EXPLORING THE EFFECTS OF CELLULAR STRESS

ON BREAST CANCER METASTASIS

By

Angela Lewis

Joseph Sucic, Ph.D.
Thesis Advisor

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Approved by:

____________________       ______________________
Joseph Sucic, Ph.D.        You-shin Chen, Ph.D.
Thesis Advisor        Committee Member

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Jessica Kelts, Ph.D.
Committee Member

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ABSTRACT

Cancer is characterized by two major features. The first is uncontrolled cell proliferation and the second is metastasis, which is the main cause of cancer related deaths. The causes of uncontrolled cell growth have been studied thoroughly and consequently the underlying mechanisms are fairly well understood. In contrast, the causes of metastasis are significantly less well understood. We are suggesting that metastasis occurs as a result of cancer cells removing themselves from the inhospitable environment inside tumors by employing normal cellular defense mechanisms. We grew MCF-7 and MDA-MB-468 breast cancer cells in cell culture and exposed them to heat and hypoxia stress. Western blot analysis of the cell lysates showed an increase in furin expression in the heat and hypoxia stress conditions. The MCF-7 cells showed a decrease in PACE4 expression in the stress conditions. Invasion assay analysis of the MDA-MB-468 and MCF-7 cells under our experimental cell culture condition showed an increase in cell migration with the stress exposed cells. The Western blot results paired with the invasion assay findings suggest that heat and hypoxia stress can promote increased invasiveness of the breast cancer cells and may be an underlying mechanism that drives cancer metastasis. Further exploration of this hypothesis using 3-dimensional cell culture and an alternative method of testing MMP activity or expression would bolster our understanding of the biological mechanisms involved in cancer metastasis, leading to the identification of novel diagnostic or therapeutic targets in treating aggressive cancers.
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Chapter One: Overview and Background of the Project

Introduction

I. Background and History

A. Breast Cancer

Cancer remains one of the most difficult medical conditions to treat in the twenty-first century and is the second leading cause of death in America. The American Cancer Society predicted that 1.9 million new cases of cancer would be diagnosed and that 609,360 deaths were expected in America in 2022 (1). This condition affects a large portion of the human population and has consequently been an area of interest to researchers throughout the past century.

The proper and efficient treatment of cancer is dependent on a thorough understanding of the biology of cancer cells. Cancer is a class of diseases that are characterized by two major features. The first is that cancer cells grow and divide at a very fast rate without normal physiological stimulants (2). These cells have been immortalized because they can undergo infinite cycles of cell division, whereas normal cells can only divide a limited amount of times. This is called uncontrolled cell proliferation or uncontrolled cell growth. The other characteristic of cancer cells is their ability to invade other tissues and areas in the body, referred to as cell metastasis. Tumor cells are not truly cancerous if they exhibit uncontrolled cell division but are not metastatic. Furthermore, non-metastatic tumors are typically not life threatening (3). In contrast, metastasis is the main cause of cancer related deaths (4).

The causes of uncontrolled cell growth have been studied thoroughly during the last 60 years and, consequently, the mechanisms are fairly well understood. Normal cell growth is regulated by the protein products of both tumor suppressor genes and proto-oncogenes (5). The protein products of tumor suppressor genes help regulate cell division in normal cells by
preventing cell division and initiating apoptosis when appropriate (6). Conversely, proto-
oncogene protein products promote cell division and inhibit apoptosis (7). When these two genes
are being expressed properly, the protein products function to control cell division and ensure
that the cell is dividing as it should. However, when this mechanism of control is disrupted by
mutation of these genes, proto-oncogenes mutate and become oncogenes, which promote cell
division and inhibit cell death inappropriately. This is usually a dominant mutation, which can
result in either an increase in the protein product or an altered protein product. In either case, the
activated oncogenes promote abnormal cell division, which results in the development of a tumor
(5, 7). Furthermore, tumor suppressor genes may also be mutated or epigenetically silenced via
methylation of their promoter regions, in which case they will no longer suppress cell growth and
induce apoptosis when the cell would normally require it. Past research has shown that mutations
in these two types of genes cause the uncontrolled cell proliferation exhibited by cancer (5, 8).

The causes of metastasis are less understood and no true consensus has been reached that
fully explains metastatic cell behavior. One common hypothesis is that cancer cells accrue a
large amount of genetic mutations which causes them to abandon normal cellular growth patterns
and invade other tissues in the body. This hypothesis attributes metastasis to the increased
mutability of the cancer cells (2, 4). We are proposing an alternative hypothesis to explain
metastasis. We are suggesting that metastasis occurs as a result of cancer cells removing
themselves from the inhospitable environment present inside the primary tumor by employing
defense mechanisms that are normally present in the cell. Cells, including cancer cells, have the
ability to react to different stressful conditions (9, 10). There are several stress conditions present
within tumors. These include heat shock, which is an elevation in temperature, and hypoxia,
which is a lack of oxygen. Hypoxia occurs due to inconsistent vasculature in tumors and densely
growing cells resulting in poor overall oxygenation (9, 11). There is also increased acidity in
tumors, acidosis, which is a result of elevated metabolic activity. We are suggesting that these
stress conditions cause the cells’ response mechanisms to activate in order to allow the cancer
cells to escape the inhospitable conditions of the primary tumor and invade an alternative, more
hospitable environment.

When cancer cells are exposed to extremely stressful conditions, there can be dramatic
consequences for the cell. Hypoxia and heat shock are known to “turn on” or activate specific
proteins which will attempt to acclimate the cell to its environment (12). If this is not possible,
then normal cells will activate proteins that will initiate apoptosis. However, apoptosis is
typically not an option for cancer cells because this pathway is not functional causing the cancer
cells to be inappropriately immortal (9, 10). The other option that the cancer cells have is to
move to a less stressful environment. Furthermore, clinical studies have shown that hypoxia in
primary tumors correlates with an increase in tumor aggressiveness and likelihood of metastasis
(9).

These observations led us to examine the effect of heat and hypoxia stress conditions on
the expression of genes often activated for metastasis. Metastasis occurs in two phases. The first
is the physical dissociation from the primary tumor. The second phase is the adaptation of the
colonizing cancer cells to the new area that it is invading (2). In order for this to occur, the
cancer cells must first break down the surrounding extracellular matrix (ECM). To invade a
neighboring tissue, they must degrade the ECM again that surrounds the cells in the target tissue
(13). This is done by specific types of proteins called matrix-degrading metalloproteinases
(MMPs). There is another class of proteins called proprotein convertases (PCs) that regulate
MMP activity. Furin and paired basic amino acid-cleaving enzyme 4 (PACE4) are two PCs that
contribute greatly to the regulation of MMPs. Most MMPs have a cleavage site where they are activated by furin. Furin activation of MMPs was displayed by Bassi et al. (14) when they performed furin transfections and inhibition experiments, which showed that furin overexpression, both at the mRNA and protein level, correlated with increased metastasis. They also showed that increased furin expression was directly linked to increased MMP activity and increased migration of squamous cell carcinoma cell lines with invasion assays (14).

