Impact of Microenvironmental Stress on miRNA Expression and Invasive Properties in Cultured Breast Cancer Cells

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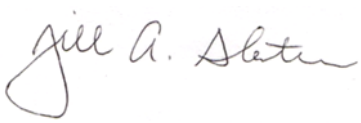

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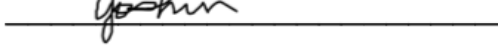

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Abstract

The relationship between miRNA expression and the onset, development, and advancement of aggressive cancers keeps miRNAs a subject of keen interest, and is the primary focus of this study. miRNAs are known to play critical roles as both proto-oncogene and tumor suppressor gene products. We are hypothesizing that stresses imposed within the tumor microenvironment of a cancer cell may become overly harsh, creating unsuitable conditions and provoking a cellular response that impacts expression levels for select miRNAs, and ultimately, the cancer cell's invasiveness. In this study, we explore how stresses affect levels of expression for miR-145 and miR-330-3p in MCF7, MB-468, and MB-231 breast cancer cell lines, and we examine the effects of these altered miRNA expression levels on the invasiveness of MCF7 and MB468 cells.

miR-145 is a tumor suppressor found to be down regulated in breast cancers. Its regulatory actions and targets can be associated with a number of critical cellular pathways. In contrast, miRNA-330-3p is a protooncogene product found to be overexpressed in breast cancers. We expected to find lowered levels of miR-145 expression and increased levels of miR-330-3p expression in breast cancer cells exposed to stress, compared with breast cancer cells grown in normal conditions. We also expected MCF7 and MB468 cells exposed to stress would prove to be more invasive than those grown in normal conditions.

Although mixed results are reported, some data collected for this study appear to support our hypothesis. miRNA expression data in agreement with this hypothesis include down-regulation of miR-145 in cells exposed to dual stress conditions, as well as upregulation of miR-330-3p in cells exposed to hypoxic stress (for all 3 cell lines), and in MCF7 and MB231 cells exposed to heat stress. Some invasion data in agreement with our hypothesis are also reported. Cell migration increased among MCF7 cells exposed to hypoxic stress, and a marked increase in cell migration was observed for MB468 cells under all stress conditions, compared with those under normal conditions. These data are all in agreement with our hypothesis, suggesting that stress conditions in the tumor microenvironment could be a trigger for metastatic cell behavior.

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Introduction

Cancer describes a complex collection of diseases that continue to threaten the lives of individuals across all demographics of society. The National Cancer Institute (NCI) has estimated that 1,806,950 new cases of cancer will be diagnosed in the US in 2020; projections have also been published for cases of all cancers that will end in death, and these numbers are reported at 606,520 (1). Sobering projections have also been broken down and established for specific types of cancers: breast cancers remain an imposing threat to women across the globe, and in the United States estimates published by the NCI for this year reflect newly diagnosed cases will number at 276,480, and 42,170 cases of breast cancers will result in death (1).

Characteristics common to all cancers include genetic abnormalities that interfere with standard restraints imposed on cell cycle progression, ultimately resulting in unchecked cell proliferation, and in the case of metastatic disease, the ability of cancerous cells to migrate to other sites in the body and establish tumors in new locations. These genetic deviations affect two large categories of genes: proto-oncogenes and tumor suppressor genes. Proto-oncogenes encompass genes whose products are associated with cell cycle progression and are important elements of cell signaling pathways, as well as those connected with cell survival. Tumor suppressor genes include those whose products forestall progression through the cell cycle, as well as those implicated in preserving genomic integrity.

Cancerous tumors may contain an expansive array of disparate mutations affecting genes from both of these categories; these mutations can alter the normal expression of the genes and the normal function of the gene products. This type of gene dysregulation, exacerbated by the faulty DNA repair pathways usually seen in cancer cells, has been shown to play a prominent role in the development and spread of cancers, as well as to create daunting challenges for

treatment. Genetic perturbations seen in cancer include point mutations, translocations, amplifications, and deletions. Point mutations are single base pair changes that can be either transitions or transversions; a transition involves the replacement of a purine with another purine (for example, changing an A to G) or a pyrimidine with another pyrimidine (for example, changing a T to C), while transversions occur when a purine is changed to a pyrimidine or vice versa. These mutations may have substantial impacts, depending on where the point mutation occurs. Translocations take place due to breaks in the sugar phosphate backbone; a section of one chromosome is broken off or separated, and then attached to another. Translocations can be very important to the development of cancers; they may affect standard regulation of gene expression or change protein structure and function, depending on where they occur.

Amplifications are known to generate many copies of the same gene, resulting in overexpression, and are also seen in many cancers. Point mutations, translocations, and amplifications are known to affect expression of protooncogenes, or the function of their products. Deletions are mutations that involve the removal of large sections of DNA that may affect several different genes; these mutations often affect tumor suppressor genes or their products and are often associated with contributing to a loss of heterozygosity, or LOH, which is often a key step in the development of cancer.

Mutations that spur deviant expression or improperly initiate action of proto-oncogene products may drive a cell to become cancerous; these are usually dominant and are referred to as 'gain of function' mutations. Additionally, those that negate expression of tumor suppressor genes or disengage the actions of their products may also drive a cell to become cancerous; these are normally recessive and are known as 'loss of function' mutations. Other factors that can predispose cells to becoming cancerous include epigenetic silencing, various exposures

encountered within an individual's environment (which can contribute to both mutations and altered epigenetic silencing), as well as exposure to certain viruses.

As mentioned above, proto-oncogene products are often involved in signaling pathways that mediate cell proliferation. Mutations that affect elements of these various cell signaling pathways may lead to uncontrolled cell proliferation and thus can have a dramatic, wide-ranging impact in cancers. At least four major signaling pathways are involved in controlling cell proliferation: the RAS MAP Kinase pathway, Wnt signaling pathways, Hedgehog signaling, and Notch signaling. I will briefly discuss each of these pathways.

The Ras MAP Kinase Pathway

The Ras MAP Kinase pathway is used by a wide variety of cells to respond to a wide variety of signaling molecules. Key components of this pathway are products of proto-oncogenes and include cell membrane receptors, the G protein or guanine nucleotide binding protein, Ras, a tyrosine kinase named Src, a serine/threonine kinase called p21 activated kinase (PAK), Growth Factor Receptor Binding protein (Grb2), a Guanine Nucleotide Release Factor (GNRF), named Sos, and serine/threonine kinases called MAP kinase kinase kinase (RAF), MAP kinase kinase (MEK), and MAP kinase (ERK), (2).

An ordered pattern of events begins with the interaction of an extracellular stimulus, such as a growth factor, with a cell membrane receptor, (2). This typically results in the receptors dimerizing and cross-phosphorylating, which prompts binding to the receptors by Grb2, Src, and PAK. Grb2 binds to the receptor through its SH2 domain, often with another protein called SHC, before bringing in Sos. The GNRF, Sos, is then able to initiate activation of Ras, and Ras can draw RAF to the plasma membrane, where it is activated through Src and PAK

phosphorylation; active RAF then initiates the action of MEK, which initiates the action of ERK, through sequential phosphorylation (2).

ERK then phosphorylates several transcription factors, including Myc, Jun, and Elk-1, which activates Jun and Elk-1 (3). Phosphorylation by ERK allows Myc to assume a more stable form, enabling Myc to bring about the transcription of a vast number of genes (3). After activation by ERK, Elk-1 stimulates transcription of the gene encoding Fos; when Fos is available to bind with Jun, they form a dimeric transcription factor called AP-1, which then activates the expression of key genes whose products are involved in cell cycle progression (2). The series of reactions in this signal transduction pathway ultimately leads to the production of copious amounts of proteins needed for cell division.

Various mutations can affect signal transduction pathways, but those occurring at significant regulatory points in a pathway produce greater consequences. A single point mutation in the G protein Ras enables constitutive activation of the Ras MAP Kinase pathway by curtailing the GTPase action (GTPase hydrolyzes GTP to GDP) that deactivates the Ras protein; in this situation, active Ras will continually recruit RAF to the membrane, and initiate the kinase cascade without an extracellular stimulus (2). Additional components of this pathway may be similarly affected by acquired point mutations that cause them to remain in a perpetually active configuration, including growth factor receptors, kinases, and transcription factors (2). In one such case, growth factor receptors will continually undergo dimerization and autophosphorylation, stimulating the kinase cascade. Transcription factors such as Myc and Jun may also be impacted by these types of mutations, remaining active without phosphorylation by ERK; Myc could acquire stability without ERK activation (3).

Signaling kinases may also be impacted by these mutations, and Dhillon *et al*. report another important example of a cancerous mutation within this pathway occurs with a particularly high frequency in the gene that codes for B-RAF, one of three mammalian isoforms of RAF (2). Most often, cancerous mutations in the gene coding for B-RAF, such as the mutation V600E, result in the product's notably increased kinase action, and consequent increase in overall efficiency in the activation of ERK; these mutations are reportedly found in thyroid, colon, ovarian, and many other different cancers, as well as in 66% of cancerous melanomas (2).

Wnt Signaling Pathways

Another signal transduction pathway that can lead to uncontrolled cell proliferation in cancers is a Wnt pathway (4). This pathway involves the protein, *B*-catenin, a protein also known for its role in cell-to-cell adhesion (4). The Wnt pathway is initiated when an extracellular protein belonging to the Wnt family binds to a receptor; Wnt proteins are associated with both cell division and differentiation, and this Wnt pathway is known to prompt transcription of a number of the same genes as those activated through the RAS MAP kinase pathway.

Receptors for this pathway are called Frizzled, and they are known to form a heterodimer with another protein called LDL-receptor-related protein (LRP), before activation of the protein Disheveled (Dvl), (4). Once the action of Dvl is initiated, it inhibits the normal activity of the protein, Glycogen Synthase Kinase 3 (GSK-3), (4). Active GSK-3 usually binds to form a complex with Adenomatous polyposis coli (APC), Axin, and various other proteins that ultimately work towards proteasome degradation of *B*-catenin, which binds to APC (4). This most often occurs through phosphorylation of *B*-catenin by GSK-3 and casein kinase 1 (CK1), although phosphorylation independent paths to *B*-catenin degradation may also take place (4).

Conversely, when GSK-3 is rendered inactive through stimulation of the Wnt pathway, *B*-catenin can disassociate from APC (4). The integrity of the protein *B*-catenin is preserved and it remains stable (4). In its stable form, *B*-catenin then penetrates the nucleus (4). A co-repressor protein called Groucho forms a complex with lymphoid-enhancer factor/T cell factor (LEF/TCF) transcription factors in the absence of the Wnt protein signal (4). Following activation of the Wnt pathway, *B*-catenin enters the nucleus and supplants Groucho; the *B*-catenin/ LEF/TCF complex then drives transcription of numerous genes, including those coding for c-Myc, c-Jun, matrilysin, CD44, peroxisome proliferator-activated receptor, cyclin D1, urokinase-type plasminogen activator receptor, and fra1, among others (4).

