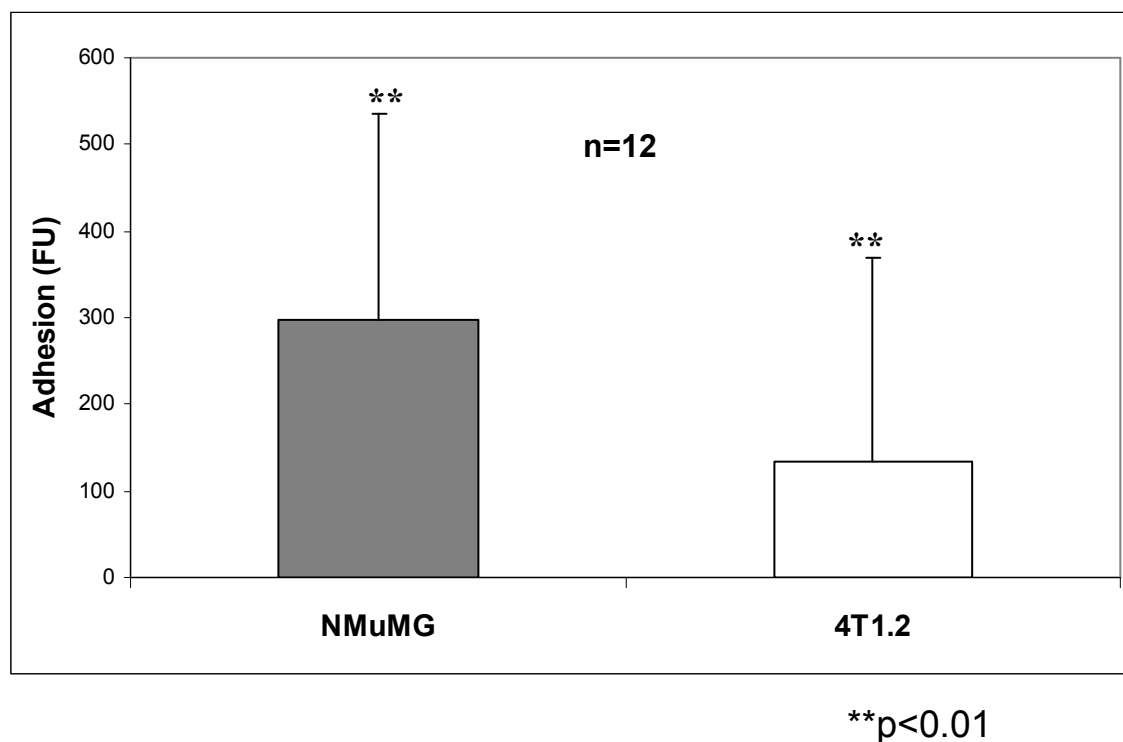
**Figure 4.3.1. Separate Adhesion Assays of NMuMG & 4T1.2 Cells on MC3T3.** This figure displays the results for the four separate adhesion assays, each with a total n=6 (n=3 for each cell type). The normal mouse epithelial cell line, NMuMG, shows statistically significant greater adhesion to MC3T3 than the 4T1.2 cell lines in each of the four assays.



\*p<0.05

\*\*p<0.01

**Figure 4.3.2. Overall Adhesion Assay of 4T1.2 & NMuMG Cells on MC3T3.** This figure displays the cumulative results for the four separate adhesion assays, with a total n=12. It is easy to see that the NMuMG, normal mouse epithelial cell line shows statistically significant greater adhesion to the MC3T3 cell line than 4T1.2 in the combination of the four assays. Overall there is a statistically significant difference between the adhesion to MC3T3 shown in the NMuMG cell line and the 4T1.2 cell line, with the NMuMG cell line showing greater than 50% more adhesion on MC3T3 than the 4T1.2 mouse breast cancer cell line.