Research regarding the roles of PACE4 in cancer metastasis is controversial. Some breast cancers, prostate cancers, and nasopharyngeal carcinomas showed that elevated PACE4 expression correlated with more aggressive cancers (15, 16). Other researchers have observed that decreased PACE4 expression correlated with increased MMP activity and increased MMP expression in MDA-MB-231 breast cancer cells (17). Another study showed a decrease in PACE4 expression in ovarian cancer tissue when compared to normal ovarian tissue (18). Although the exact role that PACE4 plays in promoting or inhibiting cancer metastasis is unclear, these previous studies show that further research of both furin and PACE4 is warranted in order to better understand how their expression and activation affects metastasis. This could help researchers understand the underlying mechanisms that drive metastasis better and ultimately identify potential novel therapeutic targets that could help decrease cancer invasiveness.

**B. MMP structure and function**

MMPs are a family of proteins that are crucial in degrading and remodeling extracellular matrix (ECM) (19, 20). MMPs were first discovered in 1962, when Jerome Gross and Charles Lapiere characterized collagenase while studying metamorphosis in amphibians (21). Later, researchers discovered collagenase-1 (or MMP-1) in mammals and discovered that it initiated the
degradation of fibrillar collagens, which are a component of ECM (19, 20). MMPs became clinically significant for researchers when they were found to be upregulated in human diseases such as rheumatoid arthritis and cancer. It was also noticed that higher expression of MMPs was correlated with more severe disease. This shifted the focus of MMP activity towards implications for pathology. Although MMPs may be a valuable target for treating human disease and possibly inhibiting cancer metastasis, it is important to understand their basic roles in physiology before seeking them out as a target for treating human disease. More recent research has illustrated that MMPs are responsible for degradation and remodeling extracellular matrix in non-pathological homeostasis, tissue development and regeneration (20, 22, 23).

All 23 MMPs are zinc-dependent endopeptidases and have similarities in their overall structure. MMPs contain a 20 amino acid long signal peptide region, which is important in targeting these enzymes to be trafficked through the endoplasmic reticulum to the surface of the cell. Because MMPs are crucial for degradation of the ECM, it is important that the active MMPs are trafficked either outside the cell or to the cell membrane. The next domain is the propeptide domain which consists of approximately 80 amino acids and contains a cysteine residue that acts as a switch to deactivate the enzyme. While in the zymogen (inactive) form, the cysteine residue interacts with the zinc ion that is located on the active site in the catalytic region, blocking enzymatic activity. Ten of the MMPs contain a PC recognition sequence near the end of the propeptide where PCs, typically furin or furin-like PCs, bind and cleave it, activating the MMP. The other MMPs are activated by extracellular proteases and are also cleaved by other activated MMPs. MMPs also contain a small hinge region that connects the catalytic region to the hemopexin C-terminal domain. The other domains vary among MMPs because they help determine substrate specificity. MMP-2 and MMP-9 both have fibronectin domain inserts in
their catalytic regions which allow them to cleave type IV collagen, gelatin and elastin. The hemopexin domain allows MMP-1 to cleave collagen. Membrane-type MMPs (MT) also contain either a glycosylphosphatidylinositol (GPI) cell membrane anchoring domain or transmembrane and cytosolic domains which are typically located near the C-terminal end (20, 24).

While researchers are often interested in studying MMPs in the context of human disease, many studies have focused on understanding the normal functions of MMPs in tissue growth and regeneration (20, 25). Because of their ability to degrade and remodel ECM, MMPs influence tissue growth and the migration of cells (26, 27). In addition to their ability to degrade components of the ECM, MMPs also cleave and activate enzymes that are involved in inflammation response and are important for endometrium tissue degradation and generation in humans (20, 24). Because of the wide-reaching influence that MMPs have on growth and tissue regeneration, severe mutations in MMPs or inappropriate regulation of MMP activity have dire effects on growth and development of organisms.

C. Furin structure and function

The fur gene was discovered in 1986; however, the endoprotease furin was not discovered until 1990. It is a member of a family of nine proprotein convertases (PCs) that have since been discovered. Furin is a 794 amino-acid type 1 transmembrane protein that is primarily located in the Golgi and in the trans-Golgi network. Furin also circulates through the endosomal system to the cell surface and back to the trans-Golgi network (28, 29). The main function of furin is to endoproteolitically cleave and activate proprotein substrates in the secretory pathway, both in the endosomes and at the cell surface (29, 30). These substrates include zymogens, receptors, hormones, and cell surface proteins (28). Additionally, furin activates proteins that are
involved in bacterial and viral infections as well as neurodegenerative disorders, diabetes, atherosclerosis and, as previously mentioned, cancer (28, 29).

PCs are similar structurally and all nine PCs have a signal peptide domain that directs translocation of the enzyme into the endoplasmic reticulum (ER). PCs also have a prodomain that is important for regulating PC activity by acting as an intramolecular chaperone. Furin has an 83 amino-acid prodomain that folds the inactive catalytic domain into its active form. After this event, the prodomain is autoproteolytically excised. The propeptide remains associated with furin and acts as an auto-inhibitor while it is being transported to the late secretory pathway, where it is cleaved at a second site in order to activate furin (31). The catalytic domain of PCs contains the aspartate, histidine and serine catalytic triad and is the most conserved domain of these enzymes. The other PCs have catalytic domains that are 54% to 70% identical to furin’s catalytic domain. All PCs except for the related bacterial PCs have a P domain located directly C-terminal to the catalytic domain, which is important for enzyme activity. Furin and most of the other PCs also have a transmembrane domain and cysteine rich domain. Furin has a 56 amino acid cytoplasmic domain that is important for localization and trafficking of the enzyme (29, 31).

Since it was discovered over thirty years ago, furin has been found to have many roles in normal physiological processes such as embryogenesis and homeostasis (29, 32). Furin cleaves and activates various substrates in different locations in the cell. In the trans-Golgi network, furin plays a role in neuronal innervation by cleaving pro-β-nerve growth factor (pro-β-NGF), activating it so it binds to Trk receptors to promote neuronal innervation and survival. Without furin activation, pro-β-NGF binds to a neurotropin receptor to promote apoptosis. Similarly, furin plays a role in cartilage and bone formation during embryonic development by processing pro-bone morphogenetic protein-4 (Pro-BMP-4) (29). Also, furin processes insulin pro-receptor
into its active form (33). Furin activates substrates in the cell secretory pathway, contributing to homeostasis and cell growth; the next section discusses the role that furin plays in pathology, specifically cancer metastasis.

II. Research on the Roles of MMPs and PCs in Metastasis

A. Expression of MMPs in Cancer

Although the focus of this research project is on breast cancer metastasis, extensive research was found describing MMP activation and expression in other types of cancers. These studies were key in identifying MMP-9 as a key players in cancer metastasis and pathology. One such study focused on the expression of MMPs in colorectal cancer (CRC) and correlated increased MMP-9 expression with more severe cases of CRC and poorer patient prognosis (34).

These researchers found that MMPs are upregulated in many different types of cancers. Although MMP-1, MMP-2, MMP-7, and MMP-13 have all shown elevated expression in CRC, the majority of experiments focus on MMP-9 because research found a positive correlation between poor prognosis of CRC patients and MMP-9 expression. This led researchers to carry out more studies that linked MMP-9 expression to CRC. Both immunohistochemistry and real time polymerase chain reaction experimentation showed significant increase of MMP-9 expression in CRC tissue when compared to normal colon tissue. Experiments using quantitative zymography yielded results that suggested that increased MMP-2 and MMP-9 activity significantly correlated with the stage and prognosis of CRC (34). This is useful because high levels of these MMPs in individuals may be indicative of early onset of CRC. This was tested in a pilot study that was carried out by Hurst et al. (35) where 300 patients were analyzed and it was found that high serum levels of MMP-9 accurately predicted CRC in 77.3% of the cases.
These researchers suggested that the MMP-9 serum test would be a useful secondary screening test for early detection of CRC (34, 35).