Mutations affecting members of this pathway are known to contribute to cancers and can stimulate tumor growth (4). Particularly impactful are the mutations occurring in genes that code for APC or *B*-catenin that prevent their binding (4). This creates a scenario where *B*-catenin is freely permitted to enter the nucleus, initiating constitutive transcription of the aforementioned genes. Increased expression of Cyclin D1 and Myc results in excessive cell division (4). CD44 (a transmembrane protein identified as an ECM adhesion molecule), is associated with metastases and angiogenesis, and is thought to contribute to drug resistance (5). Urokinase-type plasminogen activator receptor, or the receptor for uPA (a serine protease that can be found bound to axons of migrating cells) is thought to help with invasion and migration in cancer cells. Matrilysin, or matrix metalloprotease 7 (MMP7), is a member of a family of proteolytic enzymes that play crucial roles in cancer cell metastases and invasion through their degradation of ECM components; MMP7 is also reported to be critically important for the process of angiogenesis that takes place within the microenvironment of the original tumor site, as well as at developing

secondary sites (6). CD44, uPA receptor, and matrilysin overexpression are reportedly apt to contribute to the growth of existing tumors as well (4).

Indeed, authors report that cancerous mutations relevant to this Wnt pathway include defects in serine, threonine, and certain other proximate amino acid residues required for degradation of *B*-catenin, and these mutations can be seen in a wide variety of cancers (4). These cancers include melanomas, gastric and colon cancers, as well as cancers of the liver, ovaries, prostate, and pancreas, among others (4). *APC* gene mutations that result in altered binding sites for *B*-catenin or Axin render APC unable to exercise its tumor suppressive actions (through facilitating destruction of *B*-catenin), and heightened levels of both *B*-catenin and functional *B*-catenin/TCF complexes have been found to be present in cancers (4). These findings note a particularly significant rate of occurrence in colorectal cancers (4).

Hedgehog Signaling

The Hedgehog signaling pathway is another pathway having significant implications in cancers, and bears many similarities to the Wnt pathway; in fact, several integral proteins involved with the Wnt pathway also participate in regulatory roles in the Hedgehog pathway, such as kinases GSK-3 and CK1. Hedgehog proteins (Sonic, or Shh, Indian, or Ihh, and Desert, or Dhh), are morphogens playing critical roles in developmental processes and known to be associated with embryogenesis; Hedgehog signaling is implicated in the proliferation of various tissue stem cells, including those of the mammary gland, and proteins involved in this pathway are also reported to be perceptible and acting in human breast CSCs (cancer stem cells), observed specifically in $CD44^{+}CD24^{-}Lin^{-}$ cells (7).

Once Hedgehog proteins are produced, they undergo modifications involving lipids within the secretory pathway. Authors Evangelista, Tian, and de Sauvage specifically mention

some modifications in their article published in 2006: for example, cholesterol groups are known to be added to the carboxyl terminus of the protein, while palmitoyl groups are added to the amino terminus of the protein (8). Once the Hedgehog proteins have proceeded through the secretory pathway, and have been modified, a transmembrane protein called Dispatched (Disp) dictates release of the Hedgehog protein from the cell (8). The binding of a Hedgehog protein to the transmembrane receptor Patched (PTCH) activates the signaling cascade of the pathway in the receiving cell, enabling the GPCR-like transmembrane protein Smoothened (SMO) to relocate from the cytoplasm to the membrane of the cilia where Patched is found (8). Two regulating proteins called Hedgehog interacting protein (Hip), and growth arrest-specific protein (Gas-1), are able to isolate Hedgehog proteins following binding, and largely determine how much of the Hedgehog protein is accessible and can interact with the receptor Patched (9).

Early studies suggest Hip and Gas1 are able to render Hedgehog proteins unavailable upon binding due to competition for binding sites, as Gas1 binding to Sonic Hedgehog and Sonic Hedgehog binding to Patched cannot occur simultaneously (10). Cdo and Boc are two proteins thought to assist with Hedgehog ligand binding to the PTCH receptor. Following activation by Hedgehog binding, SMO then interacts with a complex of proteins within the cytoplasm to activate glioma-associated (GLI) proteins that may then enter the nucleus; there are three GLI transcription factors (9).

GLI1 is the fundamental activator of transcription and targets a diverse number of genes relevant to roles characteristic of most cancers, as well as feedback loops that influence activity within the pathway (8). These include genes coding for Platelet Derived Growth Factor Receptor (PDGFR) and Myc, as well as Cyclins D1 and D2, known to be involved with cell proliferation. Other GLI1 targets include the gene coding for the anti-apoptotic protein BCL2,

genes that code for VEGF and ANG, which are involved in generating nourishing vessels to tumors, genes coding for SNAIL and MMP9, which are associated with the epithelial-mesenchyme transition that is necessary for cell migration; authors Kwon, *et al*. also report that GLI1 is able to up-regulate expression of *MMP11* in triple negative breast cancer cells and ERα-negative breast cancer cells, promoting metastasis by increasing migration, as well as invasion (11). Genes coding for NANOG and SOX2, which are implicated in self-renewal, are also known targets of GLI1. Products involved with feedback regulation of the pathway include the targets Hip, PTCH, and GLI1 (8). GLI3's role is known to be that of a transcriptional repressor, and GLI2's function may be to aid, or repress transcription, contingent upon modifications that result in an abbreviated form of the protein (8).

Without the Hedgehog signal, PTCH inhibits the action of SMO, preventing its relocation to the cell membrane and retaining it in the cytoplasm (9). Protein kinase A, GSK-3, and Ck1 all interact with GLI through phosphorylation, which prompts selective proteolytic cleavage of GLI, regulated by the proteasome (9). The abbreviated form of the GLI protein, GLI3, then acts to repress transcription of Hedgehog pathway genes. Another protein that plays a prominent role in regulation for this pathway is called Suppressor of Fused (SUFU); SUFU is able to prevent transcription of genes through its ability to bind cytoplasmic GLI proteins as well as GLI proteins within the nucleus (9). It is worth mentioning that in the vertebrate pathway, proteins equivalent to Fused, Costal2, and Ci appear to be absent, unlike in the well-studied *Drosophila* model for the Hedgehog pathway.

Abnormal initiation of Hedgehog pathways in adult cells (long after crucial development), or aberrant and continuous activation during tissue repair, impact many different cancers through mutations resulting in elevated levels of expression of Hedgehog proteins, or

mutations within the pathway (9). Central characteristics of all cancers, including proliferation, anti-apoptotic or cell survival strategies, and the ability to form metastases have all been attributed to inappropriate activation of the Hedgehog pathway, resulting from excessive expression levels of Hedgehog proteins; overexpression of these ligands has reportedly been traced in a plethora of cancer cell lines and tumors from pancreatic cancers, prostate cancers, small cell lung cancers, and gastric cancers, among others (8). Lee, *et al*. mention the mutation H134Y occurring in the Sonic Hedgehog protein. This mutation may bring about tumorigenic effects from a lowered capacity to bind the regulatory protein Gas1, leaving an overabundance of Sonic Hedgehog, inducing large increases in signaling and ultimately leading to uncontrolled growth (10).

Mutations occurring within the Hedgehog pathway have been reported to occur in several types of cancerous tumors; for example, mutations rendering PTCH1 inactive and unable to repress SMO in the absence of Hedgehog protein allow inappropriate initiation of the signaling pathway (9). This type of mutation is culpable in Gorlin syndrome, where patients are made particularly vulnerable to basal cell carcinomas and unusual tumor types, including medulloblastomas and rhabdomyosarcomas (9). Basal cell carcinomas are also known to occur, with cogently fewer cases, when a particular mutation affecting SMO allows it to evade suppression by PTCH1, and multiple mutations found in sporadic medulloblastoma occur in PTCH1, SMO, and Supressor of Fused proteins (9).

Notch Signaling

Notch1-Notch4 are four transmembrane receptors that serve as the entry point for another molecular signaling pathway of critical significance in development (specifically regarding

signaling for lateral inhibition and cell fate), as well as having diverse and impactful roles in cancers (12). In cancers, aberrant signaling of this pathway is likely to affect cell fate and prevent cells from differentiating or maintain them in a proliferative mode (12). Initiation of this pathway occurs when Notch receptors respond to ligands Delta-like (Delta-like1, 3, and 4) and Jagged (Jagged1 and Jagged2); these ligands are from a family of proteins known as Delta, Serrate and Lag-2, or DSL (12).

A series of proteolytic modifications to the Notch receptor transpires outside of the cell and within, following recognition of a DSL ligand (12). The extracellular domain of Notch undergoes a proteolytic cleavage event by a protein known as a disintegrin and metalloprotease (ADAM), while the domains within the cell are subjected to a series of cleavage events that prepare the intracellular domain: the main intracellular cleavage event is carried out by the proprotein convertase furin, then the cytoplasmic domain undergoes further cleavage by a complex containing γ-secretases Presenilin and Nicastrin, before the truncated form of the Notch protein, or Notch intracellular domain (NICD) is free to enter the nucleus and affect transcription via interaction with the Cb binding factor (CBF1) family of transcription factors (12).

The effects of Notch signaling are diverse, and depend on NICD and CBF1 interactions with various protein complexes that either act as corepressors or coactivators of transcription (12). SKIP (Ski-interacting protein) is a protein that binds to CBF1 and is reported to have a dual role in the presence of Notch; SKIP can behave as a corepressor of transcription with SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) to recruit histone deacetylases that contribute to repression, and SKIP is also reported to behave as a coactivator of transcription (13). NICD/CBF1 complexes also interact with the transcriptional coactivator Mastermind (MAML1), (13).

Notch signaling may stimulate or quell a number of vital cellular processes or actions including determination of cell fate and differentiation, proliferation, and apoptosis, and errant signaling through this pathway is known to contribute to a variety of diseases, including cancers (14). Among the many genes targeted for transcription by CBF1 factors are Hes1, Hes5, and Hey1 (known for their roles in repression of transcription), as well as several others, many of which are associated with cancers; these include genes encoding c-myc, cyclin D1, NFkB2, and bcl-2, as well as Notch1 and Notch3 (13). As mentioned earlier, c-myc and Cyclin D1 are associated with cell cycle progression (D-cyclins complex with cdk4 and cdk6 to stimulate quiescent cells in G_0 to begin the cell cycle), while bcl-2 is an antiapoptotic protein. NFκB2 is one of a family of several NFκB proteins that play a role in immune response and are known to launch responses to both stress and chronic inflammation; an overabundance of signaling through NFκB pathways has also been reported in cancers (14).