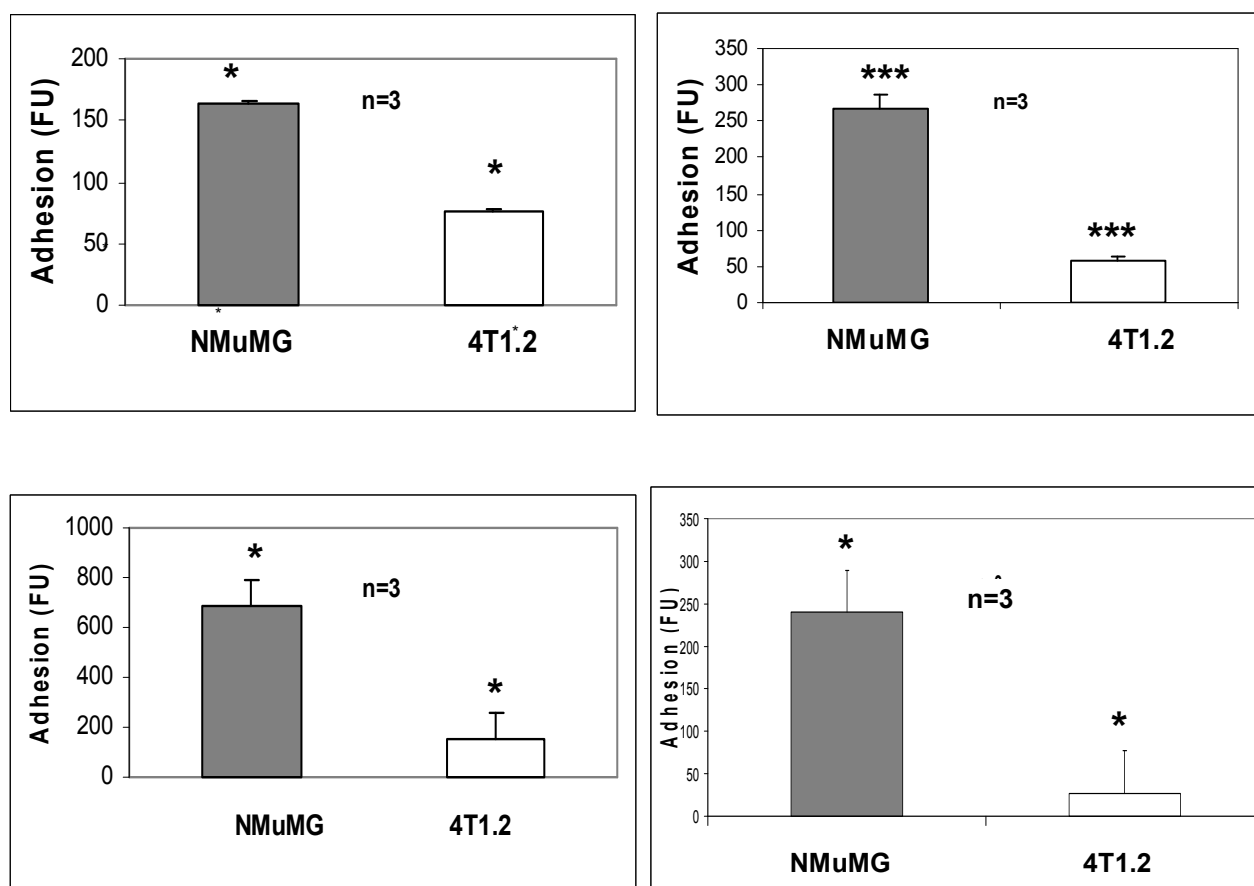
#### 4.3.2 Adhesion of Cells on NIH-3T3

Also with my adhesion assays to NIH-3T3, four separate adhesion assays were completed in this experiment, each with an n=6, and providing me with a total of n=24. Figure 4.3.3 displays the results for the four separate adhesion assays to NIH-3T3, while Figure 4.3.4 displays the combined overall results of adhesion on NIH-3T3 with n=24. Overall there is a statistically significant difference between the adhesion shown in the



NMuMG cell line and the 4T1.2 cell line, with the NMuMG cell line showing a 3 times greater adhesion to NIH-3T3 than the 4T1.2 mouse breast cancer cell line.

