QUANTITATIVE ANALYSIS OF BREAST CANCER METASTASIS TO THE BRAIN

by

Kathryn Anne Teixeira

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Biological Sciences with Distinction.

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METASTASIS TO THE BRAIN

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Kathryn Anne Teixeira

Approved: _____
Deni S. Galileo, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Gary H. Laverty, Ph.D.
Committee member from the Department of Biological Sciences

Approved: _____
Marlene G. Emara, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____
Ismat Shah, Ph.D.
Chair of the University Committee on Student and Faculty Honors

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ABSTRACT

Previous experiments have shown that MDA-MB-231 human breast cancer cells could be injected into the extra-embryonic vasculature of chick embryos, and then cells which had metastasized to the brain could be isolated by drug selection. The sensitivity of the *in vivo* chick embryo system was tested by initially injecting embryos with a large number of cells ($5x10^4$) and then decreasing the number of cells injected tenfold to see if tumors would still form. Injections of $5x10^4$ MDA-MB-231 cells produced an average of 186.6 colonies after treatment with G418, and injections $5x10^3$ cells produced an average of 37.1 colonies.

Experiments with nude mice have shown that re-injecting MDA-MB-231 cells which have been through the brain produced sublines with enhanced capacity to metastasize to the brain. In order to assess the effects of re-injection on cells, colony analysis was performed on serially injected cells. It was found that the average number of colonies detected decreased as cells were injected and recovered from the brain multiple times. It is speculated that the smaller colony numbers are due to cells dividing slower and forming smaller tumors, and not necessarily fewer cells extravasating to the brain, and re-injected cells are possibly entering a state of dormancy.

Although I was not able to demonstrate that serial injection into chick embryos resulted in a brain-specific subline, as similar experiments did in previous mouse studies, this study has shown that the chick embryo is a

useful model system for studying breast cancer metastasis to the brain. It has proven to be a sensitive system, as injection of only $5x10^3$ cells resulted in measurable brain metastases. This system, along with colony analysis, will be useful for studying the effects of the manipulation of protein expression on metastatic capability in order to gain an understanding of the molecular mechanisms of metastasis.

Chapter 1

INTRODUCTION

1.1 Breast Cancer Metastasis

Breast cancer is one of the top ten causes of death for women in the United States. The Centers for Disease Control and Prevention cited the breast as the most common cancer site for females in the United States in 2005, the most recent year for which data is available. When considering both sexes, it is second only to the prostate, as shown in Figure 1 (U.S. Cancer Statistics Working Group, 2008). It has been estimated that approximately 12% of women, or one in eight, will develop breast cancer at some point in their lives.

It is estimated that 10-16% of patients diagnosed with breast cancer will develop brain metastases. Although the brain is not the most prevalent site of breast cancer metastasis (it is fourth, below the lungs, liver, and the bones), it is extremely devastating due to the difficulty of treating brain metastases (Palmieri et al., 2006). Chemotherapy is often unsuccessful, as most chemotherapeutic drugs are unable to cross the blood-brain barrier. Radiation and surgery are the most commonly employed treatments, but their successes are also extremely limited. For patients diagnosed with brain metastases, treatment correlates with a one-year survival rate of approximately 20% (Shaffrey et al., 2004).

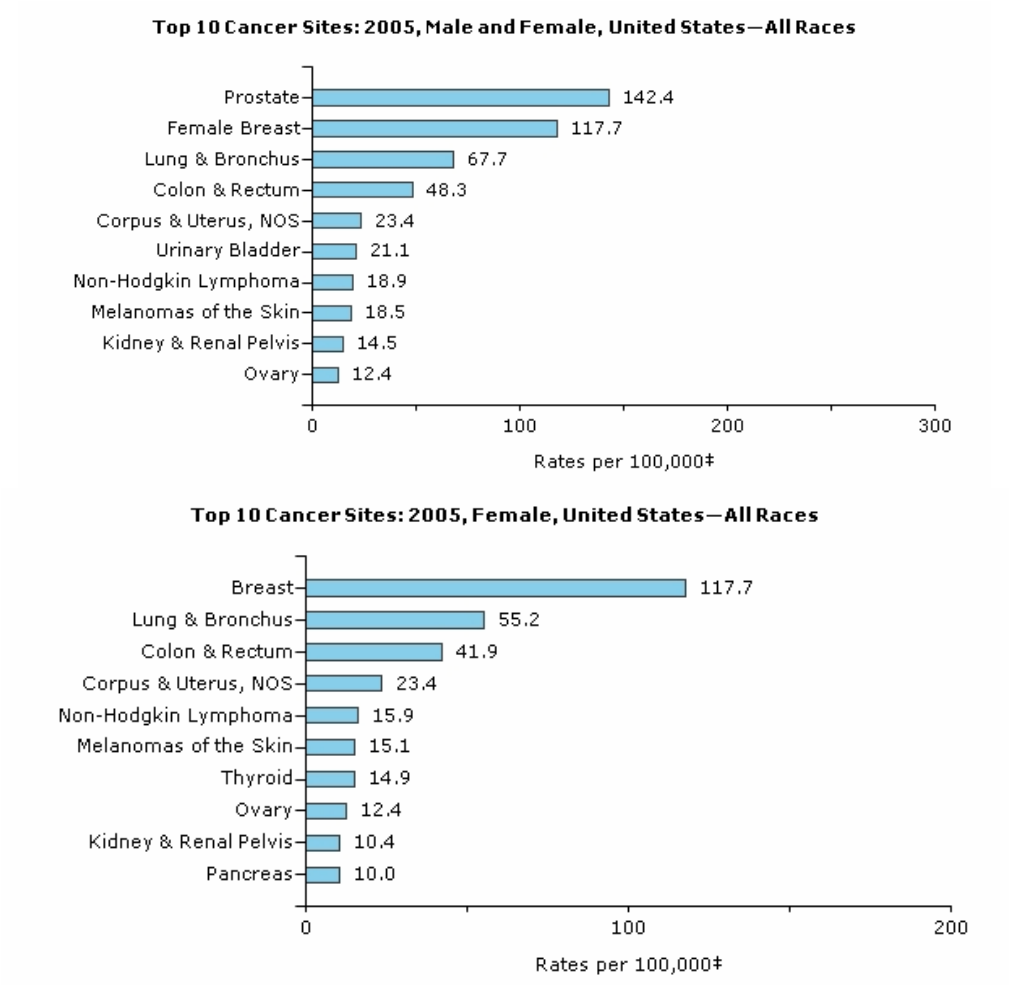

Figure 1. Cancer Site Statistics in the United States. Graphs of cancer incidence rates for the ten primary sites with the highest rates. Top: Ten sites with highest rates for males and females. Bottom: Ten sites with highest rates for females. Source: Centers for Disease Control. Available at http://www.cdc.gov/uscs

Studies of breast cancer patients with brain metastases have provided a list of characteristics associated with a high risk of development of brain metastases. These include young age, estrogen receptor negative primary tumors, and Her-2 (human epidermal growth factor receptor 2)

overexpression (Palmieri et al., 2006). It remains to be shown, however, what specific changes occur in primary tumor cells to enable migration to and growth within the brain.

The study of the mechanisms of metastasis is essential for understanding cancer and the mortality associated with it. In order for metastasis to occur, primary tumor cells break away and invade the extracellular matrix at the site of the primary tumor, migrate through blood vessel walls, and enter the bloodstream or lymphatic system, allowing migration of tumor cells into other organs where they may form a secondary tumor if a favorable microenvironment is present. A schematic representation of the phases of metastasis is depicted in Figure 2. This study aims to begin to identify the properties of a tumor cell that enable migration into the brain by selecting for breast cancer cells with a propensity to metastasize to the brain, and comparing characteristics of these cells to those of the parental cell line.

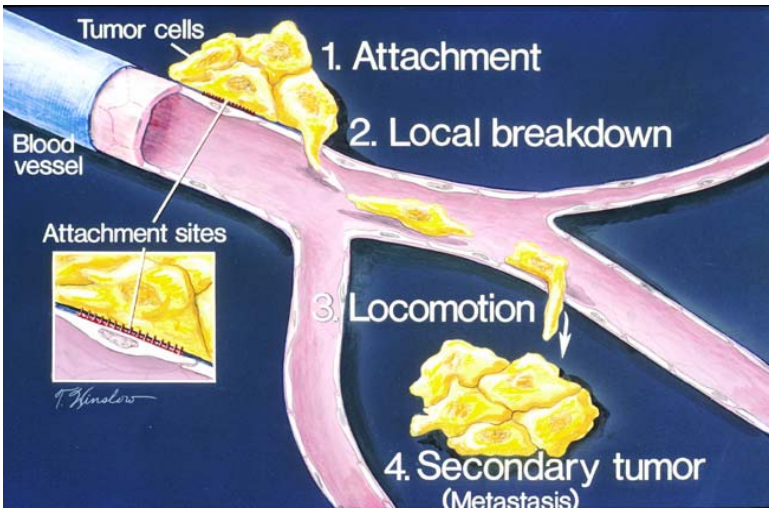

Figure 2. **Phases of Metastasis.** The four phases of metastasis: 1) Attachment, 2) Local breakdown, 3) Locomotion, and 4) Secondary tumor. Source: National Cancer Institute. Available at http://visualsonline.cancer.gov/details.cfm?imageid=2353

1.2 MDA-MB-231 Human Breast Cancer Cell Line

The MDA-MB-231 human breast cancer cell line was obtained from a pleural effusion of a 51 year old white female breast cancer patient (Cailleau et al., 1978). This cell line is very well characterized, and the expression of numerous genes by this cell line has been analyzed. The expression profile for several proteins is listed in Table 1. This table was compiled from a larger table made available by MD Anderson Cancer Center, where the cell line was originally established.

Table 1. MDA-MB-231 Expression Profile. A list of several proteins and their expression in MDA-MB-231 cells. The letter *m* means that mRNA is produced, and *p* means that protein is produced. Information Source: http://www.mdanderson.org

Factor	mRNA or protein (m/p)	Status
AP-1	p	+ (high)
Bak	p	+
Bax	p	+
b-catenin	m/p	+
bcl-2	m/p	+/+(low)
c-myc	m/p	+(high)
c-Src	p	+
E-cadherin	m/p	-
EGFR/HER1	m/p	+/+
ER	p	-
Glucocortocoid Receptor	m	+
HER2/neu	m	+(low)
p21	m	+(low)
p53	p	+ (mutation)
PTH	m	+(low)
VEGF	p	+
vimentin	m	+

The MDA-MB-231 cell line is particularly useful for studies of metastasis because of its invasive phenotype. This cell line has shown significant activity in Boyden chamber chemoinvasion assays (Thompson et al., 1992). It is capable of growth in agarose, which is considered a measure of tumorigenicity (Zhang et al., 1991). *In vivo*, injection of $1x10^6$ MDA-MB-231 cells into the tail vein of mice has produced experimental metastases (Mukhopadhyay et al., 1999).