Under circumstances where the Notch pathway has not been initiated by DSL ligands, and NICD is not present, CBF1 proteins function to repress transcription (12). Additional proteins reported to act as corepressors interacting with CBF1 include SHARP (SMRT and HDAC associated repressor protein), KyoT2, and CIR (CBF1 interacting corepressor) (13). Evidence for a distinctly separate route through the Notch pathway independent from that described above, has been elucidated in studies of *Drosophila*. Brennan and Brown (12), report this signaling route makes use of other Notch receptor domains and is conditional on the involvement of a negative regulator protein present within the cytoplasm known as Deltex. It has been suggested that a vertebrate protein equivalent to Deltex may participate in a similar role in humans; evidence suggests this route ultimately functions to abolish cell differentiation, inhibit the actions of Jun-N kinase (JNK) and also CREB binding protein (CBP) that works to

aid in activation of transcription, as well as repressing transcription of genes normally targeted downstream of the Wnt signaling pathway (12).

It is known that Notch proteins can act as products of protooncogenes (15). In addition to these early findings, further studies have established a potential role for Notch signaling in cell fate determination during normal development of human mammary tissue; these studies also established that signaling through this pathway is able to significantly increase mammary stem cell proliferation (15). This study suggests inappropriate Notch signaling could be implicated in breast cancers through deregulating self-renewal of mammary stem cells (15).

Metastasis: Progression of Cancers

Metastasis, which is the hallmark of a malignant tumor, is the most dangerous and inscrutable feature of cancers, causing problematic challenges for successful treatment and being culpable in most cancer deaths. Little is actually known about what precisely transpires to make a specific cancer cell attempt to migrate from its primary tissue site. The first of several difficulties encountered by the migrating cancer cell is breaking through its existing tissue boundaries. A cancer cell must have acquired certain features that distinguish it from other cancerous cells within the initial tumor, allowing it to fulfill this role as it responds to local stimuli (via paracrine signaling) and undergoes epithelial-mesenchyme transition. One of these features is the likely loss of E-cadherin (crucial for cell-to-cell adhesion via specialized recognition), as well as the likely initiation of expression for select mesenchymal proteins, such as N-cadherin, vimentin, fibronectin, matrix metalloproteases (MMPs) and PDGFR (16).

Vimentin is an intermediate filament protein contributing to structures in certain cells like fibroblasts, while N-cadherin is another member of the family of proteins critically involved with cell-to-cell adhesion. Fibronectin is an ECM protein composed of a dimer with subunits connected by cross-linking disulfide bonds; type III fibronectin binds to cell surface integrins, affixing matrix protein to the cellular cytoskeleton. Platelet Derived Growth Factor Receptor (PDGFR) is normally heavily involved in wound healing and associated with cell proliferation, as mentioned earlier. MMPs, or matrix metalloproteases, are a class of proteolytic enzymes that work extracellularly to locally degrade fundamental matrix components such as collagen or laminin, or fibronectin. Some MMPs work with high specificity, while others are bound to the membrane where they are needed, or limited by tissue inhibitors of metalloproteases (TIMPs). MMPs are essential for a cell to extricate itself from the extracellular matrix, allowing for cell migration.

The next difficulty presented to the migrating cancer cell that has encroached normal tissue borders is to reach or secure a path that will allow escape of its primary site. Macrophages are reported to play a vital role in facilitating the cancer cell's successful migration into blood vessels it will utilize on its path (17). It must then survive through the lymph or blood circulation to reach a secondary site, where it may entrench itself and successfully invade new tissue borders. In order to survive this journey, the migrating cancer cell must effectively evade immune response components; for example, within the bloodstream, platelets are instrumental in shielding the cancer cell from recognition by Natural Killer (NK) cells (17).

Invasion requires use of the same degradation strategy needed for escape from the tissue borders of its primary site. If this has been accomplished, then micrometastases must be established and suitably maintained for cancer cell survival, growth, and colonization or

development of a new tumor at the second site. This is a series of difficulties most migrating cancer cells are unable to navigate and survive. Lacking constant signaling and nourishment emanating from the tumor microenvironment of the primary tumor site, these challenges most often prove fatal and the migrating cancer cells die off before reaching a new site, or the micrometastases cluster fails to establish itself and is unable to grow and eventually dies. Our hypothesis is centered around the question of what prompts the cancer cell to undertake the initial challenge of migration.

We hypothesize that cancer cell migration occurs in order for a cell to escape harsh stresses imposed by a tumor microenvironment that has become too inhospitable to support it. Stresses within a tumor microenvironment are created with the involvement of multiple factors, over time. Among the stresses encountered are hypoxic conditions, an acidic pH, and, possibly, elevated temperature (17). Accumulating mutations and genetic dysregulation, together with defective DNA repair mechanisms, and aberrant cell survival mechanisms contribute to uncontrolled cell proliferation and tumor growth in cancers (18, 17). Although tumor microenvironments are known to change over time to favorably support a tumor as it increases in size, tumor growth may occur at a faster rate than the development of vasculature that will reach tumor cells located within the innermost areas of the tumor (17). Hypoxia is introduced as cells are denied necessary oxygen, heat stress occurs within tumors as cells growing and proliferating uncontrollably may begin to elevate core temperatures inside of the tumor, and increased metabolic activity may begin to alter pH, creating conditions that are more acidic (17, 18). Some stresses encountered within the tumor microenvironments of cancer cells may impact or determine crucial adaptive cellular responses contributing to levels of expression for miRNAs

that can have broad and cumulative results with respect to the invasiveness of cancer cells (18). These results may affect the onset or progression of metastatic disease.

miRNAs

miRNAs are small, single-stranded, non-coding RNAs that base pair with specific mRNAs, often affecting levels of protein expression within cells either by activating or inhibiting translation. miRNAs were originally identified in 1993, and since that time at least 2500 human miRNAs have been discovered. Recent studies project up to 60% of protein coding genes are subject to regulation by miRNAs; more than half of genes that code for miRNAs are known to be located in areas of the human genome frequently associated with cancers (19). miRNAs are known to be products of protooncogenes and tumor suppressors, and have been heavily studied due to their crucial roles in cancers. miRNAs are also known to have significant involvement in roles connected with normal development of breast tissue, as well as promotion or suppression of invasive disease in breast cancers. Many important cellular processes are subject to the regulatory actions of miRNAs with diverse and wide-ranging impacts.

miRNAs are produced from a pre-miRNA transcript known to undergo processing by at least two ribonucleases: Drosha and Dicer. RNA-Induced Silencing Complex (RISC) then becomes active, and Argonaut (AGO) proteins can then degrade the passenger strand. miRNAs most often target specific AU-rich sequences within the 3' UTR of a specific mRNA. Interactions between miRNAs and their target mRNAs do not require precise complementarity; consequently, a single miRNA may potentially target a vast number of mRNAs (19). miRNAs are also known to regulate gene expression through transcriptional repression by initiating action of the RNA Induced Transcription Silencing (RITS) complex, which prompts a conversion to transcriptionally inert heterochromatin.

Many studies have been performed to examine miRNA expression levels in relationship to invasive breast cancers compared with healthy breast tissue. Some specific miRNAs associated with metastatic disease were found to have expression levels comparable to those demonstrated during normal phases of healthy tissue development characterized by large proliferation rates (19). Le Quesne, *et al*. offer examples taking place during phases of puberty and gestation, where proliferation in ductal and alveolar breast tissues are known to be dramatically increased (19).

Classification of breast cancers based on extensive gene expression profiles, or 'signatures' performed with microarrays, have allowed researchers to categorize or establish subtypes for breast tumors (20, 21). Authors Hu, *et al*. explain that the subtypes that most breast tumors fall into include Luminal A, Luminal B, Basal-like, $Her2^{+}$, and normal breast-like, and then go on to discuss the significance of these large-scale miRNA expression studies (21). The article by Hu *et al*. details how the subsequent identification of subtypes not only sheds light on breast cancer etiology as well as associating pathological characteristics with certain tumor types, but has crucial clinical relevance regarding specific targeted treatment approaches and patients' prognoses (21).

Early observations that clarified various roles for miRNAs within normal healthy tissues have allowed for better comprehension regarding the impacts of gene dysregulation in the onset and promotion of disease. Previous studies relating to invasive breast cancers demonstrate particular miRNAs to be up-regulated, and certain others to be down-regulated.

Previous studies reflect down-regulated levels of expression for miR-145 in breast cancers, and these lowered expression levels have been associated with several pathological aspects of metastatic disease (22). miR-145 is known to participate in a wide range of cellular

processes and functions significant in many different cancers, and its numerous roles help to make it robust in its tumor suppressor effects (22). Multiple studies examining miR-145 identify its involvement in apoptosis, cancer cell differentiation, proliferation, tumor growth, angiogenesis, invasion, metastasis, as well as its role as a diagnostic and prognostic indicator; it's therapeutic potential has also been identified (23, 24). miR-145's tumor suppressive behavior is tissue and tumor-type specific; targets and functions vary in differing metastatic diseases. In breast cancers, several oncogenic targets for miR-145 include ER-α, c-Myc, RTKN, MUC1, FSCN1, OCT4, N-RAS, MMP11, Rab27a, and HBXIP (22, 24, 25).

A study that examined the effects of elevated expression levels for miR-145 has reported lowered levels of expression for EF-α, and higher levels of expression for p53 (known to also be regulated by miR-34), leading to a greater presence of the cleaved form of PARP and higher levels of the proapoptotic proteins, PUMA and p21 (23, 24). One study concluded miR-145 expression ultimately induces apoptosis in breast cancer cells (24). c-Myc is associated with proliferation, but when Myc expression is targeted by miR-145 the cell cycle is arrested (23). c-Myc is also associated with apoptosis, as well as invasion in breast cancers, while RTKN is another target associated with proliferation. Mucin1 (MUC1) is associated with invasive and metastatic features of breast cancer, occurring as a result of its ability to interact with β-catenin; when MUC1 is successfully targeted by miR-145, cancerous migration from breast to lung tissue is reportedly checked (23). Fascin homologue 1 (FSCN1) is a protein known to bind with actin and is reported to be associated with invasion in breast cancers as well. Octamer binding Transcription factor 4 (OCT4) is associated with cell differentiation and CSCs, while N-RAS promotes both cell division and angiogenesis in breast cancers (23).