Single-Nucleotide Polymorphism (SNP) analysis has also been performed on CRC and other types of cancers. The most commonly studied SNP in MMP-9 is a C to T substitution that is located -1,562 base pairs upstream of the promoter region, which increases the transcriptional expression of MMP-9. When occurrences of this particular SNP are compared to CRC susceptibility, the results are conflicting. A study that was carried out in Korea found that this SNP is found more frequently in individuals with CRC than in individuals that do not. However, several other researchers performed similar studies and found conflicting results. This SNP needs to be studied more closely in order to determine if it is important for CRC growth and metastasis (34).

As mentioned previously, the focus of this research project is to better understand the molecular mechanisms that drive breast cancer metastasis. We chose this as our focus because breast cancer is one of the most prevalent cancers in the world and is the fifth leading cause of death worldwide. It is estimated that there will be over 43,000 deaths from breast cancer in the United States alone in 2023 (3). This has made studying breast cancer a topic of interest to cancer biologists. Similar to MMP expression in CRC, increased expression of MMP-2 and MMP-9 has been detected in breast cancers. Researchers have found that detection of these MMPs in body fluid may be a valuable diagnostic tool in identifying early onset of breast cancer (36).

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells become characteristically similar to invasive mesenchymal cells. EMT activation has been linked to breast cancer tumor progression. Researchers have found that many MMPs are crucial in the
activation of EMT and therefore the development of breast cancer in many cases. MMP-1 activates protease activated receptor-1 by cleaving it. This has been shown to increase the invasiveness and migration activity of breast cancer cells. Additionally, MMP-3 and MMP-7 both target E-cadherin, which induces the formation of bioactive particles that promote cancer cell motility and invasion. MMPs have been associated with EMT in cancer growth in other mechanisms as well. Researchers have found that increased levels of MMPs in the tumor’s microenvironment induced EMT in the breast epithelial cells. Additionally, when cancer cells are undergoing EMT, more MMPs are produced in order to facilitate metastasis (37).

B. MMP Inhibitors as Cancer Treatment

Given the close relationship that MMP activity has with cancer metastasis, MMPs have become an inviting pharmacological target for treating cancers. Researchers were interested in using synthetic MMP inhibitors to reduce cancer metastasis. Several of these MMP inhibitors have been tested in Phase III clinical trials. However, most of these studies failed because they were either ineffective or had adverse side effects, and currently, MMP inhibitors are not routinely used for treating cancers. More research must be carried out in order to understand all of the functions of MMPs in normal tissue growth and development. The most common adverse side effect of MMP inhibition is musculoskeletal syndrome, which causes intense pain and immobility of the shoulder joints as well as contracture of the hands (34, 37). Many MMPs are involved in tissue remodeling in healthy individuals. Because many of the MMP inhibitors were not selective among MMPs, the use of these inhibitors affect the MMPs that are necessary for normal tissue growth and repair and causes adverse side effects (34).

Another problem with administering broad spectrum MMP inhibitors is that some MMPs actually inhibit and help prevent cancer cell metastasis. It was discovered that MMP-8
suppresses cell metastasis in breast cancer. Apparently, increasing cell adhesion to the ECM diminishes the invasive potential of the cell. By knocking down the expression of MMP-8, the cancer cells were found to be more metastatic. This clearly shows that if MMP inhibitors were to inhibit the activity of MMP-8, then this would promote increased metastasis (37). As researchers improve their understanding of the roles of MMPs in tissue growth and cancer development, more selective inhibitors may be developed and implemented in cancer therapeutics. Additionally, the use of monoclonal antibodies which are highly specific for individual MMPs is a promising area of future research (34).

Because metastasis is the main cause of cancer mortality, researchers eagerly study the mechanisms that drive metastasis and they identified that MMPs are a significant player in this process (4). Although the understanding of MMPs has improved over the years, more research needs to be carried out in order to determine the specific MMPs that can be inhibited without causing adverse side-effects. Not only must specific MMP targets be identified, but specific ways of inhibiting these MMPs must also be discovered. This is a challenge because MMPs are very similar in structure (20, 34, 37). Research has shown that MMPs may be a promising target for cancer therapeutics; however, more research is needed to determine effective and safe ways of doing this.

C. PCs and MMP-9 Increase Invasiveness of Cancer Cells

As discussed previously, PCs play crucial roles in activating and deactivating MMPs. Our main PCs of interest, furin and PACE4, appear to have opposing effects on MMP-9 activation and breast cancer invasiveness when reviewing some previous research studies. Lapierre et al. (17) conducted experiments on MDA-MB-231 breast cancer cells using specific prosegments (ppPC) and a general PC inhibitor to inhibit the activation of furin and PACE4 to determine their
effects on MMP activation and cell migration (17). They tested the invasiveness and cell
migration of MDA-MB-231 cell using Boyden chamber assays. Both migration and invasion of
the cells increased when PACE4 activation was inhibited in the ppPACE4 treated cells.
Similarly, migration and invasion of the cells also increased with the general PC inhibitor (α1-
PDX). This is in contrast to the cells that were treated with ppFurin which inhibited furin
activation. When furin was inhibited, the migration and invasiveness of the cells decreased.
These results suggested that furin inhibition correlates with decreased invasiveness of breast
cancer cells whereas PACE4 inhibition correlated with increased invasiveness. These results
illustrate the inverse roles that these PCs may play in cancer metastasis (17).

In order to test the effects of furin and PACE4 inhibition on MMP-9 activity, Lapierre et
al. (17) also performed gelatinase enzymatic activity assays on the serum-free media that was
collected from the MDA-MB-231 cells and were treated with the above described PC inhibitors
and control. These experiments showed increased MMP-9 activity with the cells that were
treated with α1-PDX and ppPACE4. Decreased MMP-9 activity was seen with furin inhibition.
This suggests that PACE4 activation correlates with decreased MMP-9 activity and furin
activation correlates with increased MMP-9 activity. This trend was not observed for MMP-2,
suggesting that these two MMPs are affected differently by PACE4 and furin expression. The
highest MMP-9 activity was seen in the α1-PDX treated cells, followed by the ppPACE4 treated
cells. The cells treated with ppFurin showed less MMP-9 activity than the control cells. The
Lapierre study is important because it suggested the opposing roles for PACE4 and furin in
breast cancer cell metastasis. This points to furin as a possible mechanism for MMP-9 activation.
Conversely, PACE4 expression is negatively correlated with MMP-9 expression and increased
invasiveness (17).
The Lapierre study first identified these relationships between furin, PACE4, and MMP-9, suggesting that more research could identify these as potential targets for cancer treatment. MMP-9 is an enzyme that is integral in the initiation of cell migration because it is able to degrade many proteins present in tissue extracellular matrix, in particular type IV collagen: a major component of the basement membrane. This must be degraded in order for cancer cells to metastasize from one tissue to another. Lapierre et al. (17) showed that furin inhibition decreased MMP-9 activity and PACE4 inhibition increased MMP-9 activity, identifying these PCs as important enzymes to study with regards to MMP-9 activation and metastasis. (17).