1.3 The Role of L1 in Metastasis

L1 is a large (200-220 kDa) cell adhesion molecule belonging to the immunoglobulin (Ig) superfamily. It contains six Ig-like domains and five fibronectin-type III repeats in the extracellular domain, a transmembrane region, and a short cytoplasmic tail. The structure of L1 is depicted in Figure 3. L1 may undergo both homophilic (L1-L1) and heterophilic binding. L1 has a RGD motif in Ig-like domain 6, composed of the amino acids Arg-Gly-Asp. The RGD motif can interact with numerous integrins, allowing for heterophilic binding (Mechtersheimer et al., 2001). L1 cell adhesion molecule is expressed in the nervous system, and functions in the development of the nervous system through numerous mechanisms. It is involved in regulating cell-cell interactions, outgrowth of axons, synaptogenesis, and neuronal cell migration (Mechtersheimer et al., 2001). L1 is a vital molecule for nervous system development, and mutation of L1 in humans leads to a devastating disorder known as CRASH syndrome (Corpus callosum hypoplasia, Retardation, Adducted thumbs, Spastic paraplegia, and Hydrocephalus) (Kenwrick and Doherty, 1998).

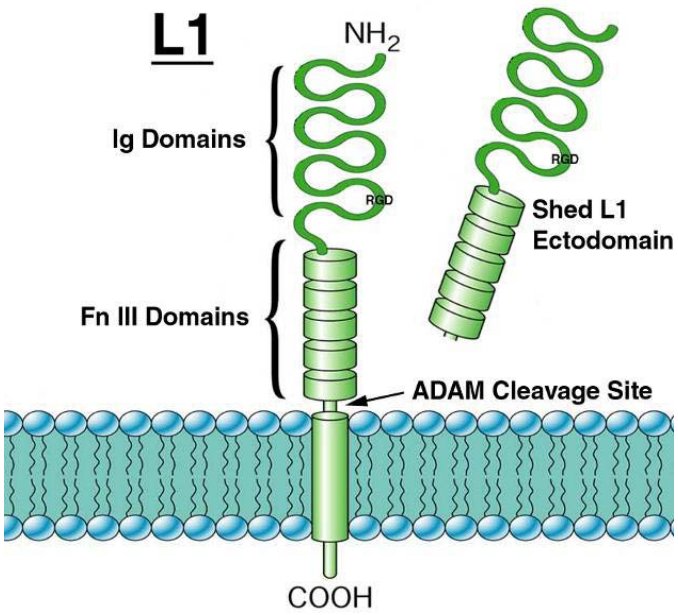

Figure 3. **Structure of L1.** Representation of L1 cell adhesion molecule, showing the immunoglobulin and fibronectin domains, as well as an ADAM cleavage site. Source: Dr. Deni S. Galileo.

L1 can be cleaved from the cell surface by a metalloproteinase known as ADAM10 to produce a large (190 kDa) soluble ectodomain. Soluble L1 ectodomain has been shown to have a role in cell migration. The migration of Chinese hamster ovary (CHO) cells transfected to express L1, which was cleaved, was blocked by a metalloproteinase inhibitor (Mechtersheimer et al., 2001).

Recently, L1 has been implicated in numerous cancers, including neuroblastoma, glioma, melanoma, ovarian carcinoma, colon cancer, and breast cancer (Raveh et al., 2009). Expression of L1 by these cancer cells is abnormal, since the tissues from which they arise do not express it. Cleavage of L1 also appears to have a role in tumor invasion. It has been shown to

occur in a variety of tumor cell lines (Fogel et al., 2003; Mechtersheimer et al., 2001). In a study of melanomas, L1 was detected in metastatic but not in non-metastatic cells. Additionally, high levels of L1 were detected in primary malignant melanomas and in metastases (Raveh et al., 2009). A clinical study has demonstrated that L1 expression is a predictor of prognostic status of melanoma, uterine, and ovarian cancer patients, and the association between L1 expression in primary tumors and metastasis was statistically significant (Thies et al., 2002; Fogel et al., 2003). Blocking L1 with an antibody against the extracellular domain (L1-11A) has been shown to inhibit migration and invasion of melanoma cells (Raveh et al., 2009).

These findings suggest a role for L1 in tumor migration and invasion, and given its normal neuronal functions, it is hypothesized that L1 may contribute to metastasis to the brain. If L1 expression aids in tumor cell migration to the brain, invasion into the brain, and/or survival within the brain, then it is hypothesized that tumor cells that form metastases in the brain will express high levels of L1.

1.4 Serial Injection Experiments

Cancer cells that migrate away from the primary site to another organ differ from those that remain in the primary tumor in some way. In order to identify the properties that allow metastasis to specific organs, researchers have recently established variants of breast cancer cell lines that migrate to these specific organs (Yoneda et al., 2001; Kim et al., 2004). Sublines have been created from tumors isolated from both brain and bone in mice, and these sublines reliably metastasize to the organs from which they were

obtained. This allows for comparison to the original tumor cell line to determine factors contributing to organ-specific metastasis.

1.4.1 Serial Injection Experiments with Mice

Using the process of serial injection and culture of tumor cells from mice, subpopulations of MDA-MB-231 cells with a predilection for metastasis to brain have been isolated from the parental cell line by two different laboratories. In the first study, the primary concern was the selection of a bone-seeking variant, but in the process a brain-seeking variant was also created (Yoneda et al., 2001). The second was primarily concerned with establishing a brain-seeking variant and used a slightly different technique (Kim et al., 2004). An outline of the general procedure used for establishing the brain-seeking variants is shown in Figure 4.

In both studies, $1x10^5$ cells were injected into female nude mice, either into the left ventricle or the internal carotid artery. The bone-seeking subline was established by isolation of parental cells from bone metastases using explant outgrowth technique. These cells were grown in culture, and the process was repeated nine times until no metastases could be detected histologically in any other organ. Using the same technique, a brain-seeking variant was established after six serial injections and isolations (Yoneda et al., 2001).

For the more recent study of a brain-seeking subline, MDA-MB-231 cells had been stably transfected with GFP and neomycin resistance, allowing for drug-selection of MDA-MB-231 cells from neural tissue. Brain metastases were identified by GFP fluorescence, and then resected and cultured with

G418. This process was repeated three times, resulting in three separate sublines, which were named MDA-231-BR1, MDA-231-BR2, and MDA-231-BR3. By this method, brain metastases were found in 82.4% of mice injected with the parental cell line, and 100% of mice injected with the MDA-231-BR2 and MDA-231-BR3 lines (Kim et al., 2004).

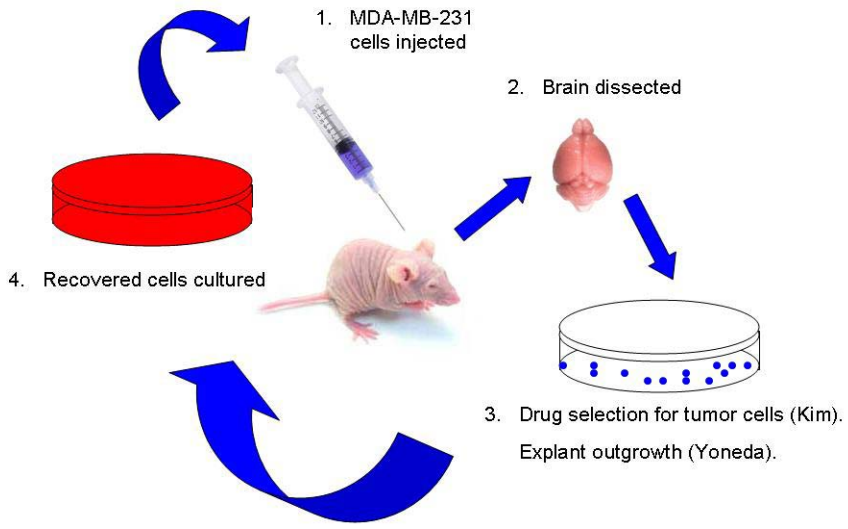

Figure 4. **Serial Injection Procedure.** 1) 10^6 cells are injected into a nude mouse. 2) The brain is dissected and analyzed for metastases. 3) Tumor cells are recovered either by drug-selection or explant outgrowth. 4) Recovered cells are cultured, and then injected again (1).

Additionally, a variant of MDA-MB-231 cells selected from experimental lung metastases, named MDA-231 LC3, was injected into nude

mice. This cell line did not show a significant difference from the parental MDA-MB-231 in the number of brain metastases which could be detected. This suggested that the increased ability of the brain-selected subline to grow in the brain is not a general property of cells isolated from experimental metastases, but rather it is a property specific to cells isolated from the brain (Kim et al., 2004).

In each case, the organ-specific variants demonstrated characteristics that differed from the parental cell line, and from cells isolated from experimental metastases to other organs. By comparison of the bone-seeking variant to the brain-seeking and parental cell lines established by the Yoneda laboratory, it was shown that the bone-seeking variant expressed higher levels of parathyroid hormone-related protein (PTH-rP) than either the brain-seeking variant or the parental cell line. PTH-rP has been shown in clinical studies to be expressed at high levels in breast cancers that metastasize to the bone. PTH-rP levels in the brain-seeking variant were similar to those of the parental cell line (Yoneda et al., 2001). The brain-seeking variant established by Kim was shown to express higher levels of vascular endothelial growth factor-A (VEGF-A) and IL-8 than the parental cell line. VEGF-A and IL-8 are both considered mediators of angiogenesis, and VEGF-A has been implicated as having a role in brain metastases (Kim et al., 2004).

Both of these experiments have shown that it is possible to isolate subpopulations of cells from experimental metastases that metastasize specifically to one and only one organ, and they have begun to identify certain

properties that enable this organ-specific growth. To further understand the mechanisms of organ-specific metastasis, these experiments must be repeated and the process of identifying properties that enable cells to migrate to and grow within the brain must be continued.

1.4.2 Serial Injection Experiments with Chick Embryos

Initial experiments using the chick embryo have shown that it is a promising model for studying breast cancer metastasis to the brain, and it may be useful for studying the selection of a subpopulation of breast cancer cells that specifically target the brain. The Galileo lab has previously shown that two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, extravasate into brain tissue following injection into the extra-embryonic vasculature of chick embryos. The optimal age for injection was determined to be E5 based on the fact that at this age, the blood vessels are an appropriate size for injection, and the vessels are still on top of the yolk. Prior to E5, blood vessels tend to be too small to inject, and at E6 and beyond, the embryo and the blood vessels sink into the yolk and are unreachable for injection (Hansen, 2006).