MMP11 is associated with invasion and metastases, and Rab27a is reported to advance tumor growth as well as metastasis in breast cancers (25). New evidence portrays Hepatits B X-interacting protein (HBXIP) as a suspected target of miR-145 in a recently published study, and it has been reported to act as an oncogenic protein in breast cancers with functions relating to cell proliferation and invasion (24).

miR-330-3p is reported to be upregulated and to function as the product of a protooncogene in breast cancers. miR-330-3p has been found to promote breast cancer metastases, and is potentially useful as a biomarker; due to its overexpression, patients' distant relapse-free survival is found to be decreased, making it a prognostic indicator (25). Mesci, *et al*. explain that miR-330-3p has roles in various other metastatic disease (including esophageal and lung cancer, as well as glioblastoma), and that potential miR-330-3p targets involved in proliferation and invasion in other cancers include CCBE1, PDCD4, SH3GL2, EGR2 (25).

Although few targets of miR-330-3p have been identified with respect to invasive breast cancers to date (compared to more extensively studied miRNAs), it has recently become of interest as an important topic of study, and further research is needed to gather a better perspective of its roles in breast cancers. One of miR-330-3p's direct targets, Collagen and Calcium Binding EGF Domains 1 (CCBE1), a protein previously studied in relationship to other disease states, has recently been found to have tumor suppressive action in breast cancers; studies report that its silencing can be correlated with lower survival rates (25).

For this investigation, heat and hypoxic stresses have been introduced in three cultured breast cancer cell lines: MCF7, MB468, and MB231. MCF7 breast cancer cells are estrogen receptor positive (ER^+) and progesterone receptor positive (PR^+), while MB468 and MB231 breast cancer cells are triple negative for estrogen, progesterone, and human epidermal growth

factor 2 receptors (ER^-, PR^-, and $HER2^-$). Triple negative breast cancer cells are known to demonstrate aggressive invasiveness, and triple negative breast cancers present unique challenges for treatment. This study was undertaken in order to examine the impact of heat and hypoxic stresses encountered within the tumor microenvironment for endogenous expression levels of miR-145 and miR-330-3p. We also wanted to analyze the effects these altered miRNA expression levels have on the invasiveness of breast cancer cells, in order to explore whether the altered miRNA expression levels are correlated with greater invasiveness.

Materials and Methods

Cell Culture

MCF7, MB468, and MB231 breast cancer cell lines were obtained from Dr. Jessica Kelts (Department of Chemistry and Biochemistry at the University of Michigan-Flint). Cells were thawed and cultured in DMEM medium with 10% FBS (Fetal Bovine Serum) supplemented with sodium pyruvate, non-essential amino acids, and fungicidal and bactericidal agents amphotericin-B (0.25 μg/ml), and gentamicin (50 μg/ml), respectively. Media prepared for MCF7 cells was also supplemented with insulin (10 μg/ml).

Cell Preparation and Treatment

MCF7, MB468, and MB231 cells were initially plated in P60 culture dishes (100,000 cells per dish). Cells were allowed to adhere overnight. Then, two dishes were placed under normal conditions (37 degrees Celsius and ambient oxygen), two dishes were exposed to heat stress of 40 degrees Celsius, two dishes were exposed to hypoxic stress of 3.5% oxygen, and two dishes were exposed to both heat and hypoxic stress conditions at 40 degrees Celsius and 3.5% oxygen. Cells were then incubated for 96 hours under each condition.

miRNA Extraction

Immediately following the 96-hour incubation, cells were lysed and miRNA extractions were performed using the miRNA isolation and enrichment protocols from the mirVana kit (Life Technologies; Gaithersburg, MD). Two sets of extractions were obtained for every cell line for each condition.

miRNA Quantification

The miRNA Quantification Assay was performed using the Quant-IT kit (Life Technologies). The microplate was prepared by loading two wells from the first set of extractions, then two wells from the second set of extractions for each cell line, in a separate row for every condition, beginning with row B. The first row of the microplate (row A) contained the standards. Fluorescence values obtained from the microplate reading were used to generate a standard curve using Excel software in order to determine concentration. Concentrations of the experimental samples were determined by using the equation of the line generated by the standards.

Reverse Transcription

Reverse transcription was performed using the Taqman miRNA RT kit (Life Technologies). 10 ng RNA was used per reaction in order to obtain cDNA samples from both sets of samples for each condition in all cell lines. Volumes per reaction were sufficient to load cDNA for subsequent Real Time PCR reactions in triplicate.

Real Time PCR

Real Time PCR amplification was performed using Taqman miRNA assays (20x primers specifically prepared for miR-145 and miR-330-3p) and Taqman PCR mastermix purchased from Life Technologies. All samples were then plated in triplicate. ΔΔCT analysis was utilized to examine results obtained from Real Time PCR performed to establish miRNA expression levels following exposure to stress conditions. Expression of each miRNA under each condition was normalized to the U6 small nuclear RNA control, giving a ΔCt value. This ΔCT value was compared against the ΔCT values obtained for each sample for normal conditions, giving a ΔΔCt value. This value represents the expression of each miRNA in each condition compared to the

U6 control and the cells grown under normal conditions, with expression levels being equivalent to $2^{\Delta\Delta Ct}$. Positive values thus indicate an increase in expression, while negative values indicate a decrease in expression.

Invasion Assays

Invasion assays were performed using Matrigel invasion chambers obtained from Fisher Scientific (Pittsburgh, PA). MCF7 and MB468 breast cancer cells were resuspended in freshly prepared serum-free medium prior to being loaded into chambers. 25,000 and 50,000 cells were plated per chamber for each condition and cells were incubated for 96 hours: two chambers contained cells exposed to normal conditions (37 degrees Celsius, and ambient oxygen), two chambers contained cells subjected to heat stress at 40 degrees Celsius, two chambers contained cells exposed to hypoxic stress of 3.5% oxygen, and two chambers contained cells exposed to both heat and hypoxic stresses (40 degrees Celsius and 3.5% oxygen). Cells that were able to degrade the artificial extracellular matrix and migrate to the bottom of the chamber were stained with a solution of 0.9% crystal violet in 10% methanol. In order to quantify the invasion assays, each invasion chamber was photographed by quadrant, then systematically counted using ImageJ software in order to examine the invasiveness of MCF7 and MB468 breast cancer cells under the different experimental conditions.

Results

MCF7, MB468, and MB231 cells were plated in P60 culture dishes (100,000 cells per dish) and allowed to adhere overnight before undergoing a 96-hour incubation under stress conditions. Two dishes were placed under each condition: normal conditions (37 degrees Celsius and ambient oxygen), heat stress (40 degrees Celsius), hypoxic stress (3.5% oxygen), and dual stress (40 degrees Celsius and 3.5% oxygen). Cells were lysed, and miRNA was extracted immediately upon completion of the 96-hour incubation period.

miRNA was quantified and reverse transcription was performed with the Taqman miRNA RT kit purchased from Life Technologies; average values for concentrations of quantified miRNA samples are listed in appendix 1. Kruskal-Wallis analysis was performed for miRNA concentrations, using SPSS statistical software; median miRNA concentrations (µg/ml) under normal conditions, heat stress, hypoxic stress, and dual stress conditions were compared within each of the three breast cancer cell types. A significant difference in median miRNA concentrations among cells exposed to normal and stress conditions can be observed within each cell type, and Kruskal-Wallis analyses can be viewed in appendix 2, (MCF7 $p<0.05$, MB468 $p<0.01$, and MB231 $p<0.05$). These results support the prediction that overall miRNA concentrations differ within breast cancer cells subjected to stress compared with cells under normal conditions.

Reverse transcription reactions were performed for each condition with miRNA extracted from MCF7, MB468, and MB231 breast cancer cells. Taqman miR-145 and miR-330-3p assays (20X) and Taqman Universal PCR Mastermix (Life Technologies) were used, as well as the cDNA produced from reverse transcription reactions, in order to perform real time PCR amplification of miR-145, miR-330-3p, and the snRNA U6, which served as the reference gene.

Some issues encountered during this experiment involved the exhaustion of necessary reagents and of original miRNA extraction samples, limiting or preventing collection of data for certain replicates. Exceptions to our experimental parameters include the elimination of the heat stress condition in the replicate PCR run for the 2nd sample-set of MCF7 extractions (due to total depletion of the RNA sample, heat stress samples could not undergo reverse transcription); also eliminated were MB468 dual stress samples from sample-set 1 for the first and replicate PCR runs. Additionally, only one sample-set of extractions could be examined for MB231 cells, due to exhaustion of reagents required for real time PCR amplification. A first and replicate run of reverse transcription and real time PCR was performed for 1 set of miRNA extractions from MB231 breast cancer cells.

Analysis of Real Time PCR

Real time PCR reactions were done in triplicate. Ct values are listed in Tables 2, 3, and 4. The two closest Ct values were averaged for each condition from every cell type for U6, miR-145, and miR-330-3p reactions, before determining the ΔCt. Ct values were then normalized to the U6 small nuclear RNA control to determine ΔCt values for each condition, from every sample cell type.