Similarly, a study carried out by Fu et al. (18) examined PACE4 mRNA expression via RT-PCR analysis in ovarian cancer. They showed that PACE4 expression was lower in the primary ovarian cancer cell lines and the established ovarian cancer cell lines when compared to normal ovarian cell lines. This study further identified promoter DNA methylation and histone deacetylation as a mechanism that decreased PACE4 expression in the ovarian cancer cells. Using bisulfite genomic sequencing, Fu et al. (18) detected 8-9% methylation of the PACE4 gene in normal ovarian cell lines and 58-92% methylation of the PACE4 gene in ovarian cancer cell lines (18). This mechanism of epigenetic silencing of PACE4 leading to overall decrease in expression in ovarian cancer could be consistent with the findings of the Lapierre study.

In contrast to the Lapierre and Fu studies, other researchers have noticed that an increase in PACE4 expression correlated with increased cancer severity, similarly to what is seen with furin expression. This illustrates the controversy surrounding the contrasting roles that PACE4 may play in cancer metastasis. Lin et al. (16) used immunohistochemistry to examine PACE4 expression in nasopharyngeal carcinomas (NPC). They found that PACE4 staining was not detected in the normal nasopharyngeal epithelial tissues but was detected in the NPCs. They also
found that PACE4 expression was positively associated with late stages of NPC with distant metastasis. They further discovered that high expression of PACE4 in the NPC samples predicted shorter overall survival of the patients (16). In another study, D’Anjou et al. (15) similarly noticed an increase in PACE4 expression in prostate cancer tissue when compared to the corresponding normal tissue. This led them to perform PACE4 inhibitor experiments with a prostate cancer cell line. They first noticed that the prostate cancer cells grew slower once the PACE4 inhibitor was introduced via transfection. They then introduced these PACE4 inhibited cancer cells into nude mice and noticed that the decreased PACE4 expression correlated with a decrease in the ability for tumors to form in the mice (15). These conflicting findings of PACE4 expression in various cancers illustrates the fact that there is still much to learn about how PACE4 affects cancer cell growth and metastasis. This merits further research of PACE4 with a variety of cell lines as its role in metastasis appears to change in different types of cancer.

Figure 1 shows our proposed mechanism of PC regulation of MMP activity which promotes ECM degradation and increased metastasis based on the literature that we reviewed. As previously stated, PCs regulate MMPs via endoproteolysis and MMPs often activate other MMPs as well. Previous studies have shown that furin cleaves the prosegment region of MMP-14 and activates it (38). Increased MMP-14 expression correlates with increased MMP-2 activation suggesting that MMP-14 may activate MMP-2 (39). MMP-2 cleaves the prosegment region of MMP-9 (40). Once active, MMP-9 degrades key components of the ECM (26, 27). We believe that PACE4 may inhibit MMP-9 activation based on the observations made by Lapierre et al. because they noted an increase in MMP-9 activity when PACE4 was inhibited (17).
III. Goals of the Metastasis Project

Our project is based on the fact that the tumor microenvironment is both hypoxic and acidic due to irregular vasculature which deprives some cancer cells of oxygen (41, 42). Upregulated metabolism and increased cell growth also could result in increased temperatures in the microenvironment of the tumors. Heat mapping studies have shown on average 1.3 °C temperature increase in breast cancer tumors cells when compared to normal body temperature (11). Unlike the other 23 MMPs, MMP-9 is almost exclusively expressed at high levels in tissues that are in inflammatory states like colitis and rheumatoid arthritis (43). Given the relationship...
between MMP-9 activity and furin and PACE4 expression, we decided to study the expression of these PCs while inducing varying levels of stress on breast cancer cells grown in cell culture (17).

During this research project we explored the effects that induced stress conditions of heat and hypoxia on MCF-7 and MDA-MB-468 breast cancer cells grown in cell culture have on furin and PACE4 expression. This was done by performing Western blot analysis on the cell lysates collected from the cells grown under stress conditions. Furin and PACE4 expression of cells grown in the stress conditions was compared to their expression in cells grown at normal body temperature (37 ℃) and ambient oxygen percentage (20%) (44). Additionally, we used Matrigel matrix invasion assays to test the effect of heat and hypoxia stress on the invasiveness of both of these breast cancer cells lines.

MCF-7 and MDA-MB-468 are breast cancer cell lines with important genetic and phenotypic differences. The MCF-7 cell line was isolated in 1970 from the pleural effusion of a 69 year old female with metastatic disease of the pleura and chest wall following a failed treatment by left breast mastectomy and post-operative radiotherapy. This cell line is unique because it expresses estrogen receptor (ER) alpha and provides a model for testing therapeutic strategies that target this hormone receptor (45). The MDA-MB-468 cell line was isolated in 1977 from a 51 year old female patient with metastatic adenocarcinoma of the breast. This cell line is a triple negative cell line, meaning that the cells do not express estrogen receptor (ER) alpha, progesterone receptor, or human epidermal growth factor 2 receptor (HER2). Triple negative breast cancers account for 10% to 22% of all diagnosed cases of breast cancer. They typically have the poorest survival prognosis due to the fact that standard hormone therapy or therapies that target specific receptors fail to treat these cancers (46). Studying both of these cell
lines will allow us to determine the effects that our experimental conditions will have on the gene expression and invasiveness on two different breast cancer cell lines with varying genetic characteristics.

We chose the experimental conditions of 40°C as the heat stress, 3% oxygen as the hypoxia stress, and a combined dual stress condition. We also tested the invasiveness of these cell lines grown in normal conditions compared to the induced stress conditions using Matrigel Matrix Invasion Assays. Our hypothesis is that there will be an increase in metastasis markers when breast cancer cell lines are grown under stress conditions. In this case, furin expression in the Western blot analysis and increased cell migration in the invasion assays are our markers for increased metastasis. If our hypothesis is correct, we expect to see an increase in furin expression, decrease in PACE4 expression, and an increase in invasiveness of the breast cancer cells under the experimental stress conditions when compared to the normal cell culture conditions.
Chapter Two: Methods and Materials

I. Cell Culture and Lysis

A. Cell Culture

We grew MCF-7 breast cancer cells, MDA-MB-468 breast cancer cells, and LoVo colon cancer cells under normal cell culture conditions, 37°C and ambient oxygen concentration, which is 20%. The MCF-7 media was DMEM (Invitrogen; Waltham, MA) containing 10% Fetal Bovine Serum (Atlanta Biologicals; Flowery Branch, GA), 55 µg/µl gentamycin (Invitrogen), 274 µg/µl Fungizone (Thermo-Fisher; Pittsburgh, PA), 0.55 µM sodium pyruvate (Invitrogen), non-essential amino acids (Invitrogen), and 2.5 µg/µl of insulin (Invitrogen). The MDA-MB-468 cells and LoVo cells were grown in the same medium as the MCF-7 cells, but without the insulin. The breast cancer cells were obtained from Dr. Jessica Kelts (University of Michigan-Flint Department of Chemistry and Biochemistry) and were already revived and growing in their respective media. The LoVo cells were obtained from Dr. Joseph Sucic (University of Michigan-Flint Department of Molecular Biology). The cells were incubated in normal conditions (37°C and 20% oxygen) and were split when the flasks became 70% confluent.