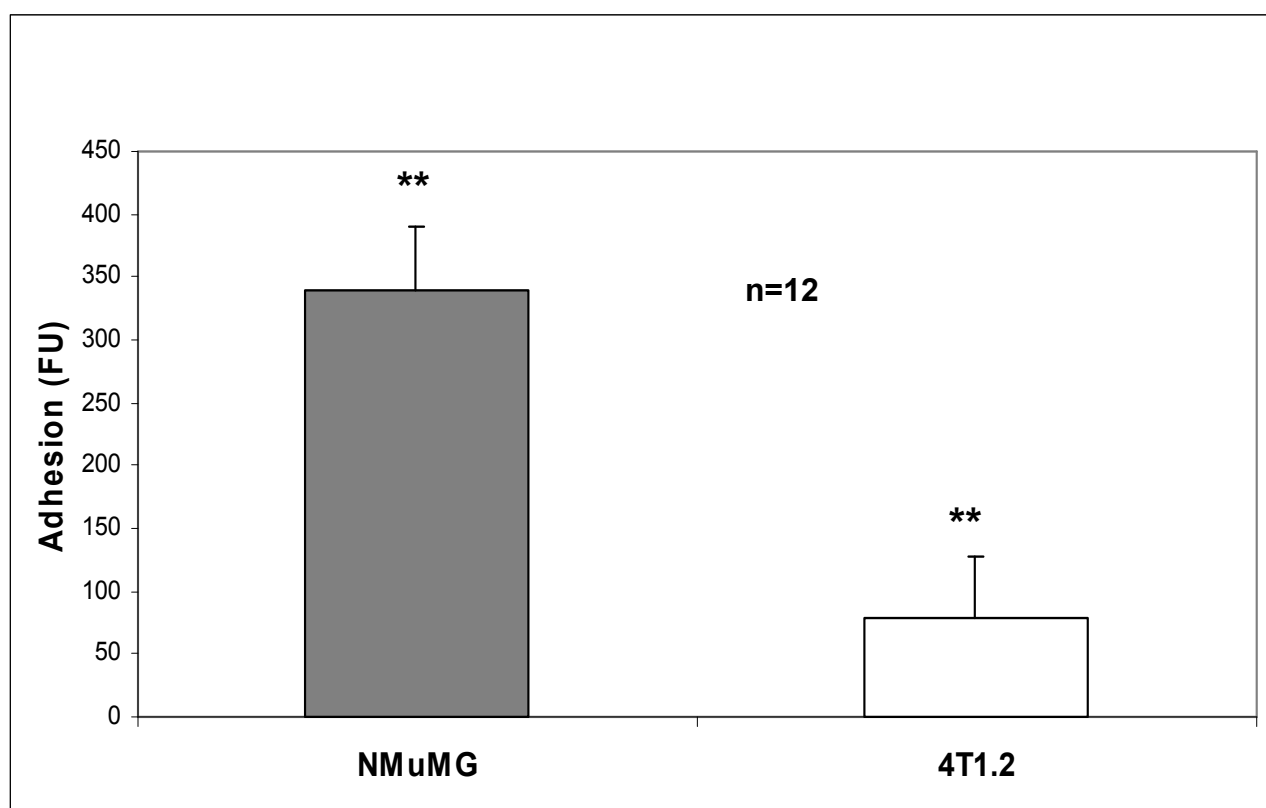
**Figure 4.3.3. Separate Adhesion Assays of 4T1.2 & NMuMG Cells on NIH-3T3.** This figure displays the results for the four separate adhesion assays, each with a total n=6 (n=3 for each cell type). It is easy to see that the NMuMG, normal mouse epithelial cell line shows statistically significant greater adhesion to NIH-3T3 than the 4T1.2 cell lines in each of the four assays.



\*p<0.05

\*\*\*p<0.001

**Figure 4.3.4. Overall Adhesion Assay of 4T1.2 & NMuMG Cells on NIH-3T3.** This figure displays the cumulative results for the four separate adhesion assays, with a total n=12. It is easy to see that the NMuMG, normal mouse epithelial cell line shows statistically significant greater adhesion to the NIH-3T3 cell line than 4T1.2 in the combination of the four assays. Overall there is a statistically significant difference between the adhesion to NIH-3T3 shown in the NMuMG cell line and the 4T1.2 cell line, with the NMuMG cell line showing about 3 times more adhesion on MC3T3 than the 4T1.2 mouse breast cancer cell line.