Cell Type	RXN	Condition	Sample-Set	PCR Run	Ct Value
MCF7	U6	Normal	Set 1	First	16.05
MCF7	U6	Normal	Set 1	Replicate	21.63
MCF7	U6	Normal	Set 2	First	21.95
MCF7	U6	Normal	Set 2	Replicate	22.85
MCF7	U6	Heat	Set 1	First	17.72
MCF7	U6	Heat	Set 1	Replicate	18.93
MCF7	U6	Heat	Set 2	First	19.66
MCF7	U6	Heat	Set 2	Replicate	*
MCF7	U6	Hypoxia	Set 1	First	19.96
MCF7	U6	Hypoxia	Set 1	Replicate	19.58
MCF7	U6	Hypoxia	Set 2	First	29.78
MCF7	U6	Hypoxia	Set 2	Replicate	17.53
MCF7	U6	Dual Stress	Set 1	First	15.46
MCF7	U6	Dual Stress	Set 1	Replicate	15.81
MCF7	U6	Dual Stress	Set 2	First	23.10
MCF7	U6	Dual Stress	Set 2	Replicate	18.56
MCF7	miR-145	Normal	Set 1	First	32.50
MCF7	miR-145	Normal	Set 1	Replicate	33.27
MCF7	miR-145	Normal	Set 2	First	30.92
MCF7	miR-145	Normal	Set 2	Replicate	29.86
MCF7	miR-145	Heat	Set 1	First	29.35
MCF7	miR-145	Heat	Set 1	Replicate	30.79
MCF7	miR-145	Heat	Set 2	First	28.63
MCF7	miR-145	Heat	Set 2	Replicate	*
MCF7	miR-145	Hypoxia	Set 1	First	30.65
MCF7	miR-145	Hypoxia	Set 1	Replicate	30.68
MCF7	miR-145	Hypoxia	Set 2	First	32.09
MCF7	miR-145	Hypoxia	Set 2	Replicate	32.20
MCF7	miR-145	Dual Stress	Set 1	First	29.74
MCF7	miR-145	Dual Stress	Set 1	Replicate	31.05
MCF7	miR-145	Dual Stress	Set 2	First	30.56
MCF7	miR-145	Dual Stress	Set 2	Replicate	30.70
MCF7	miR-330-3p	Normal	Set 1	First	33.21
MCF7	miR-330-3p	Normal	Set 1	Replicate	31.99
MCF7	miR-330-3p	Normal	Set 2	First	28.99
MCF7	miR-330-3p	Normal	Set 2	Replicate	28.87
MCF7	miR-330-3p	Heat	Set 1	First	28.86
MCF7	miR-330-3p	Heat	Set 1	Replicate	29.90
MCF7	miR-330-3p	Heat	Set 2	First	28.35
MCF7	miR-330-3p	Heat	Set 2	Replicate	*
MCF7	miR-330-3p	Hypoxia	Set 1	First	30.26
MCF7	miR-330-3p	Hypoxia	Set 1	Replicate	29.63
MCF7	miR-330-3p	Hypoxia	Set 2	First	29.35
MCF7	miR-330-3p	Hypoxia	Set 2	Replicate	30.01
MCF7	miR-330-3p	Dual Stress	Set 1	First	28.91
MCF7	miR-330-3p	Dual Stress	Set 1	Replicate	29.59
MCF7	miR-330-3p	Dual Stress	Set 2	First	27.56
MCF7	miR-330-3p	Dual Stress	Set 2	Replicate	30.09

Table 1 Ct Values from Real Time PCR Amplification of miRNAs from MCF7 breast cancer cells

Real Time PCR reactions were performed in order to examine expression levels for miR-145 and miR-330-3p in MCF7 breast cancer cells exposed to stress conditions. All samples were run in triplicate, and three Ct values were collected for each sample in both miRNA extraction sets. The two closest Ct values for every sample were averaged, and this is the value listed above. Each PCR run was then repeated (with fresh cDNA prepared from replicate RT reactions). The U6 snRNA was used as the reference. These Ct values were then used to determine the ΔCt for each condition in MCF7 breast cancer cell miRNA extractions.

*The condition for the Replicate Run for MCF7 Heat/Sample-Set 2 had to be eliminated, because the RNA sample from Set 2 was completely depleted after the first series of RT reactions were prepared.

Cell Type	RXN	Condition	Sample-Set	PCR Run	Ct Value
MB468	U6	Normal	Set 1	First	24.55
MB468	U6	Normal	Set 1	Replicate	24.62
MB468	U6	Normal	Set 2	First	25.58
MB468	U6	Normal	Set 2	Replicate	27.24
MB468	U6	Heat	Set 1	First	20.33
MB468	U6	Heat	Set 1	Replicate	29.99
MB468	U6	Heat	Set 2	First	21.67
MB468	U6	Heat	Set 2	Replicate	20.24
MB468	U6	Hypoxia	Set 1	First	34.32
MB468	U6	Hypoxia	Set 1	Replicate	34.83
MB468	U6	Hypoxia	Set 2	First	32.66
MB468	U6	Hypoxia	Set 2	Replicate	31.14
MB468	U6	Dual Stress	Set 1	First	*
MB468	U6	Dual Stress	Set 1	Replicate	*
MB468	U6	Dual Stress	Set 2	First	19.41
MB468	U6	Dual Stress	Set 2	Replicate	20.06
MB468	miR-145	Normal	Set 1	First	38.34
MB468	miR-145	Normal	Set 1	Replicate	38.04
MB468	miR-145	Normal	Set 2	First	35.50
MB468	miR-145	Normal	Set 2	Replicate	38.20
MB468	miR-145	Heat	Set 1	First	32.07
MB468	miR-145	Heat	Set 1	Replicate	33.06
MB468	miR-145	Heat	Set 2	First	29.45
MB468	miR-145	Heat	Set 2	Replicate	30.05
MB468	miR-145	Hypoxia	Set 1	First	35.88
MB468	miR-145	Hypoxia	Set 1	Replicate	*
MB468	miR-145	Hypoxia	Set 2	First	33.05
MB468	miR-145	Hypoxia	Set 2	Replicate	30.76
MB468	miR-145	Dual Stress	Set 1	First	*
MB468	miR-145	Dual Stress	Set 1	Replicate	*
MB468	miR-145	Dual Stress	Set 2	First	32.08
MB468	miR-145	Dual Stress	Set 2	Replicate	32.35
MB468	miR-330-3p	Normal	Set 1	First	35.44
MB468	miR-330-3p	Normal	Set 1	Replicate	33.99
MB468	miR-330-3p	Normal	Set 2	First	33.18
MB468	miR-330-3p	Normal	Set 2	Replicate	34.46
MB468	miR-330-3p	Heat	Set 1	First	33.12
MB468	miR-330-3p	Heat	Set 1	Replicate	33.55
MB468	miR-330-3p	Heat	Set 2	First	30.84
MB468	miR-330-3p	Heat	Set 2	Replicate	33.09
MB468	miR-330-3p	Hypoxia	Set 1	First	36.89
MB468	miR-330-3p	Hypoxia	Set 1	Replicate	37.54
MB468	miR-330-3p	Hypoxia	Set 2	First	37.17
MB468	miR-330-3p	Hypoxia	Set 2	Replicate	33.06
MB468	miR-330-3p	Dual Stress	Set 1	First	*
MB468	miR-330-3p	Dual Stress	Set 1	Replicate	*
MB468	miR-330-3p	Dual Stress	Set 2	First	38.12
MB468	miR-330-3p	Dual Stress	Set 2	Replicate	36.71

Table 2 Ct Values from Real Time PCR Amplification for miRNAs from MB468 breast cancer cells

Real Time PCR reactions were performed in order to examine expression levels for miR-145 and miR-330-3p in MB468 breast cancer cells exposed to stress conditions. All samples were run in triplicate, and three Ct values were collected for each sample in both miRNA extraction sets. The two closest Ct values were averaged, and this is the value listed above. Each PCR run was then repeated (with fresh cDNA prepared from replicate RT reactions). The U6 snRNA was used as the reference. These Ct values were then used to determine the ΔCt for each condition in MB468 breast cancer cell miRNA extractions.
*The Dual Stress condition for both the first and replicate PCR runs produced no amplification, as well as the miR-145 Hypoxia sample from the first sample-set during the replicate PCR run.

Cell Type	RXN	Condition	Sample-Set	PCR Run	Ct Value
MB231	U6	Normal	Set 2	First	24.84
MB231	U6	Normal	Set 2	Replicate	18.38
MB231	U6	Heat	Set 2	First	21.36
MB231	U6	Heat	Set 2	Replicate	23.15
MB231	U6	Hypoxia	Set 2	First	20.95
MB231	U6	Hypoxia	Set 2	Replicate	28.15
MB231	U6	Dual Stress	Set 2	First	17.08
MB231	U6	Dual Stress	Set 2	Replicate	19.32
MB231	miR-145	Normal	Set 2	First	35.21
MB231	miR-145	Normal	Set 2	Replicate	33.06
MB231	miR-145	Heat	Set 2	First	31.88
MB231	miR-145	Heat	Set 2	Replicate	32.71
MB231	miR-145	Hypoxia	Set 2	First	33.22
MB231	miR-145	Hypoxia	Set 2	Replicate	33.63
MB231	miR-145	Dual Stress	Set 2	First	32.99
MB231	miR-145	Dual Stress	Set 2	Replicate	32.78
MB231	miR-330-3p	Normal	Set 2	First	30.55
MB231	miR-330-3p	Normal	Set 2	Replicate	32.87
MB231	miR-330-3p	Heat	Set 2	First	31.46
MB231	miR-330-3p	Heat	Set 2	Replicate	32.16
MB231	miR-330-3p	Hypoxia	Set 2	First	30.46
MB231	miR-330-3p	Hypoxia	Set 2	Replicate	32.54
MB231	miR-330-3p	Dual Stress	Set 2	First	31.05
MB231	miR-330-3p	Dual Stress	Set 2	Replicate	33.16

Table 3 Ct Values from Real Time PCR Amplification for miRNAs from MB231 breast cancer cells

Real Time PCR reactions were performed in order to examine expression levels for miR-145 and miR-330-3p in MB231 breast cancer cells exposed to stress conditions. All samples were run in triplicate, and three Ct values were collected for each sample from the second set of miRNA extractions (the second set of extractions were examined first, and upon completion of Real Time PCR reactions for this set of reactions, reagents had been exhausted preventing further PCR analysis). The two closest Ct values were averaged, and this is the value listed above. Each PCR run was then repeated (with fresh cDNA prepared from replicate RT reactions). The U6 snRNA was used as the reference. These Ct values were then used to determine the ΔCt for each condition in MB231 breast cancer cell miRNA extractions.

ΔΔCt values were calculated by comparing the ΔCt value for cells grown under normal conditions to the ΔCt value for cells grown under each stress condition in miR-145 and miR-330-3p reactions from each cell type, in order to examine the specific miRNA expression levels for breast cancer cells exposed to each stress condition. ΔΔCt values represent the expression of each miRNA (in each condition, compared with both the RNA control and normal conditions), and expression levels can be expressed as $2^{\Delta\Delta Ct}$; positive values are indicative of increases in expression and negative values are indicative of decreases in expression. ΔΔCt values have been averaged for every miRNA in each condition for each cell type and are listed in Tables 5 (MCF7), 6 (MB468), and 7 (MB231).

Expression levels for miR-145 have previously been found to be downregulated in breast cancer cells, and we hypothesized that expression levels would be downregulated in MCF7, MB468, and MB231 breast cancer cells exposed to stress conditions. Additionally, we expected to find expression levels for miR-330-3p to be upregulated in MCF7, MB468, and MB231 breast cancer cells exposed to stress conditions. ΔΔCt values determined for miR-145 in MCF7 breast cancer cells were found to be positive under heat stress conditions at 40 degrees Celsius and under hypoxic stress at 3.5% oxygen (ΔΔCt 1.53 and 1.33, respectively); these data appear to suggest increased expression, which was unexpected. ΔΔCt values determined for miR-145 reactions were, however, found to be negative in MCF7 cells exposed to dual stress conditions at 40 degrees Celsius and 3.5% oxygen (ΔΔCt -1.26); these data appear to indicate downregulated expression, consistent with our expectations.