The next step was to determine the optimal age for dissection for various organ analyses. The optimal age is the one which allows the cancer cells to incubate the longest in order to allow the most metastases to form, while still maintaining a relatively high survival rate and allowing for relatively easy dissection of the brain. Based on these criteria, it was determined that E15 was the optimal age for dissection of brains to be fixed with formaldehyde, and E9 was the optimal age for dissection of brains to be used for colony

analysis. After E9, the neurons become too entangled for dissociation into single cells.

Cancer cells could be detected on the interior of the brain, just below the surface, of E15 brains that were fixed with formaldehyde and stained with X-Gal (Hansen, 2006). It was assumed that cancer cells were not detected deeper inside the brain tissue due to X-Gal failing to penetrate into the tissue. Further analysis by cryosectioning and immunostaining showed that single cancer cells could be detected deep in brain tissue, but most cancer cells remained at or near the surface of the brain. FACS analysis of E9 brains showed that a significantly larger number of GFP-expressing cancer cells could be detected in the meninges than in the brain (Hansen, 2006). The meninges are a vascularized tissue surrounding the brain (Figure 5). It was hypothesized that the large number of cells present in the meninges was due to the lack of ample time for cells to extravasate out of the blood vessels and into the brain in the short 4 day incubation period.

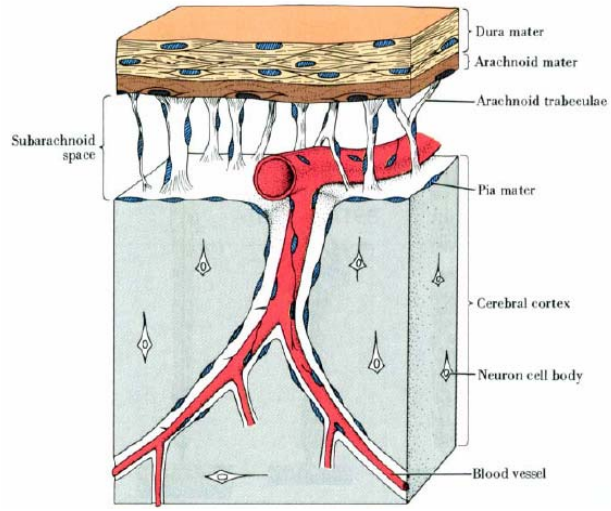

Figure 5. Layers of the Meninges. A schematic of the meninges surrounding the brain (light blue). The dura mater and arachnoid mater are represented in beige and the pia mater is white. Blood vessels running through the meninges and into the brain are red. Source: Ross et al. 1995.

By dissociating brains and selecting for drug-resistant colonies of MDA-MB-231 cells, metastasis to the brain could be analyzed quantitatively. It was found that after injection of $5x10^4$ MDA-MB-231 cells, an average of 140 colonies per $2x10^7$ brain cells in a 10cm culture dish could be detected following X-Gal staining. It was determined that colony analysis is a more sensitive and reliable method for quantifying metastases in the brain. Using drug selection, a single cell that has metastasized to the brain will form a colony and will be included in the quantitative analysis. Colony analysis detected larger numbers of cells with less variation than FACS analysis. For this reason, I used colony analysis of LacZ-expressing cells for this study rather than FACS analysis of GFP-expressing cells.

Initial re-injection studies using MDA-MB-231 cells and the chick embryo showed that after one series of injection, recovery, and re-injection (MDA-MB-231-1x), the average number of colonies that could be counted decreased slightly. The same experiment performed with MDA-MB-435 cells also showed a decrease over a series of two injections and isolations (MDA-MB-435-2x, compared with MDA-MB-435-0x) (Hansen, 2006). However, because these were only preliminary studies, and did not go beyond twice injecting and recovering the cells, it was unknown whether this trend would continue.

Fewer embryos injected with the MDA-MB-231-1x cells survived to E9 than those injected with MDA-MB-231 parental cells (71% compared with 60%). Similarly, the survival rates to E9 of embryos injected with the various MDA-MB-435 variants were decreased in comparison to the parental cell line. However, the survival rate initially dropped off significantly following one series of injection and isolation, and then gradually increased with subsequent injections and isolations. The survival rates of E9 embryos injected with MDA-MB-435 cells are shown in Figure 6, where 1.0 means 100% survival (Hansen, 2006).

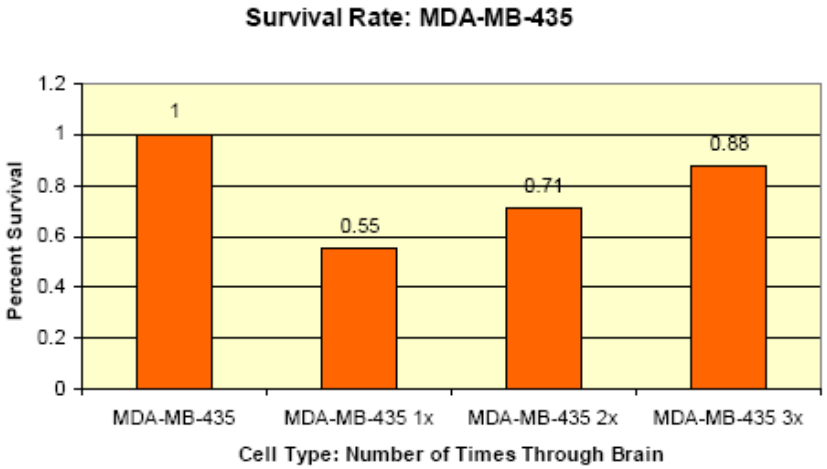

Figure 6. MDA-MB-435 Survival Rates after Serial Injections. The survival rates of embryos injected with MDA-MB-435 re-injected variants that survived to dissection age, E9 (Hansen, 2006).

1.5 LZ12 Plasmid Transfection

LacZ was stably introduced into MDA-MB-231 cells by co-transfection of a plasmid that encodes the LacZ gene along with the neomycin resistance gene (Neo^r). The neomycin gene provides the cells with a drug resistance, allowing for selection of only those cells which have incorporated the LacZ gene into their genomes. The plasmid used, pLZ12 (Figure 7), encodes an ns-LacZ fusion protein that is directed to the nucleus. This results in blue nuclei when cells are stained using X-Gal.

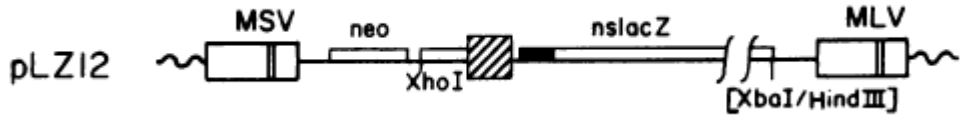

Figure 7. LZ12 Plasmid. Plasmid encoding nuclear localized LacZ and neomycin resistance. Rectangles are Moloney sarcoma virus (MSV) and Moloney leukemia virus (MLV) long terminal repeats. Nuclear signal (ns) is indicated by black box. Diagonal lines indicate Rous sarcoma virus (RSV) sequences used as an internal promoter (Galileo et al., 1989).

1.5.1 Neomycin Resistance Gene

The neomycin resistance gene encodes an enzyme that confers resistance to the antibiotic neomycin and the similar antibiotic G418. G418 is produced by the bacteria *Micromonospora rhodorangea*. It inhibits polypeptide synthesis by binding irreversibly to the 80S ribosome complex. Unlike neomycin, G418 is effective in killing eukaryotic cells, and is therefore useful for selecting for eukaryotic cells that have had the neo^r gene introduced.

1.5.2 LacZ Gene and X-Gal Staining

The LacZ gene encodes an enzyme, β-galactosidase, which is involved in metabolism in *Escherichia coli*. β-galactosidase cleaves X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside), forming a blue precipitate. The mechanism for this reaction is shown below in Figure 8. The formation of a blue precipitate allows differentiation of LacZ-transfected MDA-MB-231 cells and untransfected cells.

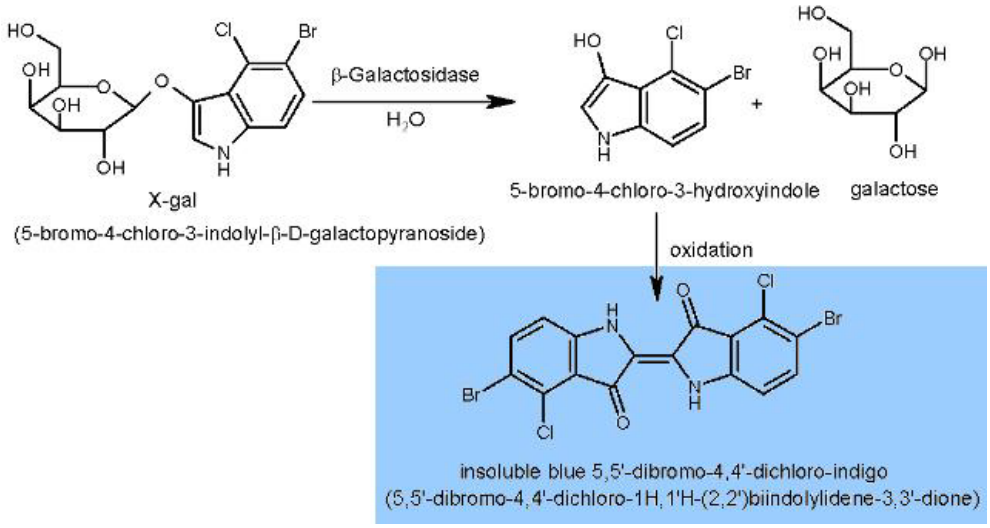

Figure 8. Mechanism of the Reaction Involved in X-Gal Staining. B-galactosidase cleaves 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole, which is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, a blue precipitate.

1.6 Protein Detection by Western Blot

Western blotting is a technique by which denatured proteins are first separated by molecular weight, and then specific proteins can be detected using antibodies. Proteins are denatured in sodium dodecyl sulfate (SDS), which gives them an overall negative charge. This allows separation of proteins by molecular weight using polyacrylamide gel electrophoresis (PAGE). Separated proteins are then transferred to a polyvinylidene fluoride (PVDF) membrane, which can then be stained using antibodies directed against a specific protein.

1.7 Objectives and Specific Aims

There were two main aims for this project: 1) testing the sensitivity of the chick embryo model system to breast cancer metastasis to the brain, and 2) investigating the serial injection and isolation of MDA-MB-231 human breast cancer cells from brain metastases in chick embryos.