To grow these cells in our experimental conditions, we quantified our cells using a hemocytometer and plated approximately 50,000 cells in each well of a standard six well plate. We grew these cells under hypoxia stress (3% oxygen), heat stress (40°C), and dual stress conditions, which combined the 40°C heat stress with each hypoxia condition, for 96 hours. We also plated 50,000 of MCF-7 cells, MDA-MB-468 cells, and LoVo cells in six well plates and grew them the normal cell growth conditions (37°C and 20% oxygen). The cells grown in both stressed and normal conditions were incubated for 96 hours prior to lysate collection.
B. Cell Lysis

After the 96 hour incubation, the cells were removed from the incubator and placed in the cell culture hood, where the medium was aspirated off. 350 µl of UVM lysis buffer (50 mM trisHCl with pH 8.0, 0.15 NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.5% SDS) was added to each well and the plates were tilted until the lysis buffer was equally distributed. The plates were placed on ice and incubated for 15 minutes while being tilted every five minutes to re-distribute the lysis buffer. The buffer was transferred to chilled microcentrifuge tubes which were centrifuged at 7,000 rpm for 20 minutes in the Eppendorf 5415D Centrifuge at 4℃. The supernatants were transferred to new chilled microcentrifuge tubes and were stored at -80°C for protein quantification and Western blot analysis.

II. Western Blot

A. BCA Protein Quantification

We used the BCA (Bicinchoninic Acid) Assay kit (Thermo-Fisher) to quantify the protein concentration in each of our cell lysate samples. We first prepared a set of protein standards that ranged in concentration from 20-2000 µg/ml. We then plated these standards in a 96 well plate with 25 µl of each of our lysate samples along with 200 µl of the working reagent. The plate was incubated for 30 minutes at 37℃ according to the Thermo-Fisher protocol. We measured the absorbance of the standards and the lysate samples at 560nm using a plate reader. We generated an Absorbance at 560 nm versus concentration curve in µg/ml using the standards provided in the kit to determine the protein concentrations of each lysate sample.
B. Protein Gel and Western Blot Analysis

We used the concentrations that we obtained from our protein quantification to determine the volumes of the lysates needed to load 10 µg of protein. The samples were prepared for the SDS-PAGE by combining them with de-ionized water to bring the volume up to 26 µl. Then, 10 µl BOLT Sample Buffer (Invitrogen), and 4 µl of reducing agent were added to the samples. The tubes were then placed into the thermocycler and heated at 70°C for 11 minutes. The samples were loaded into each well of the Bolt 4-10% polyacrylamide pre-cast gels (Invitrogen) along with pre-stained protein markers (Invitrogen). The SDS-PAGE ran for 30 minutes at 165 volts.

Once the gels were complete, they were transferred to the cassettes for the Western blot transfer. Immobilon (Millipore; Bedford, MA) was cut to 7 X 8.5 cm and was placed against the gel inside the cassette. The transfer was performed with MOPS transfer buffer (Invitrogen) at 100 volts for 1.5 hours at 4°C. Once complete, the Immobilon was transferred to a sterile container with 40 ml of 3% milk in 1X TBS-Tween for blocking. This was blocked for 1 hour at room temperature on a platform shaker at approximately 60 rpm. Next, an aliquot of the primary antibody was added to the milk solution and left to incubate overnight at room temperature on the plate agitator. Anti-furin (Thermo-Fisher) was diluted 1:1500, while anti-PACE4 (Sigma Aldrich; St. Louis, MO) was diluted 1:2500. We performed a control Western blot for each lysate using anti-β-actin (Thermo-Fisher) diluted 1:2000. After the primary antibody incubated overnight, we performed three washes using 1x TBS-Tween. We then blocked the Immobilon for 30 minutes using 3% milk in 1X TBS-Tween. Anti-rabbit secondary antibody AP conjugate (Promega; Madison, WI) was added at dilution 1:5000 to the blocking solution and incubated at
room temperature on the platform shaker for one and a half hours. The Immobilon was then washed in 1x TBS-Tween and washed again in Western Blot Substrate Buffer (0.1 M tris HCl at pH 9.5, 0.1M NaCl, and 5mM MgCl$_2$). The Immobilon was then covered in substrate development solution which was made up of 20 ml of Western Blot Substrate Buffer, 40 µl of NBT (Promega), and 20 µl of BCIP (Promega). The Western Blot was developed until the bands became visible.

**C. Quantification of Western Blots**

We used ImageJ software to quantify the band intensity of the Western blots and generated graphs showing the relative amounts of furin and PACE4 normalized to the β-actin control Western blots. The Western blots were photographed, uploaded to the computer as JPEGs and opened in ImageJ. The images were converted to 8-bit under image type. The background noise for the Western blots was decreased by adjusting the brightness and contrast of the images. The bands were then selected using the “rectangle” tool and the analyze gels function. Both the lanes for the β-actin and experimental proteins were selected. Then the intensities of the bands were graphed using the “plot lanes” tool. Each peak of the graph corresponded to an individual band on the Western blots. These were identified and the areas under the curves were isolated and selected using the “wand” tool. These areas, once selected, generated numerical values for the intensities of each band.

These values were copied and carried over to a Microsoft Excel Spreadsheet where ratios of the values for the experimental conditions to the normal condition were calculated for both β-actin and the experimental proteins. These ratios were used to calculate the β-actin to experimental protein ratios in order to normalize the protein quantification values. These ratios
were graphed to show the relative furin and PACE4 expressions in experimental stress conditions compared to the normal cell culture conditions. No statistics were performed on these values because there were not multiple Western blots for each experimental protein.

III. Invasion Assay

We performed invasion assays on MDA-MB-468 and MCF-7 cells grown under normal cell growth conditions and the previously described experimental stress conditions using the BD BioCoat Matrigel Invasion Chamber kit (Thermo-Fisher). First, we removed the wells for the invasion chambers from the freezer and thawed them for 30 minutes. Then, 500 µl of serum-free DMEM was added to the inserts and 500 µl of DMEM containing FBS was added to the outer chambers to serve as a chemoattractant for the cells. This was allowed to stand for 2 hours. We plated 5,000, 10,000, 25,000, and 50,000 cells in the 24 well plate invasion chambers. The cells were re-suspended in 500 µl of serum-free media and added to the inserts in the invasion chambers. The serum-free media in the inserts was dumped out prior to adding the re-suspended cells. 250 µl of serum-containing media was added to the outer chambers of each insert. These invasion chambers were incubated in the previously described normal cell growth conditions and stress conditions for 96 hours.