Positive ΔΔCt values were determined for miR-330-3p reactions in MCF7 cells exposed to heat and hypoxic stresses (ΔΔCt 1.25 and 2.05, respectively); these data seem to support our expectations for increased miR-330-3p expression under stress conditions. Negative ΔΔCt

values were determined for miR-330-3p reactions in MCF7 cells under dual stress conditions (ΔΔCt -2.64); this would suggest downregulation of miR-330-3p, which was unexpected.

Some mixed findings can also be reported for MB468 cells: positive ΔΔCt values were determined for miR-145 reactions in MB468 breast cancer cells exposed to heat stress, and hypoxic stress (ΔΔCt 3.92, and 11.03, respectively). These data appear to suggest increased expression levels for miR-145 under heat stress, and under hypoxic stress. Although this is not what we expected to find for cells exposed to individual stress conditions, ΔΔCt values determined for miR-145 in MB468 cells under dual stress conditions were negative (ΔΔCt -1.26), seeming to indicate downregulated expression for miR-145, which would be consistent with our hypothesis. Surprisingly, negative ΔΔCt values were reported for miR-330-3p reactions in MB468 cells exposed to heat stress (ΔΔCt -0.82), as well as for cells exposed to dual stress conditions (ΔΔCt -10.27); a positive ΔΔCt of 5.84 was determined for miR-330-3p in MB468 cells subjected to hypoxic stress, which appears to support our expectation that increased expression of this miRNA would be observed under stress conditions.

Positive ΔΔCt values were determined for miR-145 reactions in MB231 cells subjected to heat stress (ΔΔCt 2.49), and hypoxic stress (ΔΔCt 3.65); although this is not what we expected to observe in cells exposed to individual stresses, cells exposed to dual stress conditions were determined to have a ΔΔCt of -2.16. This appears to support our expectations for decreased expression of miR-145 under stress conditions. Seemingly in agreement with our expectation, positive ΔΔCt values were reported for miR-330-3p reactions in MB231 cells under heat stress (ΔΔCt 0.55) and under hypoxic stress (ΔΔCt 3.15); this appears to suggest increased expression for miR-330-3p in cells exposed to these stresses. Unexpectedly, a negative ΔΔCt

value of -3.81 was calculated for miR-330-3p expression in MB231 cells subjected to dual stress conditions, seeming to indicate downregulation of miR-330-3p in MB231 cells under dual stress.

Cell Type	RXN	Condition	ΔΔCt	Change in Expression
MCF7	miR-145	Heat	1.53	2.89
MCF7	miR-145	Hypoxia	1.33	2.51
MCF7	miR-145	Dual stress	-1.26	-2.39
MCF7	miR-330-3p	Heat	1.25	2.34
MCF7	miR-330-3p	Hypoxia	2.05	4.14
MCF7	miR-330-3p	Dual stress	-2.64	-6.23

Table 4 ΔΔCt values for miR-145 and miR-330-3p in MCF7 breast cancer cells exposed to stress conditions

Expression levels for miR-145 and miR-330-3p in MCF7 breast cancer cells exposed to stress conditions were examined through ΔΔCt analysis following Real Time PCR. All ΔΔCt values obtained for MCF7 miRNA samples were averaged for each miRNA in each condition, and are listed here. In MCF7 cells, ΔΔCt values appear to reflect increased expression for miR-145 under heat stress conditions at 40 degrees Celsius and also under hypoxic stress at 3.5% oxygen; however, under dual stress conditions at 40 degrees Celsius and 3.5% oxygen, miR-145 reactions in MCF7 cells seem to suggest down-regulation. ΔΔCt values appear to reflect increasing expression levels for miR-330-3p in MCF7 cells under heat stress (ΔΔCt 1.25) as well as hypoxic stress (ΔΔCt 2.05), although data seem to suggest lower levels of expression under dual stress conditions (ΔΔCt -2.64).

Cell Type	RXN	Condition	ΔΔCt	Change in Expression
MB468	miR-145	Heat	3.92	15.14
MB468	miR-145	Hypoxia	11.03	2091.03
MB468	miR-145	Dual Stress	-2.04	-4.11
MB468	miR-330-3p	Heat	-0.82	-1.77
MB468	miR-330-3p	Hypoxia	5.84	57.28
MB468	miR-330-3p	Dual Stress	-10.27	-1234.75

Table 5 ΔΔCt values for miR-145 and miR-330-3p in MB468 breast cancer cells exposed to stress conditions

Expression levels for miR-145 and miR-330-3p in MB468 breast cancer cells exposed to stress conditions were examined through ΔΔCt analysis following Real Time PCR. All ΔΔCt values obtained for MB468 miRNA samples were averaged for each miRNA in each condition, and are listed here. In MB468 cells, ΔΔCt values for miR-145 reactions appear to depict increased expression levels under heat stress and seem to suggest an increase in expression under hypoxic stress; under dual stress conditions, miR-145 expression levels again appear to reflect down-regulation in MB468 cells. miR-330-3p reactions in MB468 cells show slightly decreased expression levels under heat stress, although expression levels under hypoxic stress are shown to be increased, miR-330-3p expression under dual stress conditions seems to be markedly decreased.

Cell Type	RXN	Condition	ΔΔCt	Change in Expression
MB231	miR-145	Heat	2.49	5.62
MB231	miR-145	Hypoxia	3.65	12.55
MB231	miR-145	Dual Stress	-2.16	-4.47
MB231	miR-330-3p	Heat	0.55	1.46
MB231	miR-330-3p	Hypoxia	3.15	8.88
MB231	miR-330-3p	Dual Stress	-3.81	-14.03

Table 6 ΔΔCt values for miR-145 and miR-330-3p in MB231 breast cancer cells exposed to stress conditions

Expression levels for miR-145 and miR-330-3p in MB231 breast cancer cells exposed to stress conditions were examined through ΔΔCt analysis following Real Time PCR. All ΔΔCt values obtained for MB231 miRNA samples were averaged for each miRNA in each condition, and are listed here. In MB231 cells, miR-145 reactions appear to have increased expression under heat stress and under hypoxic stress, while miR-145 expression seems to suggest down-regulation under dual stress conditions. miR-330-3p reactions in MB231 cells appear to reflect upregulation of expression under heat stress and under hypoxic stress, although expression levels are seemingly decreased under dual stress conditions.

Invasion Assays: Invasive Properties in Breast Cancer Cells Exposed to Stresses

Matrigel invasion chambers were utilized for this portion of the project. MCF7 and MB468 cells were plated at densities of 25,000 or 50,000 cells per well (cell densities were chosen based on optimal cell densities shown for previous studies with MCF7 cells and recommendations, calling for use of multiple densities, within the Matrigel protocol). Cells were incubated for 96 hours. Invasive cells are able to digest through a layer of extracellular matrix material and migrate through pores to the "outside" of the chamber. Cells that remained in the chamber were scrubbed away, then cells that migrated through the matrix and pores were stained with a solution of crystal violet. Invasion chambers were then individually photographed by quadrant through a microscope, and were systematically counted using Image J software in order to quantify the invasiveness of the cells under normal and stress conditions. Invasion assay data are expressed as percentage cell migration in Figures 1 and 2. Invasion data were collected and quantified as described above; percentage cell migration has not been supported by further statistical analysis, due to the scale of this portion of the experiment.

We hypothesized that MCF7 and MB468 breast cancer cells exposed to heat, hypoxia, and dual stress conditions would prove to be more invasive than cells under normal conditions. In MCF7 cells (Figure 1), invasion chambers plated with 50,000 cells have 19% cell migration, and chambers plated with 25,000 cells reflect 15% cell migration under normal conditions. MCF7 cells exposed to heat stress reflect 15% cell migration for both chambers. MCF7 cells exposed to hypoxic stress show a 5% cell migration in the chamber containing 50,000 cells, but have a 29% cell migration in the chamber plated with 25,000 cells. MCF7 cells exposed to dual stress conditions have a 1% and 3% cell migration for the 50,000 and 25,000 cell chambers, respectively. There was a marked increase in migrating MCF7 cells under hypoxic stress (29%

compared to 15% under normal conditions). The remainder of the invasion assay data collected for MCF7 cells reflects equivalent or lesser percentage cell migration under heat stress, and appears to suggest lesser percentage cell migration under dual stress conditions compared with percentage cell migration found under normal conditions.

Invasion assays performed with MB468 breast cancer cells (Figure 2), have 7% and 2% cell migration under normal conditions for chambers plated with 50,000 and 25,000 cells, respectively. MB468 cells exposed to heat stress suggest a 42% cell migration for the 50,000 cell chamber, and a 17% cell migration for the 25,000 cell chamber. MB468 cells exposed to hypoxic stress have a 21% and 17% cell migration for the 50,000 cell and 25,000 cell chambers, respectively. MB468 cells exposed to dual stress conditions have a 6% cell migration for the chamber containing 50,000 cells, and have a 7% cell migration for the chamber containing 25,000 cells.

Data collected for all invasion assays performed with MB468 breast cancer cells exposed to heat and hypoxic stresses appear to show increased percentage cell migration when compared with cells under normal conditions, in agreement with our hypothesis. MB468 cells exposed to dual stress conditions seem to reflect slightly lesser cell migration (6%) for the 50,000 cell chamber when compared with cells under normal conditions (7%); the dual stress chamber containing 25,000 cells appears to have increased cell migration (7%) when compared with cells under normal conditions (2%), in agreement with our hypothesis.