1) In order to test the sensitivity of the chick embryo, the colony analysis technique was used to quantitate brain metastases. Initially, a large number ($5x10^4$) of cells was injected into the vasculature of chick embryos and metastases were quantified. Then, the number of cells injected was reduced ten-fold ($5x10^3$) to see if brain metastases would still be detectable. If brain metastases formed after injection of a relatively small number of human breast cancer cells ($5x10^3$), then this would show that the chick embryo is a sensitive model system for studies of metastasis to the brain.

2) In order to investigate the possibility of isolating a brain-specific subline of human breast cancer cells, MDA-MB-231 cells were injected into the vasculature of chick embryos, and then isolated from brain metastases by dissociation of the whole brain and drug selection using G418. This process was repeated multiple times. Properties of the MDA-MB-231 sublines (numbered according to the number of times they had been isolated from the brain) were compared to the original cell line. The properties that were investigated included morphology, growth rates, L1 expression, and tumor formation (as measured by the number of colonies detected by drug selection following injection).

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

MDA-MB-231 human breast cancer cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, CellGro) supplemented with 10% bovine growth serum (BGS, Hyclone), 1% penicillin/streptomycin stock solution (pen/strep, 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, CellGro), and 1% L-glutamine stock (CellGro). They were incubated in a humidified atmosphere maintained at 5% CO_2 and 37^{o}C. These cells had been stably transfected with a retroviral vector encoding nuclear localized LacZ and neomycin resistance, provided by Dr. Deni S. Galileo (Galileo et al., 1990).

2.2 Chick Embryos

Fertilized White Leghorn eggs were provided by the Department of Animal and Food Sciences at the University of Delaware. Eggs were incubated in a humidified atmosphere at 37.5^{o}C to the desired embryonic stage. The first 24 hours of incubation are considered embryonic day 0 (E0). Once embryos are at the desired embryonic stage (E5 for injection, E9 or E15 for dissection of organs), a hole is cut at the top of the egg at the air pocket so that the embryo

and extra-embryonic blood vessels are exposed. The hole can be sealed with tape, and the embryo can be incubated to later stages.

2.3 Injection of MDA-MB-231 Cells into Vasculature

MDA-MB-231 cells were prepared for injection by first trypsinizing cells in 0.05% trypsin/0.02% EDTA (CellGro) until they had detached from the bottom of the culture dish and separated into single cells (approximately 5 minutes). 10mL of media with serum was added, and the number of cells in the solution was counted using a hemacytometer. Following centrifugation at approximately 800rpm for 5 minutes, cells were resuspended in the necessary volume of medium to achieve a cell concentration of either $1.0x10^6$ or $1.0x10^7$ cells per milliliter of medium. Approximately 70μL of Fast Green (Sigma) was added so that the cell solution could be visualizing during injection.

Holes were cut into the air pocket of eggs that had been incubated to E5, and the top membrane covering the embryo and the yolk was removed after wetting the membrane with Medium 199 (CellGro). Using a pressurized microinjector (PV380 Picopump; World Precision Instruments) attached to a nanomanipulator (NM3D-25VP; Discovery Technology International) (Figure 9) approximately 5μL of cell solution (either $5x10^3$ or $5x10^4$ total cells) were injected into the extra-embryonic vasculature of an E5 chick embryo, as shown in Figure 10. Approximately 5 drops of 50mg/mL ampicillin (a broad spectrum antibiotic similar to penicillin) in water was added over the site of injection, and the hole in the egg was sealed with tape. Embryos were incubated at 37.5^oC to the embryonic stage necessary for organ preparation.

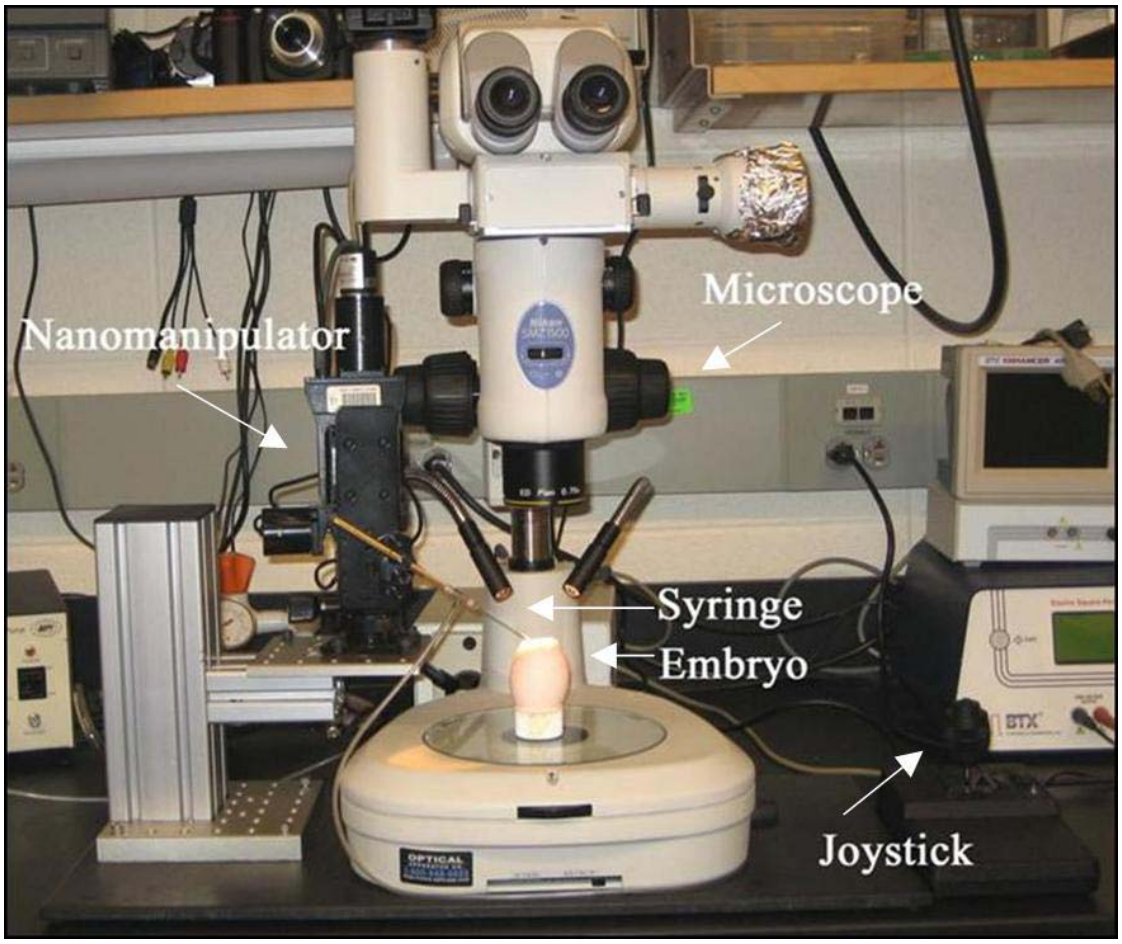

Figure 9. **Microinjection Station.** Nanomanipulator used to maneuver syringe attached to pressurized microinjector for injection into chick embryo vasculature. The joystick is used to control movement of the syringe, which is pointing into the air hole at the top of an egg containing an E5 chick embryo.

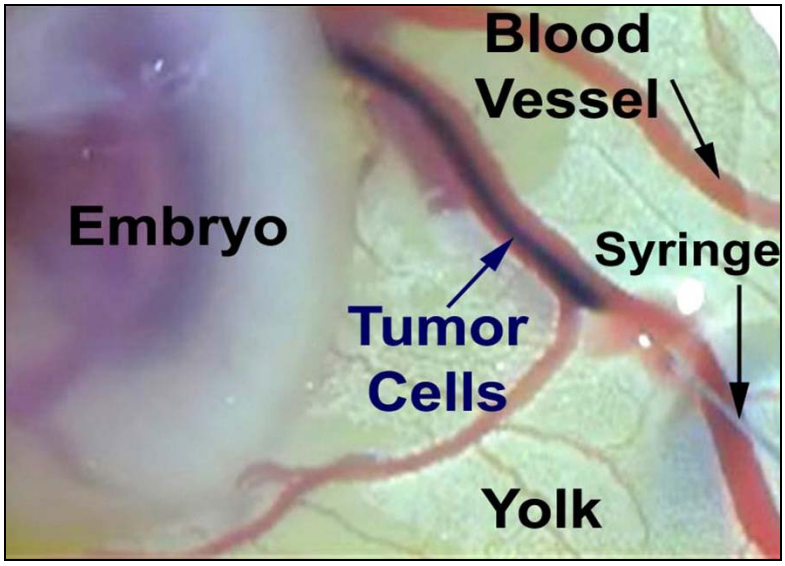

Figure 10. Injection of Cells into Chick Embryo Vasculature. An E5 chick embryo being injected with tumor cells colored with fast green. The embryo and blood vessels sit on top of the yolk at E5, allowing for injection into the extra-embryonic blood vessels. Source: Hansen, 2006.

2.4 Recovery of MDA-MB-231 Cells from the Brain and Liver

2.4.1 Preparation of E9 Brains

Embryos which had been injected at E5 were sacrificed at either E9 or E15 for colony analysis. The whole brain was removed. The pia mater, the innermost layer of the meninges surrounding the brain was left intact on brains of embryos injected with $5x10^4$ cells, but was removed in later experiments from the brains of embryos injected with $5x10^3$ cells. Figure 11a shows an E9 brain with the pia removed. Brains were minced in 1x calcium and magnesium free Tyrode's saline solution (CMF). The minced brains were centrifuged at 800rpm for approximately 5 minutes and the CMF solution was removed.

1.5mL of 0.25% trypsin was added, and the brains were incubated for 20 minutes in a 37^{o}C water bath. Trypsinized organs were placed on ice for several minutes to cool. 1.5mL of cold soybean trypsin inhibitor/DNase I (SBTI/DNase) was added, and brains were again centrifuged and the supernatant aspirated. 2.0mL of SBTI/DNase was added, and brains were dissociated to single cells by trituration (pipetting up and down using a sterile Pasteur pipette).

The dissociated tissue was plated in DMEM with 10%BGS, 1% pen/strep stock, 1%L-glutamine stock, and 200µg/mL G418 (CellGro). Tissue was cultured in G418 for enough time that the neural tissue was killed by G418, and the remaining neomycin resistant MDA-MB-231 cells formed colonies large enough to be counted (approximately 10-14 days). Figure 11b shows a plate of X-Gal stained colonies of MDA-MB-231 cells recovered from an E9 brain, after approximately 14 days of treatment with G418, and Figure 11c is a magnified image of a single colony of LacZ expressing MDA-MB-231 cells showing blue nuclei following staining.