After the incubation period, the Matrigel was removed from the invasion chamber using a cotton swab moistened with DMEM. This also removed all non-invading cells. The cells on the invasion chamber inserts were stained with 100% methanol and 1% Toluidine blue for 30 minutes. After this, the stain was rinsed with deionized water to remove the excess stain. Six different students counted the cells found in the most representative field of view for the 50,000
cell plated invasion chambers, visualized under 10X magnification. These counts were averaged together. The invasion assays were performed in duplicate to compare results.
Chapter Three: Results and Discussion

I. Results

A. Protein Gel and Western Blot Results

The Western blots of the lysates collected from the MCF-7 cells are shown in Figure 2A. β-actin, the control protein, was used to normalize the protein quantification values that were determined using ImageJ software. The molecular weight of the bands were estimated by comparison with a molecular weight ladder (not shown) and corresponded with the expected molecular weights of β-actin, furin, and PACE4. Visual analysis revealed that the bands for β-actin were similar in intensity with the exception of the dual stress exposed lysates, which showed a less intense band. (The varying intensities of the β-actin bands are problematic for our controls because we would expect that the expression of the control would not change under our experimental conditions. However, this was the only control anti-body that we had access to that would result in a successful Western blot with our cell lysates. The β-actin Western blot was repeated and it showed a decrease in band intensity in the dual stress condition multiple times. Because this was the only control Western blot available to us, I used it as my control to normalize the furin and PACE4 Western blots for quantification.) The band for furin was the least intense in the normal condition and the band was darker for the heat, hypoxia, and dual stress exposed lysates. Inversely, the PACE4 band showed the highest intensity in the normal condition lysate, followed by the hypoxia stress exposed lysate. The heat and dual stress exposed lysates showed incredibly faint bands.

The results of the ImageJ quantification of the furin MCF-7 lysate Western blots are shown in Figure 2B. When normalized to the β-actin Western blot, furin showed highest
expression in the dual stress condition. The next highest expression of furin was seen with the
heat stress condition, followed by the hypoxia stress condition. The lowest furin expression was
seen in the normal cell culture condition. The ImageJ analysis corresponded with our initial
visual analysis of the Western blots.

Figure 2A: Western blots of β-actin, PACE4, and furin generated for the lysates collected from MCF-7 cells grown for 96 hours in cell cultures under normal and experimental conditions. Molecular weights were estimated from the molecular weight marker standard (not shown).
Figure 2B: Quantification of MCF-7 furin Western blot results using ImageJ. The highest protein expression is seen in the dual sample. All stress conditions show higher furin expression when compared to the normal cell culture condition.

The results of the ImageJ quantification of the PACE4 MCF-7 lysate Western blots are shown in Figure 2C. When normalized to the β-actin western blot, PACE4 showed the highest expression in the normal cell culture condition. All three MCF-7 lysates from the experimental stress exposed cells showed low PACE4 expression. The ImageJ analysis corresponded with our initial visual analysis of the Western blots.
Figure 2C: Quantification of MCF-7 PACE4 Western blot results using ImageJ. The highest protein expression is seen in the cells grown in the cell culture conditions when compared to the lysates collected from the cells grown in the different stress conditions.

The Western blots of the lysates collected from the MDA-MB-468 cells are shown in Figure 3A. β-actin was used to normalize the protein quantification values that were determined using ImageJ software. The molecular weight of the bands was estimated using a molecular weight ladder (not shown) and corresponded with the expected molecular weights of β-actin, furin, and PACE4. Visual analysis revealed that the bands for β-actin were similar in intensity with the exception of the dual stress exposed lysates, which showed a less intense band. (This is similar to what was seen in the MCF-7 Western blot in Figure 2A) The bands for furin were similar in intensity in the normal condition and the heat, hypoxia, and dual stress exposed lysates. Inversely, the PACE4 band shows the highest intensity in the heat and hypoxia stress exposed lysates. The MDA-MB-468 Western blot results were different from the MCF-7 Western blot results upon visual analysis.
The results of the ImageJ quantification of the furin MD-MB-468 lysate Western blots are shown in Figure 3B. When normalized to the β-actin Western blot, furin showed highest expression in the dual stress condition. The next highest expression of furin was seen with the normal condition lysates, followed by the hypoxia stress condition. The lowest furin expression was with the heat-exposed cell lysates. ImageJ analysis corresponded with our initial visual analysis of the MD-MB-468 Western blots. The PACE4 bands for the MD-MB-468 lysate Western blots were too faint with relation to the background for ImageJ quantification.

Additionally, we performed cell culture on LoVo colon cancer cells and subjected them to our experimental stress conditions. Like the Western blot results performed on the breast cancer cell lines, furin showed more intense bands in the stress-exposed cell lysates when compared to the lysates collected from the LoVo cells grown in the normal cell culture conditions. PACE4 also showed a decrease in band intensity in the stress-exposed cell lysates when compared to the cell lysates collected from the LoVo cells grown in the normal cell culture conditions. The LoVo furin and PACE4 Western blot results are shown in Figure 4. ImageJ band intensity quantification was not performed on these Western Blot because we did not perform a β-actin control Western blot on these samples. (All Western blots were performed with 10 µg of protein loaded into each lane.)
Figure 3A: Western blots of β-actin, PACE4, and furin generated for the lysates collected from MDA-MB-468 cells grown for 96 hours in cell culture under normal and experimental conditions. Molecular weights were estimated from the molecular weight marker standard (not shown). The bands from PACE4 are not dark enough relative to the background to quantify using ImageJ software.

Figure 3B: Quantification of MDA-MB-468 furin Western blot results using ImageJ. The highest protein expression is seen in the dual sample, followed by normal conditions. This is different from the MCF-7 Western blot results, which showed higher furin expression in all experimental conditions when compared to normal conditions.
Figure 4: Western blots of PACE4 and furin generated for lysates collected from LoVo cells grown for 96 hours in cell cultures in normal and experimental conditions. Molecular weights were estimated from the molecular weight marker standard (not shown). PACE4 showed a decrease in band intensity in the stress conditions when compared to the cells grown in normal cell culture conditions, mirroring the results seen in the MCF-7 lysate Western Blots.

B. Invasion Assay Results

Duplicate trials of MCF-7 and MDA-MB-468 cells invasion assay were performed with the normal cell culture conditions, heat stress (40°C), hypoxia (3% oxygen), and dual stress (40°C with 3% oxygen). The number of cells that migrated through the Matrigel matrix were visualized at 10X magnification and were counted by six different students. The MCF-7 invasion chambers with 50,000 cells and the MDA-MB-468 invasion chamber with 25,000 cells were counted. The invasion chambers were encoded by a third party who was not involved in counting
the cells to eliminate bias in reporting the number of cells that migrated under the experimental conditions. The highest and lowest count for each sample were excluded and the resulting four counts were averaged and reported in the tables below. Two-tailed t-test was performed to determine the statistical significance of the number of cells that migrated in the experimental stress conditions when compared to the number of cells that migrated in the normal cell culture condition. Statistical significance was reported with p-value < 0.05.

Figure 5A shows the total number of MCF-7 cells that migrated for each experimental condition through the Matrigel invasion chambers in the first trial of the invasion assay experiments. The highest number of cells migrated in the hypoxia condition and this was found to be statistically higher than the number of MCF-7 cells that migrated under the normal cell culture condition in this trial, with a p-value of 0.033. Dual stress-exposed MCF-7 cells showed a slightly higher amount of migration than the normal cell culture exposed cells; however, this difference did not achieve statistical significance with a p-value of 0.748. The lowest number of MCF-7 cells that migrated in trial one is seen in the heat-exposed cells and this also is found to be statistically lower than the cells that migrated in the normal cell culture conditions with a p-value of 0.010. The average number of cells migrated for each experimental condition, the variance, standard deviations, and p-values for the first trial of MCF-7 cells are shown in Table 1A.
Figure 5A: The average number of cells that migrated in the invasion assay is shown for the first trial of MCF-7 cells grown under normal and experimental conditions. Hypoxia showed the highest number migration when compared to the cells grown in normal conditions. The cells under heat stress showed the migration when compared to the cells grown in normal conditions. Both of these conditions showed statistical significance with $p$-values $\leq 0.05$ with two-tailed t-test analysis. The cells grown in the dual stress conditions showed slightly higher migration when compared to the cells grown in normal conditions. The migration in the dual condition did not achieve statistical significance with $p$-value of 0.748.