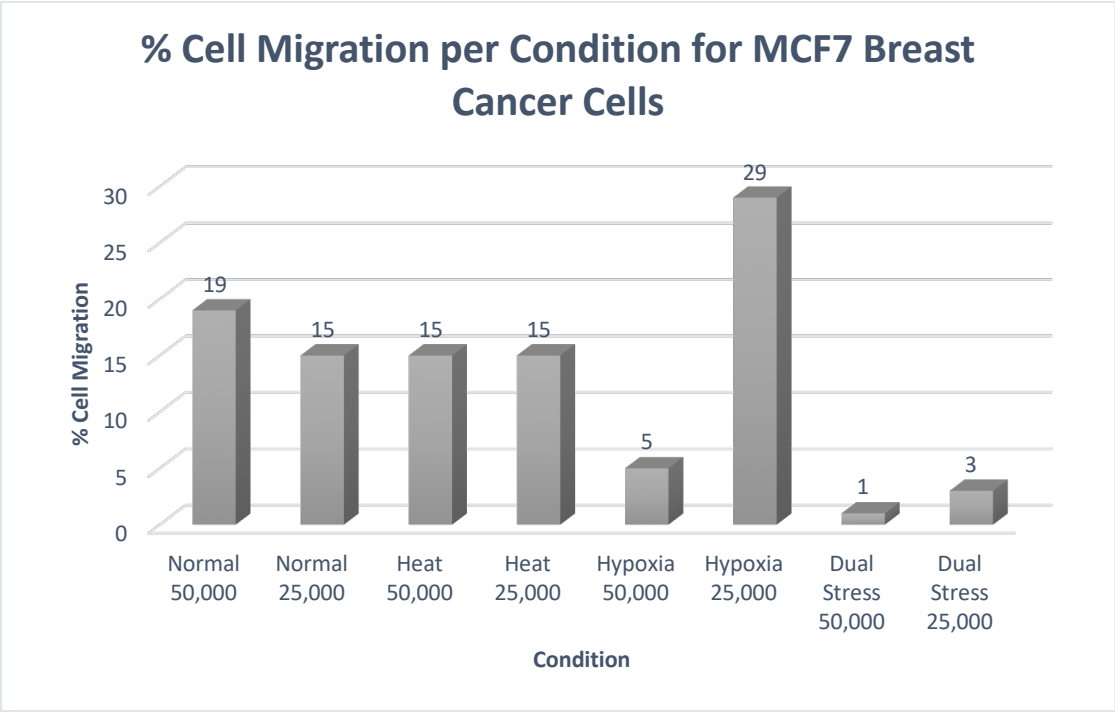


Figure 1 Percent Cell Migration per Condition for MCF7 Breast Cancer Cells

25,000 and 50,000 cells (that were resuspended in serum-free medium) were plated per invasion chamber for each condition for assays performed with MCF7 breast cancer cells, and then subjected to a 96-hour incubation. Two chambers were exposed to normal conditions (37 degrees Celsius, and ambient oxygen), two chambers were exposed to heat stress at 40 degrees Celsius, two chambers were exposed to hypoxic stress at 3.5% oxygen, and two chambers were exposed to dual stress conditions (heat at 40 degrees Celsius, and hypoxic stress at 3.5% oxygen). Invasive cells are able to digest through the matrix and migrate through the pores to the outside of the chamber. Cells remaining in the interior of the chamber were scrubbed away, before migrating cells were stained with a crystal violet solution. Chambers were then photographed by quadrant using a microscope and systematically counted, using ImageJ software. Percentage cell migration data is not supported by further statistical analysis. The invasion assay performed with MCF7 breast cancer cells under hypoxic stress (25,000 cell chamber) appears to have a marked increase in migrating cells (29%) compared with cells under normal conditions (15%), in agreement with our hypothesis. The remainder of the invasion assay data collected for MCF7 cells seem to reflect equivalent or lesser percentage cell migration under heat stress and lesser percentage cell migration under dual stress conditions, compared with percentage cell migration under normal conditions.

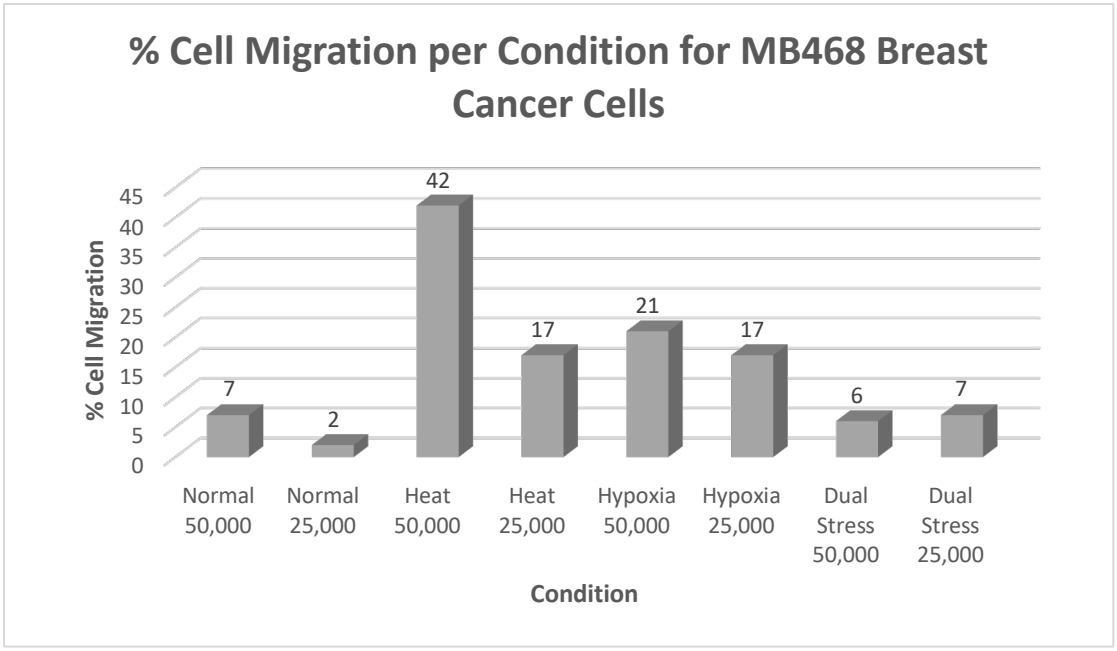


Figure 2 Percent Cell Migration per Condition for MB468 Breast Cancer Cells

25,000 and 50,000 cells (that were resuspended in serum-free medium) were plated per invasion chamber for each condition for assays performed with MB468 breast cancer cells, and then subjected to a 96-hour incubation. Two chambers were exposed to normal conditions (37 degrees Celsius, and ambient oxygen), two chambers were exposed to heat stress at 40 degrees Celsius, two chambers were exposed to hypoxic stress at 3.5% oxygen, and two chambers were exposed to dual stress conditions (heat at 40 degrees Celsius, and hypoxic stress at 3.5% oxygen). Invasive cells are able to digest through the matrix and migrate through the pores to the outside of the chamber. Cells remaining in the interior of the chamber were scrubbed away, before migrating cells were stained with a crystal violet solution. Chambers were then photographed by quadrant using a microscope and systematically counted, using ImageJ software. Percentage cell migration data is not supported by further statistical analysis. Data collected for all invasion assays performed with MB468 breast cancer cells exposed to heat and hypoxic stresses appears to have increased percentage cell migration when compared with cells under normal conditions, in agreement with our hypothesis. MB468 cells exposed to dual stress conditions seem to reflect slightly lesser cell migration (6%) for the 50,000 cell chamber when compared with cells under normal conditions (7%); the dual stress chamber containing 25,000 cells appears to have increased cell migration (7%) when compared with cells under normal conditions (2%), in agreement with our hypothesis.

Discussion

Cancers are a deadly assortment of diseases characterized by uncontrolled cell proliferation and cell migration, invasion, and secondary tumor development in other areas of the body. Metastatic disease presents obstacles for treatment, resulting in poor patient outcomes and lower rates of survival. Our study explored the impact of heat and hypoxic stresses that occur within tumor microenvironments, on endogenous miRNA expression levels, and ultimately a cancer cell's invasiveness. We hypothesized that a cancer cell's immediate tumor microenvironment may become too inhospitable to support it due to harsh stresses. Some stresses encountered by the cancer cell may contribute, or be decisive in a cell's adoption of adaptive responses, affecting miRNA expression and ultimately a cell's invasiveness, which is impactful in both the onset and advancement of metastatic disease (17).

Cancers are frequently associated with tumors characterized by a wide variety of mutations, occurring in two primary classes of genes: proto-oncogenes and tumor suppressor genes. These mutations may affect gene expression and/or the function of their products. miRNAs are recognized as products of both proto-oncogenes and tumor suppressor genes, having important roles in cancers, and are known to exercise regulatory effects on many cell processes. Through activation or inhibition of translation, miRNAs are able to influence protein expression; additionally, miRNAs may regulate expression of genes by means of repressing transcription. Interactions between miRNAs and their target mRNAs do not require perfect precision, so a single miRNA may successfully target a large number of mRNAs, with significant and broad effects.

The purpose of this study was to analyze miR-145 and miR-330-3p expression levels in breast cancer cells exposed to stresses known to occur in tumor microenvironments, then to

examine if any altered miRNA expression levels may have impacted the invasiveness of the cancer cells. miR-145 is known to be the product of a tumor suppressor gene and is reported to be down-regulated in breast cancers, while proto-oncogene product miR-330-3p has been found to have increased expression in breast cancers. For this investigation, cells from MCF7, MB468, and MB231 breast cancer cell lines were exposed to heat stress, hypoxic stress, and dual stress conditions. We hypothesized that breast cancer cells exposed to stress would have lower levels of expression for miR-145, and increased levels of expression for miR-330-3p, when compared to miRNA expression analyzed in breast cancer cells grown in normal conditions.

Some data collected for miRNA expression in this study appear to support our hypothesis, although mixed results are reported. Data seem to reflect down-regulation of miR-145 in samples from all three breast cancer cell lines exposed to dual stress conditions, in agreement with our hypothesis. Surprisingly, miR-145 expression levels for cell samples exposed to heat and hypoxic stress appear to indicate increased expression in all three breast cancer cell lines for each of these individual stresses, which is a pattern we did not expect to observe.

Down-regulation of tumor suppressor miR-145 has been associated with multiple facets of metastatic disease, and it has been suggested that this change in expression levels indicates advancement of disease (24). Results of at least one study report that lower expression found in triple negative breast cancers may be attributed to effects of methylation on *miR-145* (24). Although miR-145 targets and functions vary in different tissues and tumor types, several identified oncogenic targets of miR-145 are reported to be involved with invasion and metastasis, including MUC1, FSCN1, MMP11, and Rab27a, among others.

It has been reported that as a result of miR-145's successful targeting of MUC1, migration of breast cancer cells to lung tissue is curtailed (19). FSCN1 has also been implicated in breast cancer brain metastasis, and one study associates increased expression of FSCN1 with breast cancer cell migration and penetration of the blood brain barrier; this study also indicates the use of FSCN1 as a biomarker for potential breast cancer brain metastasis, one of the most treatment-resistant and deadly features of metastatic breast cancers (26). MMP11, a member of a family of matrix metalloproteases known to contribute to metastasis, is a critical driver of cell migration and invasion and is over-expressed in breast and many other cancers. Rab27a is also associated with advancing metastasis. Rab27a is reported to drive secretion of IGFII (insulin like growth factor II), which is then able to mediate the expression of several factors involved in both invasion and metastasis, including MMP9, UPA, VEGF, cyclin D1, cathepsin D, and p16 (27).