\*\*p<0.01

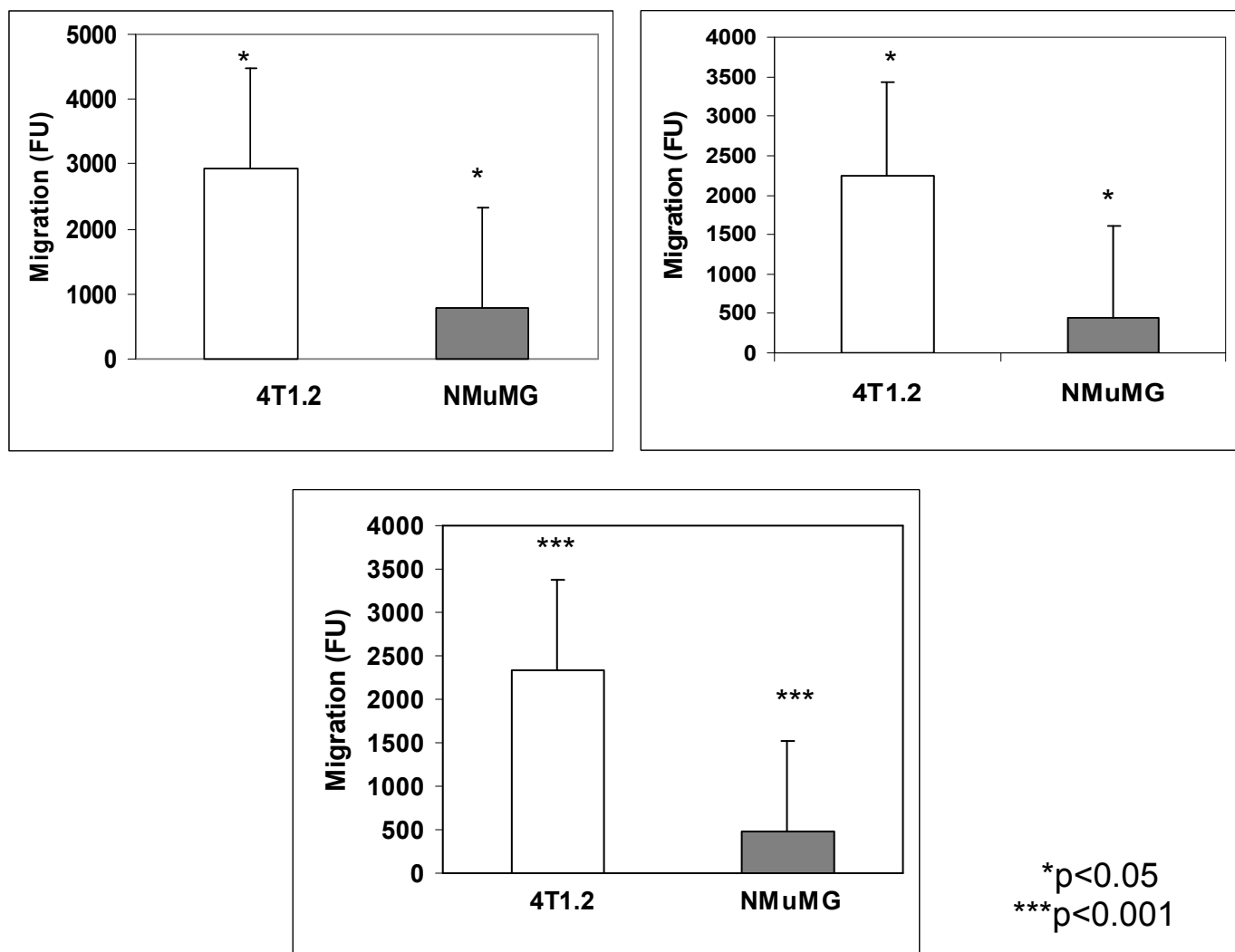
#### 4.4 **MIGRATION ASSAY**

Before carrying out my migration assays and obtaining my results, I anticipated that murine breast cancer cells will display greater levels of migration than normal murine breast epithelial cells. Because migrating from the site of original development is a pivotal part of the characteristic nature of tumor cells and the process of metastasis, I reasoned that 4T1.2, the murine breast cancer cell should display increased levels of migration. To verify this hypothesis, I quantified the levels of migration in both of these cell types. Migration was tested using 24-well plates, insert wells with a membrane, and both serum-free media and chemoattractant supplemented media.

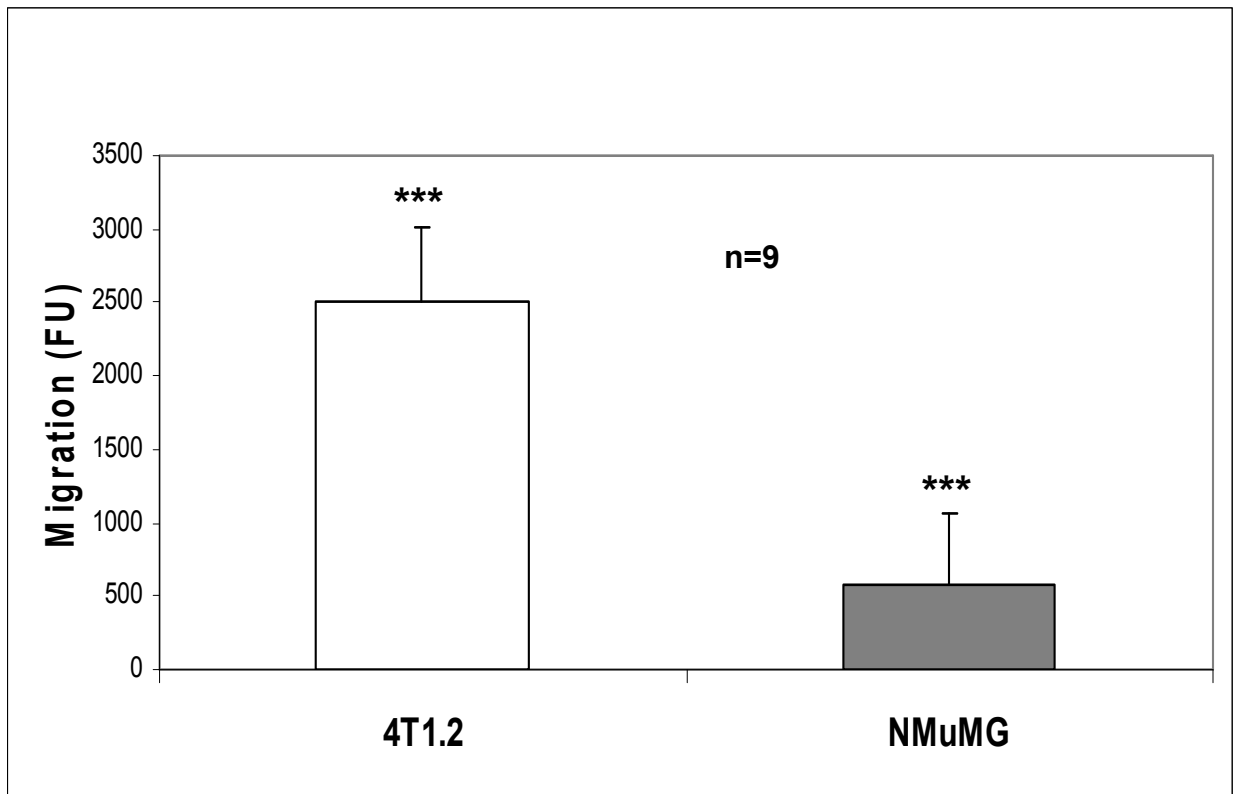
##### 4.4.1 **Migration in 4T1.2 and NMuMG**