Table 1A: Two-tailed t-test analysis of the total number of cells migrated for the first trial of MCF-7 cells grown under normal and experimental stress conditions. The $p$-values in bold show a statistical significance in the number of cells that migrated in the experimental stress conditions when compared to the number of cells that migrated under normal conditions.
The results from the duplicate trial of the MCF-7 cells grown in the Matrigel invasion assay are shown in Figure 5B. These results were different from the results reported from the first trial because the highest number of cells migrated in the dual stress condition followed by the hypoxia condition. Both of these experimental stress conditions showed statistically higher migration when compared to the cells grown in the normal stress conditions. The p-value for dual is 0.012 and the p-value for hypoxia is 0.002. Similar to the first trial, the lowest number of MCF-7 cells migrated in the heat stress condition. The average number of cells migrated for each experimental condition, the variance, standard deviations, and p-values for the second trial of MCF-7 cells are shown in Table 1B.

**Figure 5B:** The average number of cells that migrated in the invasion assay is shown for the second trial of MCF-7 cells grown in the normal and experimental conditions. Dual followed by hypoxia shows the highest migration when compared to the cells grown under normal conditions. Both of these conditions showed statistical significance with p-values < 0.05 after two-tailed t-test analysis. The cells grown under heat stress show the lowest migration when compared to the cells grown in normal conditions. The migration under heat stress did not achieve statistical significance with p-value of 0.402.
<table>
<thead>
<tr>
<th>Cell Culture Condition</th>
<th>Average # of Cells Counted</th>
<th>Variance</th>
<th>Standard Deviation</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
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<td>752</td>
<td>27.4</td>
<td>NA</td>
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<tr>
<td>Heat</td>
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<td>100.9</td>
<td>10.0</td>
<td>0.402</td>
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<td>Hypoxia</td>
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<td>9478</td>
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<tr>
<td>Dual</td>
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<td>129514.9</td>
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<td>0.012</td>
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</table>

Table 1B: Two-tailed t-test analysis of the total number of cells migrated for the second trial of MCF-7 cells grown in the normal and experimental stress conditions. The p-values in bold show a statistical significance in the number of cells that migrated in the experimental stress conditions when compared to the number of cells that migrated in normal conditions.

The MDA-MB-468 Matrigel Invasion Assay results for the first trial are shown in Figure 6A. The highest number of cells migrated in the hypoxia stress-exposed cells, followed by the dual stress-exposed cells. Both of these experimental conditions showed statistically higher migration when compared to the number of cells that migrated under normal cell culture conditions, with p-values of 0.002 for both conditions. The lowest number of cells migrated in the heat exposed cells and this was also shown to be statistically significant when compared to the number of cells that migrated in the normal cell culture conditions, with a p-value of 0.021. The average number of cells migrated for each experimental condition, the variance, standard deviations, and p-values for the first trial of MDA-MB-468 cells are shown in Table 2A.
Figure 6A: The average number of cells that migrated in the invasion assay is shown for the first trial of MDA-MB-468 cells grown under normal and experimental conditions. Hypoxia and dual stress showed the highest migration when compared to the cells grown under normal conditions. The heat stress-exposed cells showed the lowest migration when compared to the cells grown in the normal conditions. All three of the experimental conditions show statistical significance with p-values < 0.05 with two-tailed t-test analysis.

<table>
<thead>
<tr>
<th>Cell Culture Condition</th>
<th>Average # of Cells Counted</th>
<th>Variance</th>
<th>Standard Deviation</th>
<th>P-value</th>
</tr>
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<td>329</td>
<td>1148.7</td>
<td>33.9</td>
<td>0.002</td>
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Table 2A: Two-tailed t-test analysis of the total number of cells migrated for the first trial of MD-MB-468 cells grown under normal and experimental stress conditions was performed. The p-values in bold show a statistical significance in the number of cells that migrated in the experimental stress conditions when compared to the number of cells that migrated in normal conditions.
The results for the duplicate trial of MDA-MB-468 cells grown in the Matrigel matrix invasion chambers are shown in Figure 6B below. The highest number of cells migrated in the hypoxia stress condition and when compared to the cells grown in normal cell culture conditions the difference is found to have statistical significance with a p-value of 0.001. This directly correlated with the results from the first trial. However, the cells grown under normal conditions showed the next highest cell migration, which was different from the first trial of this experiment. Dual stress and heat-exposed MDA-MB-468 cells showed the lowest cell migration and both achieve statistical significance with p-values of 0.001. For both trials, the lowest migration was seen in the heat-exposed MDA-MB-468 cells. The average number of cells migrated for each experimental condition, the variance, standard deviations, and P-values for the second trial of MD-MB-468 cells are shown in Table 2B.

**Figure 6B:** The average number of cells that migrated in the invasion assay is shown for the second trial of MDA-MB-468 cells grown in normal and experimental conditions. Hypoxia showed higher cell migration compared to the cells grown under normal conditions. Both the dual stress and heat-exposed cells showed lower cell migration when compared to the normal cell culture conditions. All three of the experimental conditions show statistical significance with P-values < 0.05 with two-tailed t-test analysis.
<table>
<thead>
<tr>
<th>Cell Culture Condition</th>
<th>Average # of Cells Counted</th>
<th>Variance</th>
<th>Standard Deviation</th>
<th>P-value</th>
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<td>0.033</td>
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Table 2B: Two-tailed t-test analysis of the total number of cells migrated for the second trial of MD-MB-468 cells grown under normal and experimental stress conditions. The p-values in bold show a statistical significance in the number of cells that migrated in the experimental stress conditions when compared to the number of cells that migrated in normal conditions.

II. Conclusion

A. Discussion

Despite being in the forefront of medical research, cancer remains one of the most difficult diseases to treat because the molecular mechanisms that drive metastasis remain poorly understood. In order for cancer cells to metastasize, they must degrade the ECM that surrounds the primary tumor and also the ECM that surrounds the target tissues. MMP-9 is key in this process, as it degrades key components of the ECM, like collagen IV and collagen V. Studies have shown that blocking or inhibiting MMP-9 in cancer cells and tissue remodeling significantly inhibits cell invasion (47, 48). Recent research identified furin and PACE4 as enzymes of interest because they appeared to help regulate MMP-9 activity (17). It was observed that inhibiting furin decreased MMP-9 activity and cell migration in MDA-MB-231 breast cancer cells. This was contrasted by the increase in MMP-9 activation and cell migration they observed when PACE4 expression was inhibited (17). Most studies agree that furin is
upregulated in many cancers including lung cancer, breast cancer, and head and neck cancer (49).

The conflicting literature that surrounds PACE4’s role in MMP activation and cancer metastasis identified it as a potential PC of interest in our research project. Some research studies on prostate cancer and nasopharyngeal carcinomas correlated increased PACE4 expression with increased cancer metastasis and poorer patient outcomes (15, 16). A study that was carried out on human ovarian cancer cells correlated increased PACE4 methylation and deacetylation with tumor growth when compared to normal ovarian cells. This agrees with the Lapierre findings where PACE4 expression was shown to have an inverse relationship with MMP-9 activity and increased cell migration (17).