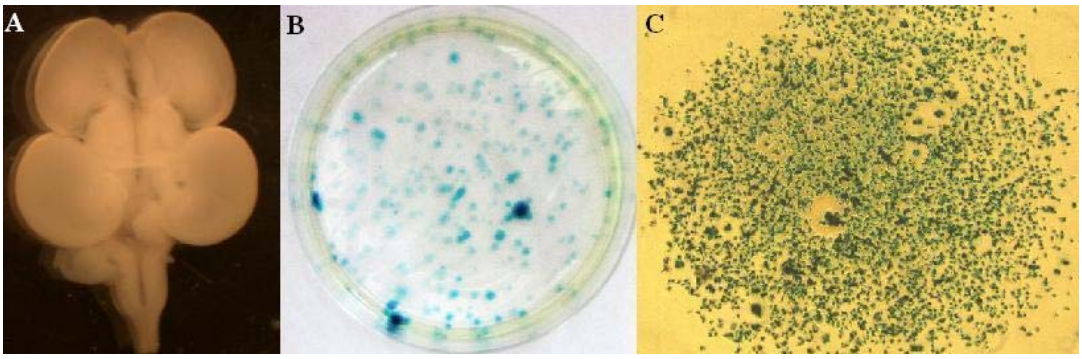

Figure 11. Analysis of Whole Brains. A) A brain dissected from an E9 embryo with the pia removed. B) A plate of G418-resistant colonies of MDA-MB-231 cells fixed and X-Gal stained. C) A magnified image of a single colony of G418-resistant MDA-MB-231 cells after X-Gal staining.

2.4.2 Preparation of E15 Brains and Livers

Whole brains and whole livers were removed from embryos sacrificed at E15. The pia mater was removed from the brains. Organs were minced in 1x CMF-Tyrode's solution and prepared in the same manner as E9 brains with one additional step to aid in dissociation. Prior to trypsinizing, 2mL of the neutral protease dispase (5mg/mL; Boehringer Mannheim Corporation) was added to each organ. Dispase is, like trypsin, a protease, but, unlike trypsin, it is gentle enough that tissue can be left in a dispase solution for a longer time without harming cells. Tubes containing the minced organs in dispase were placed on a rocker at room temperature for one hour. Following dispase treatment, E15 organs were trypsinized in the same manner as E9 brains.

Livers were diluted 1:10 so that 10% of the liver tissue was cultured, and whole dissociated brains were cultured in DMEM with 10%BGS,

1% pen/strep, 1%L-glutamine, and 200µg/mL G418. Organs were plated to allow sufficient time for drug selection of neomycin resistant MDA-MB-231 cells (approximately 12-16 days).

2.4.3 Drug Selection for Serial Injections

To establish a brain-seeking subline of cells, E5 chick embryos were injected with $5x10^3$ cells from the parental cell line, MDA-MB-231-0x. Whole brains of E9 embryos were dissected and the pia was removed. Brains were dissociated to single cells and cultured with G418 to allow for drug-selection of the resistant cell line. These cells were maintained in medium supplemented with 200µg/mL G418 for several weeks to ensure that only MDA-MB-231 cells remained, and then re-inoculated into the extra-embryonic vasculature of an E5 chick embryo. This procedure was repeated several times, resulting in several subpopulations of cells, MDA-MB-231-1x, -2x, -3x, and -4x (where #x indicates the number of times the cells have been isolated from the brain).

2.4.4 X-Gal Staining and Quantitation of Colonies

Following drug selection with G418, plates of colonies of MDA-MB-231 were stained with X-Gal for visualization. Plates of colonies were fixed in 2mL of 1% formaldehyde (ACS grade) in 1x PBS for 30 minutes. The formaldehyde was removed, and plates were rinsed twice with 1x PBS. Fixed plates were stained with 3mL of X-Gal solution (Roche Diagnostics; 1mg/mL X-Gal, 16mM potassium ferricyanide, 16mM potassium ferrocyanide, 2mM $MgCl_2$ in 1x PBS) overnight at room temperature. The number of stained

colonies on each plate was counted. The number of colonies detected was assumed to be representative of the number of MDA-MB-231 cells that extravasated into the brain. This method allowed for a quantitative comparison of metastasis between different variables.

2.5 Analysis of L1 Expression by Western Blot

Total cell extracts from MDA-MB-231-0x and MDA-MB-231-4x cells were prepared by lysing cells in RIPA (Radio Immuno Precipitation Assay) lysis buffer with a Complete Mini Protease Inhibitor Cocktail tablet (PI; Roche Diagnostics). Protein concentrations were determined using the bicinchoninic acid (BCA) assay kit (Pierce). Serial dilutions of 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0µg/mL bovine serum albumin (BSA) in RIPA/PI were prepared. Absorbance was measured and a standard curve was constructed. Absorbance of the prepared cell lysates were measured and compared to the standard curve in order to determine the total protein concentration.

Once the total protein concentration had been determined, the cell lysates were prepared with lithium dodecyl sulfate (LDS). Proteins were denatured in a 70^{o}C water bath for 10 minutes. Equal amounts of total protein (16.2µg) were loaded into each lane. NuPage MOPS SDS PAGE Running Buffer (Invitrogen) diluted 1:20 with distilled water was used in running the gel.

Prepared protein samples and MagicMark (Invitrogen) were loaded into the lanes of a Nupage 4-12% Bis-Tris gel (Invitrogen), and the gel was run at 120V for 2 hours. Following electrophoresis, a PVDF membrane (Invitrogen) was soaked in methanol and sandwiched with the ge in NuPage

Transfer Buffer (Invitrogen) and 10% methanol in distilled water. The transfer was run at 30V overnight in the cold room at 4^{o}C.

The next day, the membrane was placed in blocking solution of 1xPBS + 0.01% Tween-20 (PBST) and 5% non-fat powdered milk for 2 hours at room temperature on a rocker. The membrane was then incubated with a 1:1000 dilution of the primary antibody, mouse monoclonal antibody to L1, UJ127 (GeneTex), at room temperature for one hour on a rocker. The membrane was rinsed three times for 15 minutes each in PBST at room temperature on a rocker. The membrane was then placed in blocking solution with a 1:5000 dilution of peroxidase-labeled goat anti-mouse secondary antibody for 2 hours at room temperature on a rocker, and then rinsed three times for 15 minutes each in PBST.

The membrane was stripped using Restore Plus Western Blot Stripping Buffer (Pierce). The membrane was then re-probed with anti-β-tubulin antibody (5.7mg/mL, ascites, Developmental Studies Hybridoma Bank) as a loading control (1:10000 dilution).

Enhanced chemiluninescence (ECL) blotting substrate was prepared (Pierce) by mixing equal parts of luminol enhancer solution and peroxide solution. The membrane was immersed in ECL for approximately one minute. The membrane was developed onto an autoradiography film (ISC BioExpress).

2.6 Determination of Doubling Times

MDA-MB-231 cells were trypsinized in 0.05% trypsin until the cells had detached from the bottom of the culture dish and separated into single

cells (approximately 5 minutes). 5mL of media were added, and the number of cells in the solution was counted using a hemacytometer. The cell solution was centrifuged at 800rpm for 5 minutes, and the supernatant aspirated. The cells were resuspended in the necessary volume of medium to achieve a final cell concentration of $6x10^5$cells/mL.

1mL of cell solution was added to a 60mm dish and 5mL of media added. Cells were incubated in a humidified chamber at 37^oC and allowed to proliferate for 4 days. After 4 days, the cells were trypsinized and counted as before, and the growth over four days analyzed. Each experiment was performed in triplicate.

Chapter 3

RESULTS

3.1 Assessment of the Sensitivity of the Chick Embryo System

The sensitivity of the chick embryo system to breast cancer metastasis to the brain was analyzed by initially injecting a large number of MDA-MB-231 cells ($5x10^4$ cells in 5μL) into the extra-embryonic vasculature and performing colony analysis as described in Materials and Methods, and then reducing the number of cells injected ten-fold ($5x10^3$ cells in 5μL) and comparing the number of colonies detected. The number of colonies detected was assumed to represent the number of MDA-MB-231 cells which extravasated to the brain. Chick embryos were injected at E5 and brains were dissected at E9. For this set of experiments, the pia mater (inner layer of the meninges) was left intact.

It was expected that when the number of cells injected into the vasculature was decreased ten-fold, the number of colonies detected after drug-selection would also decrease approximately ten-fold. However, as Figure 12 shows, the number of colonies detected following injection of $5x10^3$ cells versus that detected following injection of $5x10^4$ cells was not decreased ten-fold. Rather, the number of colonies was decreased approximately five-fold, from an average of 186.6 when $5x10^4$ cells were injected to an average of 37.1 colonies when $5x10^3$ cells were injected.

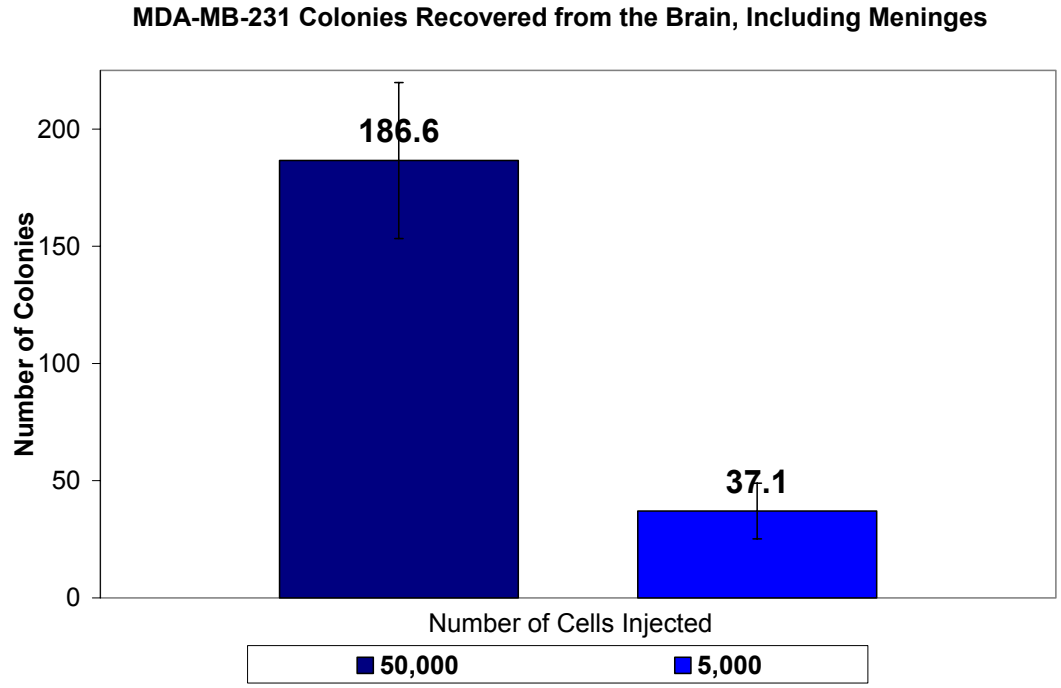

Figure 12. Colonies of MDA-MB-231 Cells Isolated from Brain and Pia. A graph of the average number of colonies present following drug-selection of E9 brain and pia tissue. The bar on the left represents the average number of colonies after injection of $5x10^4$ cells, and the bar on the right represents the average number of colonies after injection of $5x10^3$ cells. $5x10^4$ cells: n=16. $5x10^3$ cells: n=14. Bars shown are SEM.