Migration assays are slightly less time-consuming experiments than adhesion assays, each one taking 2 days to complete. Three separate migration assays were completed in this experiment, each with an n=6, and providing me with a total n=18. Figure 4.4.1 displays the results for the three separate adhesion assays, while Figure 4.4.2 displays the combined overall results with n=18. Overall there is a statistically significant difference between the migration shown in the NMuMG cell line and the 4T1.2 cell line, with the 4T1.2 murine breast cancer cell line showing over five times greater migration rates than the NMuMG normal mouse epithelial cell line.

**Figure 4.4.1. Separate Migration Assays of 4T1.2 & NMuMG Cells.** This figure displays the results for the three separate migration assays, each with a total n=3. It is easy to see that the 4T1.2, murine breast cancer cells show statistically significant greater migration than the NMuMG, normal mouse epithelial cell line.

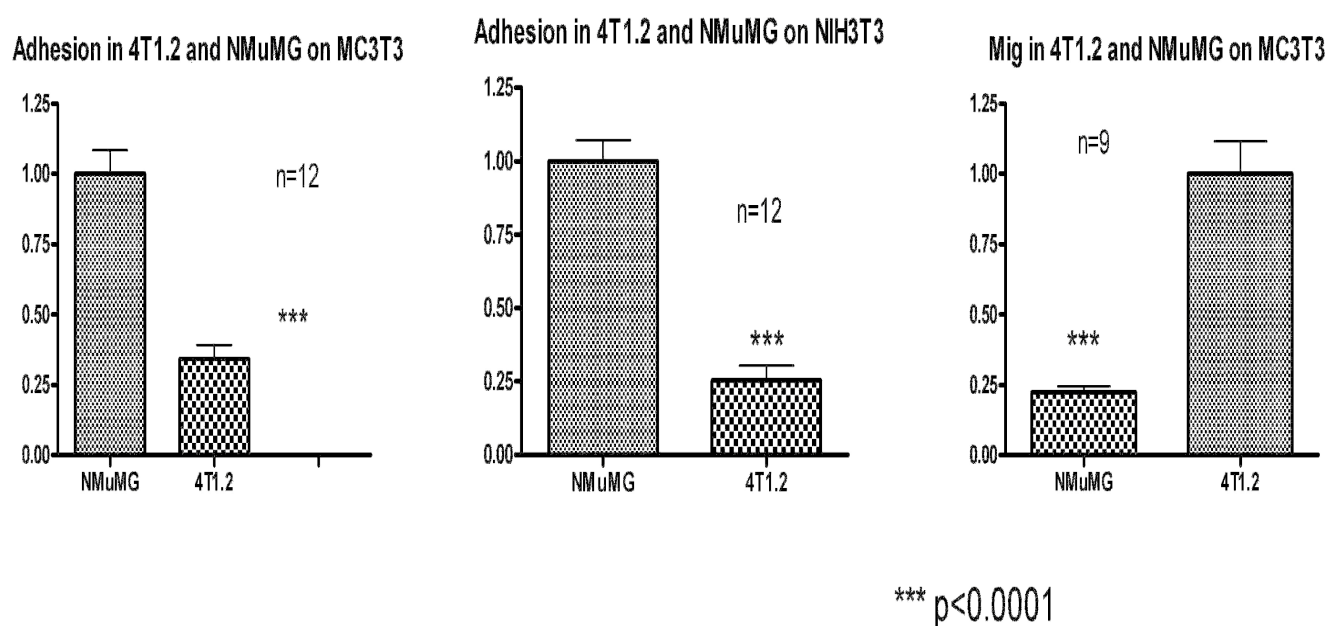


**Figure 4.4.2. Overall Migration Assay of 4T1.2 & NMuMG Cells.** This figure displays the cumulative results for the three separate migration assays, with a total n=9. It is easy to see that the 4T1.2, mouse breast cancer cell line, shows statistically significant greater migration than NMuMG, normal mouse epithelial cell line, in the combination of the three assays. Overall there is a statistically significant difference between the migration of the 4T1.2 cell line and the NMuMG cell line, with the 4T1.2 mouse breast cancer cell line showing about 5 times more migration than the NMuMG normal mouse epithelial cell line.