Previous research suggested the role of PACE4 differs with MMP-9 activity and cell migration in different cancers and cell types. Our study analyzed furin and PACE4 expression in triple a triple negative breast cancer cell line (MDA-MB-468), a breast cancer cell line that expresses estrogen receptors (MCF-7), and a colon cancer cell line (LoVo) exposed to hypoxia and heat stress. We observed that the MCF-7 cells showed increased furin expression in all of the experimental stress conditions when compared to the normal cell culture conditions. As we predicted, we detected the highest furin expression in the highest stress condition. Similarly, the MDA-MB-468 lysate Western blots showed highest furin expression in the dual stress condition. The PACE4 Western blot results for the MCF-7 cell lysates match our prediction of decreased expression in the stress experimental conditions when compared to the normal cell culture conditions. However, the MDA-MB-468 lysate PACE4 Western Blot results do not match this prediction, showing the highest level of expression in the heat and dual stress exposed lysates. The LoVo, a colon cancer cell line, PACE4 Western blot results matched the MCF-7 results,
where the increased furin was observed in the experimental stress conditions when compared to the normal condition and PACE4 expression was substantially decreased in the stress conditions.

The effect of experimental stress conditions on the relative invasiveness of the MCF-7 and MDA-MB-468 cell lines was tested using invasion assays. These experiments were repeated in duplicate to determine if the results were repeatable. Both trials of the MCF-7 invasion assays showed increased cell migration in the hypoxia and dual experimental stress conditions when compared to the cells grown in normal cell culture conditions. Trial two showed that increased migration of the cells exposed to hypoxia and dual achieved statistical significance with two-tailed t-test analysis with a p-value < 0.05. Trial one showed slightly higher migration with the hypoxia stress exposed MCF-7 cells when compared to the normal cell culture conditions; however, this was not statistically significant with two-tailed t-test analysis. The number of cells that migrated in each trial for each experimental condition differed greatly across duplicate trials. This may have been due to an error in plating or due to contamination. No evidence of contamination or cell stress was observed during this experiment. These drastic differences in cells counts are problematic and could be addressed by repeating this experiment a third time if time and funds allow.

The MDA-MB-468 cells showed increased migration in the hypoxia stress exposed cells in both trials and both values achieved statistical significance with a p-value of < 0.05. The two trials for the MDA-MB-468 invasion assays showed differing results in the dual stress exposed cells with regards to invasiveness. The second MDA-MD-468 invasion assay trial showed lower migration in the dual stress exposed cells than the cells grown in normal cell culture which achieved statistical significance with a p-value < 0.05. Similarly to what was observed with the MCF-7 invasion assays, there is also a drastic difference in cells counts across the duplicate trials.
for the MDA-MB-468 invasion assays. Again, this is problematic because the trials were plated with the same number of cells and were subjected to the same experimental conditions. These differences in results may be due to an error in cell counting while plating or due to contamination of the cell culture. While performing these experiments, we did not observed any evidence of cell stress or contamination. If time and funds allow, the invasion assays experiments should be repeated a third time with the MDA-MB468 cells in order to verify the results. All trials for both cell lines consistently showed the lowest level of migration in the heat exposed stress conditions. (There may have been an error in plating the correct number of cells for the heat stress-exposed cells; however, this seemed unlikely because this phenomenon was observed in four different trials between the two cell lines. It seems more likely that the heat stress condition was too stressful for the cells to thrive.) Although the results from these experiments show some variability in their results, there appeared to be an upward trend in cell migration in the heat and hypoxia exposed cells when compared to the cells grown in normal cell culture conditions. These results showed that the cells became more invasive \textit{in vitro} when subjected to increased hypoxia and heat stress combined.

The increased furin expression that was detected with the Western blots for the MCF-7 correlates with the increased migration that was observed under the hypoxia and dual stress conditions. This agrees with previous research that reported increased furin expression in cancer cells typically correlates with increased invasiveness. This further supports our hypothesis that the stress condition promotes increased invasiveness. The decreased PACE4 expression detected in the experimental stress conditions for the MCF-7 Western blots also correlated with increased in migration seen in the invasion assays. This is consistent with the previous research carried out by Lapierre \textit{et al.} (17) and Fu \textit{et al.} (18) that linked increased breast and ovarian cancer
metastasis with decreased PACE4 expression. Their findings suggested that PACE4 may have inhibitory properties for MMP activation and cell metastasis. When exposed to heat and hypoxia stress conditions, the MCF-7 cell lines showed decreased PACE4 expression and increased cell migration. This also supports the hypothesis that PACE4 expression inversely correlates with increased cancer metastasis and that the cells have increased migration and therefore invasiveness when treated with dual heat and hypoxia stress.

Although the MDA-MB-468 PACE4 Western blots did not show decreased expression in the stress conditions when compared to the normal conditions, they did show increased invasiveness. This further supports the theory that PACE4 expression and its role in metastasis is complex and differs among the different cancer cell lines. As previously discussed, research carried out by Lin et al. (16) and D’Anjou et al. (15) correlated increased PACE4 expression with increased cancer metastasis in nasopharyngeal carcinoma and prostate cancer cell lines. Two out of the three cell lines we studied showed contrasting expression for furin and PACE4 in the stress conditions. To our knowledge, this project is novel in that it is the first to study the effects of cellular stress on breast cancer cell metastasis and PC expression. The results suggest that heat and hypoxia stress may promote furin expression and increased cell migration. The results also suggest that heat and hypoxia stress may decrease PACE4 expression in MCF-7 breast cancer cells and LoVo colon cancer cells. However, this was not observed in the MDA-MB-468 breast cancer cells which suggests that PACE4 expression may vary across different cell lines in our experimental stress conditions.
C. Continued Experimentation

We continued to test our hypothesis on the effects of induced cellular stress conditions on breast cancer cell lines and expanded the cell lines we investigated to include MDA-MB-231 cells, which is a triple negative, highly aggressive, and invasive breast cancer cell line (17). Invasion assays were repeated by different students and similar results were reported with increased migration seen in breast cancer cells grown in experimental stress conditions compared to normal conditions. These results encouraged us to test our hypothesis with 3-dimensional cell culture, which was a more accurate model for testing invasiveness of breast cancer cells as it mimicked how cells grow in vivo more closely than growing the cells on a flat cell culture plate. I assisted in growing MDA-MB-468, MDA-MB-231, and MCF-7 cells in 3D culture with exposure to stress conditions similar to the conditions that were used in the experiments described here. Preliminary results were promising and showed increased invasion in the experimental stress conditions compared to the cells grown in normal cell culture conditions. If the 3D cell invasion cell culture experiments corroborate our preliminary results, then we could confidently suggest that heat and hypoxia cellular stress promote increased invasiveness in breast cancer cell lines.

These results would have significant implications for possible treatments of cancer that may decrease or inhibit cancer cell metastasis. Treatment of breast cancer patients with furin or MMP-9 inhibitors may help decrease cancer invasion from the primary tumor (49). Because reports of PACE4 expression in breast cancer cells lines are not consistent, PACE4 is not a likely candidate for therapeutic intervention in preventing or treating cancer metastasis (15, 16, 17, 18). The results from this research project support the hypothesis that induced heat and hypoxia stress
contribute to increased cancer metastasis and warrants further research to determine new novel therapies to treat breast cancer.
References