The pia was removed for all dissections performed after this experiment (including serial injection analyses of E9 and E15 brains). Figure 13 shows the average number of colonies detected after injection of $5x10^3$ cells when the pia was removed compared with the number of colonies

detected after injection of the same number of cells with the pia intact. When just the brain tissue was dissociated and G418-treated, the number of colonies was markedly lower than when both the brain and pia mater were included for colony analysis.

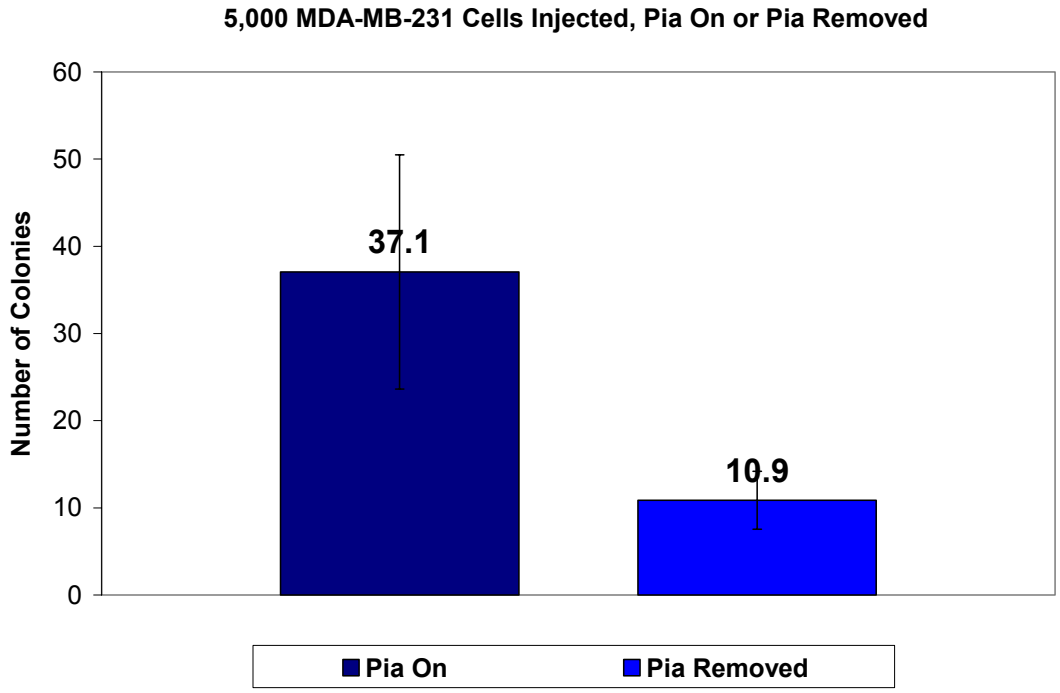

Figure 13. Colonies Analysis with Pia On or Removed. A graph of the average numbers of colonies present following drug-selection of E9 brains, either with the pia intact or removed. The bar on the left represents the average number of colonies after injection of $5x10^3$ cells when the pia is left on, and the bar on the right represents the average number of colonies after injection of the same number of cells with the pia removed. Pia on: n=14. Pia removed n=8. Bars shown are SEM.

3.2 Serial Injection of MDA-MB-231 Cells

Serial injection experiments were performed as described in Materials and Methods. For this set of experiments, $5x10^3$ cells were injected into each E5 chick embryo. This particular number of cells was decided on based on the numbers of colonies isolated from the parental cell line when $5x10^4$ cells were injected. After injection of $5x10^4$ MDA-MB-231 cells, an average of 186.6 colonies was isolated. It was unknown whether the number of colonies of re-injected cells would increase, decrease, or remain the same. If the number of colonies increased to greater than 186.6 following serial injections, it would become difficult to count and colonies may overlap one another.

Embryos were dissected at E9 and the pia was removed. MDA-MB-231 cells were successfully isolated from the brains of E9 chick embryos by drug-selection using G418. These cells isolated from brains could then be re-injected and isolated multiple times. Plates of dissociated brains yielded colonies of cells which could be quantified using the previously described procedure.

3.2.1 Colony Analysis of E9 Brains

In order to begin to understand the changes that occur in a cell population that has been isolated from the brain multiple times, quantitative colony analyses were performed. The numbers of colonies detectable following drug-selection of dissociated E9 brains were compared. Most plates of colonies were analyzed after being cultured with G418 for approximately 14 days. Due to differences in the growth rates of the serially injected cells (see

section 3.3), some plates of cells were cultured with G418 for more than 14 days to ensure that colonies were large enough to count following fixation and X-Gal staining.

When $5x10^3$ parental MDA-MB-231 cells were injected into the chick embryo vasculature, an average of 10.9 colonies was counted. Cells that had been injected and isolated from the brain twice (MDA-MB-231-2x) produced an average of 11.2 colonies after drug-selection when $5x10^3$ cells were injected. Surprisingly, re-injection of cells that had been through the brain three times (MDA-MB-231-3x) resulted in only an average of 4.3 colonies per brain, and injection of MDA-MB-231-4x cells resulted in an average of 6.0 colonies. However, the 4x injection experiments could not be repeated as many times as the 0x, 2x, and 3x injections due to time constraints, and the average of 6.0 colonies is representative of only 4 brains (for 0x, 2x, and 3x, the n's are 8, 13, and 12, respectively). The average colony numbers are displayed in Figure 14 below.

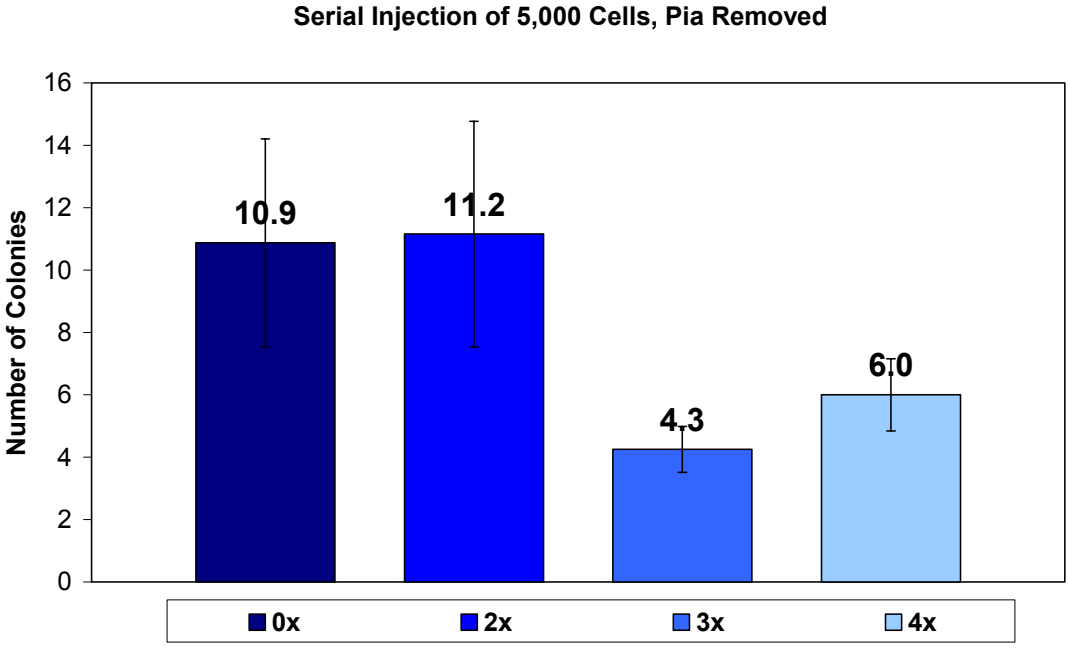

Figure 14. Colony Analyses from Serial Injections. A graph of the average numbers of colonies present following serial injection and isolation of MDA-MB-231 cells from E9 brains. 0x refers to the original parental cell line; 2x refers to cells that have been injected and isolated from the brain twice; 3x, three times; and 4x, four times. 0x: n=8. 2x: n=13. 3x: n=12. 4x: n=6. Bars shown are SEM.

3.2.2 Colony Analysis of E15 Brains and Livers

In order to determine whether cells that have been isolated from brain metastases and re-injected metastasize specifically to the brain (that is, metastasize only to the brain and not to other organs), colony analysis was attempted on livers and brains of E15 embryos. The liver was chosen for this comparison because it is one of the top three sites of breast cancer

metastasis. E9 embryos could not be used for this experiment because the liver is not yet developed at E9. Because colony numbers from E15 livers cannot be directly compared to E9 brain colony numbers (due to the additional 6 days of incubation of cancer cells), E15 brains were also analyzed for a direct comparison.

Colony analysis of E15 livers and brains was unsuccessful. Most embryos that were injected with $5x10^3$ cells died before reaching E15, whether they were injected with the parental cell line or with re-injected cells. The brains and livers of those that survived were initially analyzed by the same method as E9 brains.

When dissociated liver tissue was cultured with G418, the plate immediately became so full of cells that they formed a confluent layer over the entire plate. These cells did not appear to be liver tissue, as they were not killed by G418 even after several days in culture. These plates were fixed and stained with X-Gal, and the entire plate of cells turned blue, suggesting that a very large number of MDA-MB-231 cells had metastasized to the liver. G418 killed everything in the plates of dissociated E15 brains after several days. These plates were left for several more days (a total of 14-21 days) and X-Gal stained in case there were any colonies that were not observed under a microscope. However, after staining, no blue colonies were detected. No differences were observed between MDA-MB-231-0x and MDA-MB-231-3x subpopulations in these experiments. For both subpopulations, liver analysis showed high numbers of cancer cells, and brain analysis did not work because everything in the cultures died.