\*\*\*p<0.001

**Figure 4.4.3. Overall Statistical Analysis of Adhesion & Migration Assays in 4T1.2 & NMuMG cell lines.** This figure displays the cumulative results from three separate divisions of my project, including adhesion of 4T1.2 & NMuMG on MC3T3, adhesion of 4T1.2 & NMuMG on NIH-3T3, and migration of 4T1.2 & NMuMG. Since each of these three divisions involved fluorescence dyes, there were variations in the dye factor depending on the age of the dye. Because the dye factor is an arbitrary factor, it has been removed from these statistical analyses. Overall there is a statistically significant difference between the adhesion of the NMuMG cell line and the 4T1.2 cell line with NMuMG displaying much greater levels of adhesion than 4T1.2. Overall there is a statistically significant difference between the migration of the 4T1.2 cell line and the NMuMG cell line, with the 4T1.2 mouse breast cancer cell line showing more migration than the NMuMG normal mouse epithelial cell line. Since, a value of  $p < 0.05$  was considered statistically significant and since the p value for this less than 0.0001, we can consider the outcomes statistically significant.



## Chapter 5

### DISCUSSION

#### **5.1 Connexin Expression Profile**

It was necessary to characterize and confirm the connexin expression profile in murine breast cancer cell lines relative to normal murine breast epithelial cells in order to facilitate future use of transgenic mouse models. Many studies have been completed analyzing the connexin expression profile in human models, but little investigation has been carried out in murine models. At the initiation of this experiment, it was hypothesized that murine breast cancer cell lines would express a different connexin expression profile than normal breast epithelial cells. Western blot and RT-PCR analyses were utilized to investigate expression of specific connexins of interest, including: connexin 43, connexin 32, and connexin 26. The results of these analyses have also allowed for comparison between the human and murine connexin expression profiles. Based on these results, assessments were carried out on the effectiveness of mouse models as alternatives to human models.

Cx43 is expressed as the predominant connexin in both breast and bone tissue. Results showed that both murine breast cancer cells and normal murine epithelial cells displayed expression of Cx43 protein, but the murine breast cancer cell line expressed 50% more Cx43 protein than normal breast epithelial cells. These results appear to contradict multiple human studies which concluded that expression of Cx43 was shown to decrease metastatic potential (Li *et al.*, 2008). The positive expression of Cx43 protein

in the murine breast cancer cell line and the decreased metastatic potential of human cells that express Cx43 also reveals that murine and human models express different connexin expression profiles.

Connexin 32 is expressed predominantly in breast tissue and has also been shown to be highly expressed during lactation (Donahue *et al.*, 2003). Results showed that the normal murine breast epithelial cell line expressed Cx32, while the murine breast cancer cell line did not reveal Cx32 protein expression. Multiple human studies have shown that connexin 32 expression is decreased in the presence of connexin 43 protein and therefore human breast cancer models display increased levels of Cx32 protein (Li *et al.*, 2008). The negative expression of Cx32 protein in the murine breast cancer cell line also reveals that murine and human models express different connexin expression profiles.

Connexin 26 is also expressed predominantly in breast tissue and has been shown to be highly expressed during pregnancy (Donahue *et al.*, 2003). Results demonstrated that both murine breast cancer cells and normal murine breast epithelial cells express connexin 26 protein, but murine breast cancer cells display five times greater connexin 26 expression relative to normal murine breast epithelial cells. These results appear to contradict multiple human studies which concluded that human breast cancer cells display decreased levels of connexin 26 protein (Li and Welch *et al.*, 2008).

The variations shown in the connexin expression profiles of murine breast cancer cells and normal murine breast epithelial cells reveal that there is an altered gap junctional intercellular communication and connexin expression profiles between metastatic cells and non-metastatic cells (Eghbali *et al.*, 1991). Therefore we can confirm



that altered connexin expression relates to metastatic potential (Saunders *et al.*, 2001). There is also an altered connexin expression profiles displayed between various animal models, such as murine and human models. Future studies on these topics should investigate whether specific connexin expression contributes to metastases in specific secondary locations or whether specific connexin expression profiles contribute to metastatic invasiveness.

## **5.2 Cadherin Expression Profile**

Cadherins are defined as calcium-dependent adhesion molecules that have been implicated in causing altered cell-cell adhesion in neoplastic cell lines (Behrens, 1999). Western blot and RT-PCR analyses were used to investigate the expression profile of murine breast cancer cells, relative to normal murine breast epithelial cells. Specifically this study focused on the demonstration of three cadherins, including: E-cadherin, N-cadherin, and OB-cadherin. The results of these analyses also allowed for comparison between the human and murine cadherin expression profiles.

Epithelial cadherin or E-cadherin is a cell-cell adhesion protein that fulfills a prominent role in epithelial differentiation (Behrens, 1999). A loss of E-cadherin expression has been found to correlate with increased invasiveness and metastatic potential in human models (Berx and Van Roy, 2001). Results indicate that E-cadherin was highly expressed in the murine breast cancer cell line and minimally expressed in the normal murine breast epithelial cell line. These results appear to contradict multiple human studies that implicate a loss of E-cadherin expression correlates with metastatic

cell lines. The positive expression of E-cadherin protein in the murine breast cancer cell line and the decreased metastatic potential of human cells that express E-cadherin also reveal that murine and human models express different cadherin expression profiles.

Neural cadherin or N-cadherin is a cell-cell adhesion protein that has also been implicated to have a role in tumor metastasis. Human studies have shown that with tumor metastasis, there is a marked increase in N-cadherin expression levels, concurrent with a decrease in E-cadherin expression (Martinez-Orozco *et al.*, 2010). Murine studies have concluded that N-cadherin expression is found to be highly expressed in invasive tumor cell lines (Hazan *et al.*, 2000). Results indicated that N-cadherin was highly expressed in the murine breast cancer cell line and minimally expressed in the normal murine breast epithelial cell line. These results appear to support multiple human and murine studies that implicated N-cadherin expression in correlation with enhanced metastatic properties. The positive expression of N-cadherin protein in both murine and human breast cancer cell lines reveals a distinctive similarity in the cadherin expression profiles of human and murine models and a critical relationship for investigation in future studies.

Osteoblastic cadherin or OB-cadherin is a cell-cell adhesion protein that has been related to breast cancer's distinctive property of preferential metastasis to bone (Okazaki *et al.*, 1994). Multiple human studies have shown that increased expression of OB-cadherin is associated with aggressive breast cancers and is believed to enhance bone metastases (Tamura *et al.*, 2008). Results indicated that OB-cadherin was not expressed in the murine breast cancer cell line or the normal murine breast epithelial cell line.

These results appear to contradict multiple human studies that implicated OB-cadherin expression correlates with bone metastasis. The negative expression of OB-cadherin protein in the murine breast cancer cell line and the increased metastatic potential of human cells that express OB-cadherin also reveal that murine and human models express different cadherin expression profiles.

### **5.3 Adhesion & Migration**

Metastasis is defined as the spread of tumor cells to establish a secondary tumor mass (Welch et al., 2003). Many studies have concluded that the processes of cell-cell adhesion and cell migration play an important role in this process. Adhesion assays were carried out utilizing both the MC3T3 and NIH-3T3 cell lines as cellular substrates. Migration assays were also conducted utilizing cell culture inserts to mimic the process of cellular migration through a membrane. Specifically this study focused on the differences between the adhesion and migration levels in murine breast cancer cells and normal murine breast epithelial cells. The results of these analyses also allowed for comparison between the human and murine cadherin expression profiles.

Cell-cell adhesion has been investigated in many previous human and murine studies. In human studies, an increase in adhesive strength was observed in non-metastatic cell lines and a decrease in adhesive strength was shown in metastatic cell lines (Moss *et al.*, 1999). It has been shown that the initial step of tumor metastasis is the migration of tumor cells from the primary tumor mass and a concurrent loss of the ability to adhere or attach to various substrates (Lin *et al.*, 2000). Results indicated that murine

breast cancer cells displayed decreased levels of adhesion to MC3T3 cells and NIH-3T3 cells, relative to normal murine breast epithelial cells. These results appear to support many previous studies in both murine and human models, which have shown a marked decrease in adhesion in metastatic cell lines. The prominent decrease in adhesion in both murine and human breast cancer cell lines reveals a distinguishing similarity in the adhesive characteristics of human and murine models and a critical relationship for investigation in future studies.

Many human studies have shown increased levels of cell migration among breast cancer cells (Long *et al.*, 2010). Results indicated that murine breast cancer cells displayed increased levels of migration, relative to normal murine breast epithelial cells. These results appear to support many previous studies in both murine and human models, which have shown a marked increase in migration in metastatic cell lines. The prominent increase in migration in both murine and human breast cancer cell lines reveals a distinguishing similarity in the adhesive characteristics of human and murine models and a critical relationship for investigation in future studies.

#### **5.4 Human and Murine Models**

The field of science has made major gains in the area of breast cancer research, allowing for survival rates among patients in the United States and United Kingdom to increase steadily over the last fifteen years (Kim *et al.*, 2003). Future advances will require new preventative and therapeutic strategies, as well as a better understanding of the genetics and biology of human breast cancer (Fantozzi & Christofori, 2006).

Through the use of proteomics and transcriptional profiling, insights are constantly being gained, but these findings must be tested into a setting in which potential clinical responsiveness can be assessed (Kim *et al.*, 2003). This in turn requires better *in vitro* and *in vivo* models of human breast cancer.

The most widely used model for pre-clinical evaluation is *in vitro* culture of established breast cancer cell lines, but it is poorly representative of real cancers because this model lacks a three-dimensional structure and contains no stromal cells (Kim *et al.*, 2003). Animal models provide a useful alternative to *in vitro* culture because they possess intact stroma and structure as well as genetic and biomarker abnormalities similar to their human counterparts (Fantozzi & Christofori, 2006). This study focused on *in vitro* cultures of several established murine breast cancer cell lines and allowed for results to be compared to findings from human cell lines. Through gaining a clear understanding of the similarities and differences between these cellular models, it is thought that a better *in vivo* model can be developed, which would be useful for clinical and therapeutic experiments.