3.3 Changes in Morphology and Growth

The morphology and growth rates of MDA-MB-231 cells changed drastically after being re-injected multiple times. The most dramatic changes occurred in the MDA-MB-231-4x subpopulation of cells. MDA-MB-231-2x cells did not show any obvious difference from MDA-MB-231-0x cells. MDA-MB-231-0x cells were fibroblastic in appearance. They were often only slightly flattened with one or two very long extensions protruding from the cytoplasm. Figure 15 shows the differences in cell morphology after re-injection, and Figure 16 shows the differences in growth rates. MDA-MB-231-2x and -3x cells appeared slightly smaller than MDA-MB-231-0x cells, and they tended to cluster together. These cells showed a faster growth rate than the original cell line. MDA-MB-231-4x cells divided much slower than MDA-MB-231-0x cells, and they did not grow well in sparse conditions, in contrast to MDA-MB-231-0x cells which grew quickly whether the plate was sparsely or densely populated. Additionally, MDA-MB-231-4x cells were much flatter than MDA-MB-231-0x cells.

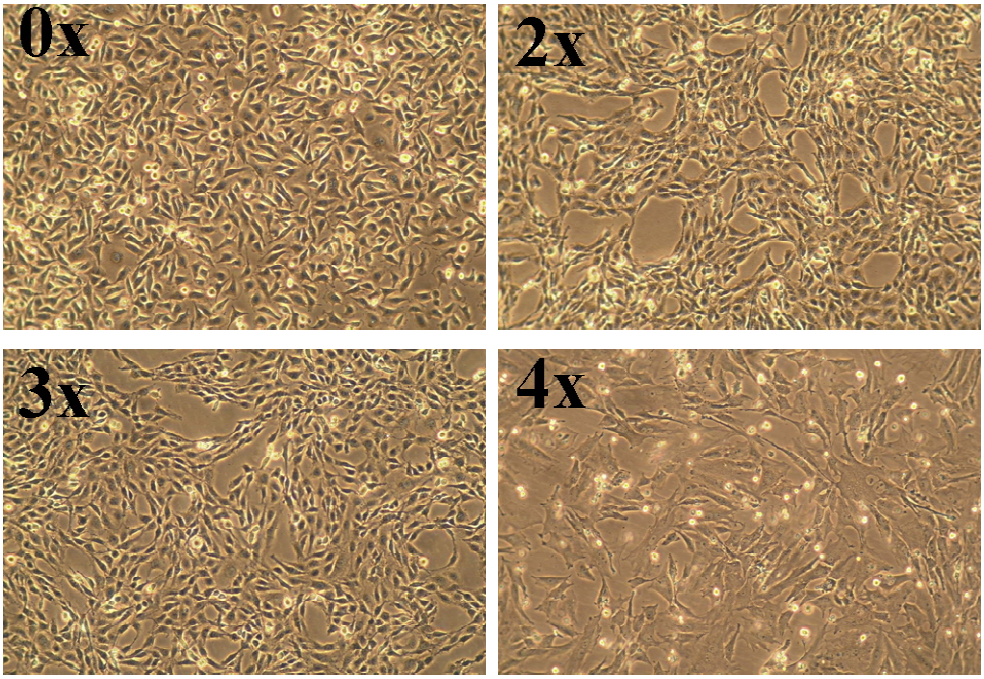

Figure 15. Morphological Changes. Images of parental cell line and re-injected cells 48 hours after $6x10^5$ cells were plated, showing changes in morphology and differences in cell density. MDA-MB-231-4x showed the most distinct changes in cell morphology and growth rate.

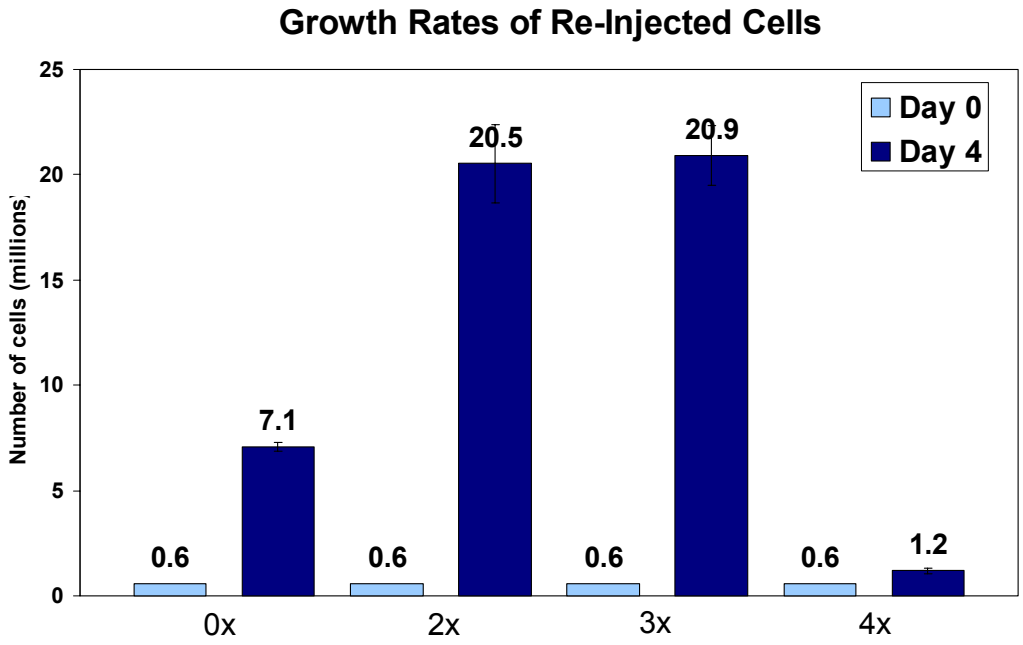

Figure 16. Changes in Growth Rates. $6x10^5$ cells were plated (Day 0) and grown for four days. The dark blue bars represent the average number of cells present on Day 4. Each experiment was performed in triplicate. Plates of MDA-MB-231-2x and -3x cells had higher numbers of cells than either MDA-MB-231-0x or -4x. Plates of MDA-MB-231-4x cells showed the smallest number of cells on Day 4. Bars shown are SEM.

3.4 L1 Expression

In order to determine whether the cell adhesion molecule L1 contributes to brain-specific metastasis, expression of L1 by the original cell line (MDA-MB-231-0x) and the population of cells which had been isolated from the brain the most times (MDA-MB-231-4x) was compared by western blot.

Following electrophoresis, the separated proteins were transferred to a PDVF membrane and probed using a monoclonal antibody to L1, UJ127. MDA-MB-231-0x cells showed detectable levels of L1, while MDA-MB-231-4x cells appeared to have lost L1 (Figure 17).

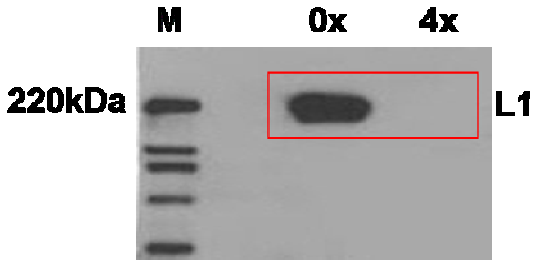

Figure 17. Western Blot for L1. Cell extracts of MDA-MB-231-0x and -4x cells were separated and probed using anti-L1 antibody. Each lane contained 16 µg total protein. M is a protein molecular weight standard; the top band is 220kDa. Exposure time 1 minute.

The membrane was reprobed for β-tubulin, a housekeeping protein, used as a loading control, using an antibody that detects human β-tubulin (mouse anti-human β-tubulin). While β-tubulin was detected in the MDA-MB-231-0x cell lysate, it could not be detected in the MDA-MB-231-4x cell lysate sample, for unknown reasons.

Chapter 4

DISCUSSION

4.1 Sensitivity of the Chick Embryo Model System

A previous study performed by Patricia Hansen in the Galileo lab showed that when MDA-MB-231 and MDA-MB-435 cells were injected into the extra-embryonic vasculature of chick embryos, tumors would form in the brain and in other organs, such as the heart, kidney, and lungs, and that these metastases could be quantitated by colony analysis and FACS analysis (Hansen, 2006 and unpublished results). FACS analysis of GFP-expressing MDA-MB-435 cells recovered from brain metastases provided results that were highly variable. Colony analysis of LacZ and Neo^r expressing MDA-MB-231 and MDA-MB-435 cells proved to be a more sensitive method. Larger numbers of cells could be counted, and there was less variability in the results from colony analyses than from FACS analyses.

In order to determine the usefulness of the chick embryo *in vivo* model system, the sensitivity of the system was tested by injecting both large and small numbers of cells and comparing the number of colonies that could be counted after drug-selection. Injection of only $5x10^3$ MDA-MB-231 cells produced metastases that could be counted after dissociation of E9 brains and drug-selection for the G418 resistant cells. This suggests that the chick embryo is a sensitive system for studies of breast cancer metastasis to the

brain. In similar studies of metastasis using mice, 10^6 cells are typically injected into the vasculature (Mukhopadhyay et al., 1999; Price and Zhang, 1989). The results of this study show that far less cells are required for metastases to form in the brains of chick embryos.

A comparison of the numbers of colonies that formed following injection of $5x10^4$ or $5x10^3$ cells per embryo showed a decrease from an average of 186.6 colonies to an average of 37.1 colonies when the pia was left covering the brain during dissection. It was expected that the number of colonies would decrease by ten-fold when the number of cells injected was decreased ten-fold. This was not the case, however, as the number of colonies detected following injection of $5x10^3$ cells was approximately 5-fold fewer than the number detected following injection of $5x10^4$ cells. It was hypothesized that the larger than expected number of colonies was due to cells localized within the meninges. In order to test this, the pia was removed during dissection of the brain, and the number of colonies that formed was compared to the number that formed when both the brain and the pia were analyzed. When the pia was removed, an average of 10.9 colonies was detected, compared to 37.1 with the pia on. This demonstrated that a larger number of cells were in the meninges than in the brain itself, but cells could still be detected deeper in the brain tissue following injection of only $5x10^3$ MDA-MB-231 cells.

The results of Hansen's previous study also showed that MDA-MB-231 cells preferentially localized to the meninges. Qualitative analysis of whole brains (meninges intact) stained with X-Gal showed that more MDA-

MB-231 cells were present in the meninges than in the deeper brain tissue. It was unknown, however, if this was due to breast cancer cells preferentially extravasating into the meninges, or if it was due to X-Gal failing to reach cells deep within the brain parenchyma. The quantitative results of the current study show that MDA-MB-231 cells do, in fact, appear to preferentially localize to the meninges.