Connexin expression profile results for connexin 43, connexin 32, and connexin 26 were substantially different between murine and human models. Murine breast cancer cells displayed an increased expression of Cx43 protein relative to normal breast epithelial cells, while there was a decreased metastatic potential in human cells that express Cx43. Murine breast cancer cells did not express Cx32 protein, while human breast cancer cells displayed increased levels of Cx32 protein. Murine breast cancer cells

displayed increased expression of Cx26 relative to normal murine breast epithelial cells, while human breast cancer cells displayed decreased levels of Cx26 protein.

Cadherin expression profile results for E-cadherin, N-cadherin, and OB-cadherin were also significantly different between murine and human models. Murine breast cancer cells displayed an increased expression of E-cadherin relative to normal murine breast epithelial cells, while human metastatic cell lines display a marked loss of E-cadherin expression. Murine breast cancer cells displayed increased expression of N-cadherin relative to normal murine breast epithelial cells, while human metastatic cell lines also display increased N-cadherin expression in metastatic cell lines. Murine breast cancer cells did not express OB-cadherin, while human studies revealed an increased metastatic potential in cell lines expressing OB-cadherin.

Unlike the variations of connexin and cadherin expression shown in murine and human models, the characteristics of adhesion and migration appear to be similar between these models. Murine breast cancer cells displayed decreased levels of adhesion to MC3T3 cells and NIH-3T3 cells relative to normal murine breast epithelial cells, which supports human studies showing a similar marked decrease in adhesion in metastatic cell lines. Murine breast cancer cells also displayed increased levels of migration relative to normal murine breast epithelial cells, supporting previous human studies that displayed a marked increase in migration in metastatic cell lines.

The use of murine models is imperative for clinical studies investigating the genetic and functional mechanisms by which breast cancer works in an animal system. In order for results to lend towards development of effective treatments and medications,

we must understand the differences in protein expression and metastatic properties. The variety of differences between models discussed above may cause some to wonder if murine models are an appropriate model for metastasis in humans. However, though protein expression is not identical, it is still an effective model to be continually utilized in breast cancer research. There are a plethora of experimental avenues that may be tested through the use of transgenic mouse models and from this results that will contribute to effective breast cancer treatments.

## **5.5 Limitations of Study**

In this study, all Western blot analysis and RT-PCR results are dependent on the specificity of the antibodies and the relative levels of proteins available in total cell preparations. All adhesion and migration assay results were dependent on levels of fluorescence. Depending on the age of the fluorescence, how long it was stored or when it was prepared higher or lower levels of fluorescence may have been shown by the plate reader. Also certain assay plates could have been exposed to slightly more fluorescent light than others while the fluorescent dye was in the cells. This may have had a slight bleaching effect, which could have slightly skewed the results. In statistical analyses fluorescence units were removed so not to skew the results.

This study characterized the similarities and differences between murine breast cancer cells and murine breast epithelial cells. To carry out these experiments, the 4T1.2, murine breast cancer cell line and the NMuMG, normal murine breast epithelial cell lines were used, which had been previously shown to be representative of human

models. There may be limitations of this study associated with extrapolating conclusions from one tumor and one normal cell line. However, we believe that our results are likely to be representative of a larger sampling of normal and tumor mouse mammary cell lines.

Though future studies will have a primarily *in vivo* focus, this study focused on *in vitro* studies of cells grown in a two-dimensional culture. Tests were completed to characterize the connexin and cadherin proteins present in these cell lines. There may also be limitations associated with characterizing these proteins from cells grown in a two-dimensional culture because it may not be representative of protein levels present in the animal model. Future studies will investigate protein levels and metastasis to bone *in vivo*.

## **5.6 Closing Remarks & Future Directions of Research**

The completion of this project marks the first chapter to our plan to complete *in vitro* studies characterizing the connexin and cadherin expression of the cell types and to determine the manner in which they carry out the process of metastasis through migration and adhesion. These results would determine how we would progress into using this information to test results in animal models. After determining the connexin and cadherin expression profiles in mice, future studies may involve development of mouse models of both wild-type mice and connexin-specific knock-out mice. Various GFP-expressing (green-fluorescence protein) breast cancer cell lines into mice may then be intracardially injected into these mouse models (Naumov *et al.*, 1999). After time for development of breast cancer cell lines, femurs may then be harvested, dissected, and



subjected to fluorescence microscopy (Naumov *et al.*, 1999). This experimental method will allow comparison of breast cancer metastasis to bone in mice with specific connexins present and mice with specific connexins knocked-out (Kuperwasser *et al.*, 2005). Future studies involving siRNA may also be conducted to knock down specific genes, such as Connexin 43 and N-Cadherin, in order to gauge the effects on migration and adhesion when expression of these genes is damped down. Conducting these future murine experiments will continue to probe at the phenomenon of breast cancer and its preferential metastasis to bone in a more accessible and efficient animal model.

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