A possible explanation for the large number of cells localized within the meninges is that MDA-MB-231 cells are within the blood vessels of the meninges. Due to the short 4-day incubation period, it could be that the cells have not had ample time to extravasate into the brain. Another possibility is that the cells preferentially localize to the meninges. It may be easier for MDA-MB-231 cells to extravasate into the meninges compared to deeper into the brain parenchyma due to favorable microenvironment conditions. The meninges are largely composed of connective tissue, and thus there are large numbers of fibroblasts in the meninges. Meningeal fibroblasts have been shown to express basic fibroblast growth factor (bFGF) (Mercier, 2001). MDA-MB-231 cells have been shown to express a receptor for bFGF, FGFR1 (Cailliau, 2005). This may create a favorable tumor-microenvironment interaction between MDA-MB-231 cells and the meninges. Although meningeal metastasis is a rare occurrence in humans, breast cancer is the primary tumor most frequently associated with meningeal carcinomatosis, so it is possible that the MDA-MB-231 human breast cancer cell line preferentially metastasizes to the meninges (Boogerd, 1995).

4.2 Serial Injections

One of the main goals of this study was to isolate a subpopulation of MDA-MB-231 cells that would metastasize specifically to the brain in the chick embryo. Although colony analyses of metastases to liver and brain in E15 chick embryos did not work as well as hoped, it appeared that even MDA-MB-231 cells that had been through the brain four times (-4x) were still capable of metastasizing to the liver, and were not brain-specific. Despite this, there were differences between the original cell line and the re-injected sublines that could be measured by various mechanisms. It would be useful to further analyze these sublines, especially the expression of specific proteins in order to determine which characteristics of the re-injected sublines may impart them with enhanced metastatic capability.

4.2.1 Colony Analyses of Serially Injected and Isolated Cells

Initially, re-injected cells did not show a significant difference in the number of colonies detected following drug selection compared to the original cell line. Injection of $5x10^3$ MDA-MB-231-0x cells resulted in an average of 10.9 colonies, and injection of the same number of MDA-MB-231-2x cells resulted in an average of 11.2 colonies. Although it seems counter-intuitive that re-injection would result in fewer colonies being detectable after drug-selection, the results of this study are in accord with the preliminary work performed by Patricia Hansen using MDA-MB-435 cells (Hansen, 2006). Hansen showed that the number of colonies detected following injection of MDA-MB-435 cells steadily decreased as the cells were isolated from the brain more times (up to two times).

In order to determine whether re-injected cells would metastasize specifically to the brain, and not to any other organs, colony analyses were attempted on E15 livers and brains. It was hypothesized that after multiple injections and isolations, fewer cells would reach the liver than the brain, and fewer colonies would be counted when the liver was dissociated compared to the brain. However, these experiments were unsuccessful, and while it appeared that a large number of MDA-MB-231 cells were present in the liver, G418 treatment of dissociated E15 brains did not result in countable colonies of drug-resistant cells but rather it seemed that everything in culture died.

It is hypothesized that the apparent lack of MDA-MB-231 cells in the E15 brain was due to the difficulty of dissociation of E15 brain tissue. At E15, neurons are tightly intertwined, making dissociation difficult. MDA-MB-231 cells may have been within tangles of neurons that were not separated by trypsinization, and may have died due to a lack of nutrients. To compensate for this, dispase was used in later experiments in addition to trypsin to aid in separation of cells from one another. Following treatment with dispase and trypsin, tissues were much easier to dissociate by trituration, but the results were not any different than when treated with trypsin alone.

Experiments with nude mice have demonstrated that subpopulations of cells that have been isolated from the brain multiple times metastasize specifically to the brain, and do not form metastases in other organs (Yoneda et al., 2001; Kim et al., 2004). Based on this, it was expected that eventually serially injected cells recovered from chick embryo brains would metastasize only to the brain, and at this point no colonies would be

detectable after analysis of the liver. Although these experiments were largely unsuccessful, it appears that MDA-MB-231-3x cells (the most re-injected subpopulation used for the E15 experiments) do still metastasize to organs other than the brain. Since it took three serial injections and isolation of MDA-MB-231 cells in one study (Kim et al., 2003) and six in another study (Yoneda et al., 2001) before cells metastasized specifically to the brain, it may be that more injections and isolations from brain metastases are necessary for establishment of a brain-seeking subline.

Another possible explanation for the failure of serially injected and recovered MDA-MB-231 cells to metastasize specifically to the brain is that, due to differences between the chick embryo system and rodents, cells will not become brain-specific. This could be tested by injecting brain-specific MDA-MB-231 cells, such as those isolated from nude mice by the Yoneda and Kim labs, into the chick embryo. Then, different organs could be analyzed to see if these cells metastasized specifically to the brain in the chick embryo.

4.2.2 Changes in Cell Morphology and Growth Rates

It was initially thought that the lower colony numbers observed following injection of MDA-MB-231-3x and -4x cells were due to slower growth. $6x10^5$ cells from each subpopulation were plated and incubated for four days in order to compare growth rates of the different subpopulations. After four days, plates of MDA-MB-231-2x and MDA-MB-231-3x cells had significantly more cells than plates of MDA-MB-231-0x cells, demonstrating faster growth than the original cell line. This does not correlate with the lower colony numbers observed after injection of MDA-MB-231-3x cells. However, these

growth rate experiments only compare growth *in vitro*, and it is possible that growth differs *in vivo*, in a different microenvironment. In contrast, MDA-MB-231-4x cells showed less growth *in vitro* than MDA-MB-231-0x cells after four days of growth, as well as fewer colonies detected after injection *in vivo*.

Although testing *in vitro* showed an increase in growth rates of re-injected cells compared to previous un-injected cells (except in the case of MDA-MB-231-4x), these cells may demonstrate different characteristics *in vivo*. This could be tested by creating an *in vitro* environment that more closely resembles that of the chick embryo brain. For example cells could be grown on ECM more similar to that of the chick embryo brain, or growth rates could be measured in the presence of factors that may be present in the chick embryo brain that are not present *in vitro*. If this were the case, and re-injected cells grow slower *in in vivo* than the original cell line, this could mean that the re-injected cells are beginning to enter a state of dormancy. This would explain the smaller colony numbers detected following injection of cells isolated from brain metastases. Once in the brain, the re-injected cells may form as many micrometastases as the original cell line, but the tumors may not grow as large in the four day incubation period as those formed from the original cell line. Colony analysis does not measure the number of tumors present in the brain, but rather the total number of cells, so the smaller colony numbers may be an effect of the re-injected cells producing smaller tumors than the original cell line. One possibility is that these cells are becoming dormant.

Dormancy is a poorly understood phenomenon in which tumor cells remain within an organ for a long period of time with little or no net growth (Naumov et al., 2002). Changes in the microenvironment may then stimulate the cells to re-enter the cell cycle and begin to divide. Angiogenic factors are also stimulated, and cells may become metastatic (Almog et al., 2009). It is possible that some factor in the growth medium of the sublines has stimulated the growth of cells that would otherwise remain dormant in the microenvironment of the chick embryo brain, and the growth rates measured *in vitro* are not representative of growth in the chick embryo brain.

4.2.3 L1 Expression

While the original MDA-MB-231 cell line showed detectable L1 protein, MDA-MB-231-4x cells appeared to have lost L1 expression, as no protein was detected by western blot. However, probing of the PVDF membrane for the housekeeping protein β-tubulin showed that the MDA-MB-231-4x cells appeared to have lost expression of this protein. One possible explanation for this, other than a loading problem, is that chick cells escaped G418 treatment, and the MDA-MB-231-4x cells are not pure MDA-MB-231 cells. The antibody used for this assay has not been tested in chick cells, and it may not detect the chick form of β-tubulin.

To test whether this subpopulation may contain cells other than MDA-MB-231 cells, a confluent plate of MDA-MB-231-4x cells was fixed in formaldehyde and stained with X-Gal. After staining, most of the plate was white with a few clusters of blue cells, indicating that the culture contained a mixed population of LacZ+ MDA-MB-231 cells and LacZ- cells of unknown

origin. A solution for this problem would be to either culture the cells with a higher concentration of G418 and see if the LacZ- cells died off, or to re-inject the MDA-MB-231-3x cells and begin the MDA-MB-231-4x subline over again.

MDA-MB-231-2x and -3x cells did not show the same X-Gal staining pattern as MDA-MB-231-4x cells, and these plates appear to contain only LacZ+ MDA-MB-231 cells. In order to determine whether re-injection has an effect on L1 expression, and therefore whether L1 plays a role in metastasis to the brain, another western blot will need to be run to compare MDA-MB-231-0x cells to MDA-MB-231-2x and -3x cells. However, there was not time to perform this experiment.

4.3 Conclusions

Based on the results of the liver and brain colony analysis comparisons, it seems that more rounds of injection and isolation are necessary before a brain-seeking subline of cells can be established. Studies with mice required either three or six rounds of injection and isolation in order for brain-specific cells to be isolated. Because of differences between the chick embryo system and mice, for example differences in the microenvironment of brain or blood vessels, it could be that more rounds of injection are required in the chick embryo in order for a brain-specific cells to be isolated, or it could be that it is not possible to isolate brain-specific MDA-MB-231 cells using the chick embryo.

Even though brain-specific MDA-MB-231 cells could not be isolated in this study, re-injection did appear to cause changes to the properties of MDA-MB-231 cells. Injection of MDA-MB-231 cells that had been isolated

from the brain multiple times resulted in fewer colonies detectable after selection with G418. Despite the faster growth rates of MDA-MB-231-2x and -3 cells compared to the original cell line, the best explanation for the smaller colony numbers is that the cells are entering a state of dormancy, characterized by slower growth *in vivo*. Conditions *in vitro* are vastly different from those *in vivo*, and in order to determine whether these cells grow slower *in vivo*, *in vitro* conditions would need to more closely resemble those in the chick brain microenvironment, or growth would need to be directly measured *in vivo*.

Additionally, this study has demonstrated that the chick embryo is a sensitive model system for studying metastasis and allows for relatively quick quantitative analysis of breast cancer metastasis to the brain. This model system would be useful for investigating the molecular mechanisms of metastasis to the brain. Using the colony analysis method, expression of proteins suspected of having a role in metastasis could be manipulated and the effects on metastasis could be quantified. The effect of L1 on metastasis to the brain could be directly measured by introducing a vector that expresses high levels of L1 protein into cells, and by knocking down expression of L1 with siRNA or blocking its function with antibodies, and then injecting into the chick embryo. Brain metastases could be quantitated and compared by colony analysis. If higher levels of L1 resulted in more metastases, and lower levels of L1 resulted in fewer brain metastases, this would demonstrate that L1 has a role in metastasis to the brain. Additionally, cleavage of L1 by ADAM10 could be blocked with metalloproteinase inhibitors, as has been done with

melanoma cells, to determine whether shed L1 is necessary for metastasis to brain.

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