These direct miR-145 targets and factors are only some that we know to be associated with invasion and metastasis in malignant breast cancers, although there may be many more direct targets as well as indirect factors that remain unknown. A single miRNA could potentially have significant effects, as it may work on a large number of these targets. miR-145 expression data suggest down-regulation of the tumor suppressor for all three breast cancer cell lines grown under dual stress conditions. This indicates that key oncogenic targets associated with invasion and metastasis (in breast cancer cells) may not be silenced; for example, without regulation from miR-145, MUC1 and FSCN1 would be free to promote advancement of metastatic breast cancer cells to the lungs and brain, respectively. This could have devastating consequences for treatment options and patient outcomes.

Data suggest up-regulation of miR-330-3p expression for samples from all three breast cancer cell lines exposed to hypoxic stress, in agreement with our hypothesis. Additionally, data

appear to suggest an increase in miR-330-3p expression in MCF7 and MB231 samples exposed to heat stress, also in agreement with our hypothesis. Other data reflect some unexpected results for miR-330-3p expression levels in MB468 samples exposed to heat stress; expression levels for miR-330-3p exposed to heat stress seem to be decreased. Although differences in behavior among different cell lines are known to occur, this is not a pattern we expected to observe. Additionally, we did not expect to see down-regulation of miR-330-3p expression in all three breast cancer cell lines exposed to dual stress conditions.

Although protooncogene product miR-330-3p has not been studied as extensively, several direct targets relating to invasion and metastasis in various cancers have been identified for this miRNA. In invasive breast cancers, CCBE1 is known to have tumor suppressive effects, and has been identified as one direct target specifically identified for miR-330-3p. Data suggest upregulation of pro-metastatic miR-330-3p for all 3 breast cancer cell lines grown under hypoxic stress conditions, and for MCF7 and MB231 breast cancer cell lines grown under heat stress. Up-regulation of miR-330-3p in breast cancer cells indicates that the tumor suppressor actions of CCBE1 could be silenced, which has been correlated to lower patient survival rates.

Issues mentioned earlier prevented sufficient data collection for further statistical analysis of miRNA expression data; consultation with statistician Dennis Viele (Biology Department, University of Michigan-Flint) confirmed sample sizes for miRNA expression data were insufficient for meaningful statistical analysis. For this reason, we are not able to report these findings as conclusive, but believe they provide a basis for further scientific investigation.

There are some limitations to bear in mind for this experiment, involving differences between conditions that occur within the body, and those that can be recreated in the lab. The parameters of our experiment included a 96-hour incubation of cells subjected to specific

stresses, while the duration of exposure for cells subjected to stresses occurring within a tumor microenvironment could potentially persist for years *in vivo*. Additional considerations associated with variability should also be weighed: possible dynamic fluctuations of those stresses within microenvironments, timing of introduction of additional or multiple stressors, and frequency of remodeling within microenvironments.

We expected that invasion data collected for MCF7 and MB468 breast cancer cells would reflect higher percentage cell migration for cells exposed to stresses, compared with cells exposed to normal conditions. Surprisingly, MCF7 breast cancer cells from the 50,000 cell chambers reflect a 19% cell migration under normal conditions, a 15% cell migration under heat stress, a 5% cell migration under hypoxic stress, and a 1% cell migration under dual stress conditions. Although the MCF7 breast cancer cell line is known to be one of the least invasive lines, these data were not consistent with our expectations. MCF7 breast cancer cells from the 25,000 cell chambers reflect a 15% cell migration under normal conditions, a 15% cell migration under heat stress, a 29% cell migration under hypoxic stress, and a 3% cell migration under dual stress conditions. While it was curious that cells grown under dual stress conditions did not behave as expected, we could speculate that perhaps there is a difference in the viability of these cells. It may be possible that fewer cells survived the harsh dual stress conditions, impacting the number of migrating cells observed under dual stress conditions. Although we expected to see greater percentage cell migration among MCF7 breast cancer cells exposed to stress conditions compared with normal conditions, data for cells from the 25,000 cell chamber subjected to hypoxic stress (29%) compared with cells under normal conditions (15%), support our expectations.

Invasion data collected for MB468 breast cancer cells from 50,000 cell chambers reflect a 7% cell migration under normal conditions, a 42% cell migration under heat stress, a 21% cell migration under hypoxic stress, and a 6% cell migration under dual stress conditions. While it is curious that cells under dual stress conditions reflect a small decrease in percent cell migration compared with cells under normal conditions for 50,000 cell chambers, a dramatic increase in migration was observed for cells under heat and hypoxic stresses, consistent with our expectations. MB468 breast cancer cells from 25,000 cell chambers reflect a 2% cell migration under normal conditions, a 17% cell migration under heat stress, a 17% cell migration under hypoxic stress, and a 7% cell migration under dual stress conditions. These data are all in agreement with our hypothesis.

We expected to find that breast cancer cells subjected to stresses found in tumor microenvironments would reflect up-regulation of miR-330-3p and prove more invasive. MCF7 and MB468 breast cancer cell lines subjected to hypoxic stress suggest an increase in expression for miR-330-3p as well as increased invasiveness. Although MCF7 breast cancer cells are known to be among the least invasive of breast cancer cell lines, an increase in percentage cell migration was observed for the 25,000 cell chambers under hypoxic stress conditions (versus cells under normal conditions), where our data seems to reflect higher levels of miR-330-3p expression. This appears to suggest MCF7 breast cancer cells subjected to stress and having up-regulated miR-330-3p expression are more invasive, consistent with our expectations.

Data also appear to support our expectation that MB468 cells subjected to stress and reflecting increased expression levels of miR-330-3p would be more invasive; increases in percentage cell migration for both 25,000 and 50,000 cell chambers were observed for these cells under hypoxic stress, where miR-330-3p expression appears to be up-regulated. Although we

had also predicted that breast cancer cells subjected to stress and having downregulated expression levels for miR-145 could be correlated with greater invasiveness, we only saw this reflected in the data for one cell line and condition. Greater percentage cell migration was reported for MB468 cells subjected to dual stress conditions, as well as downregulation for miR-145.

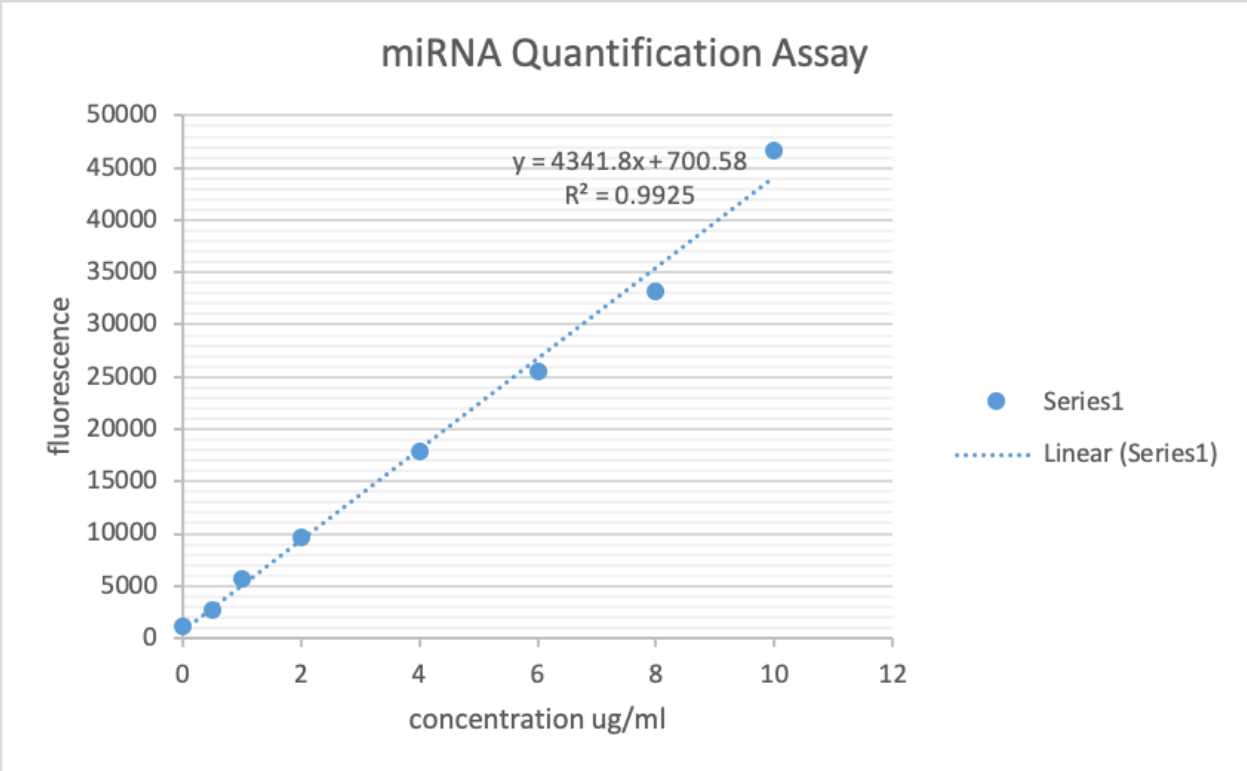
Some data in this study support our predictions, and we feel these data present an interesting and potentially important direction for future scientific investigations. Consideration for future studies should include protocol recommendations that necessitate plating for normal conditions with each stress condition for a simultaneous reaction run, in the event that PCR reactions are run separately for different stress conditions. Future studies designed to further explore the direction of our experimentation might include RNA knockout techniques.

Appendices

Appendix I

Total miRNA Concentrations during stress conditions

A miRNA quantification assay was performed using the Quant-IT kit from Life Technologies in order to confirm presence of miRNAs following extractions and to determine miRNA concentrations for each sample. Row A of the microplate was loaded with miRNA standards provided as a component of the Quant-IT kit, while Rows B-E were loaded with unknown RNA samples. Fluorescence values were obtained from a microplate reading, and Excel software was used to generate a standard curve (shown below), from which concentrations for unknown RNA samples were determined: first, amount versus fluorescence was plotted for the miRNA standards using Excel software, and the equation for the line y=4341.8x+700.58 was obtained and used to determine concentrations for the unknown RNA samples. This table (shown on the following page), lists concentrations for every sample condition for each of the 3 breast cancer cell lines. All samples were plated in duplicate, and the concentration values listed are the result of averaging the two values for each cell type and condition.

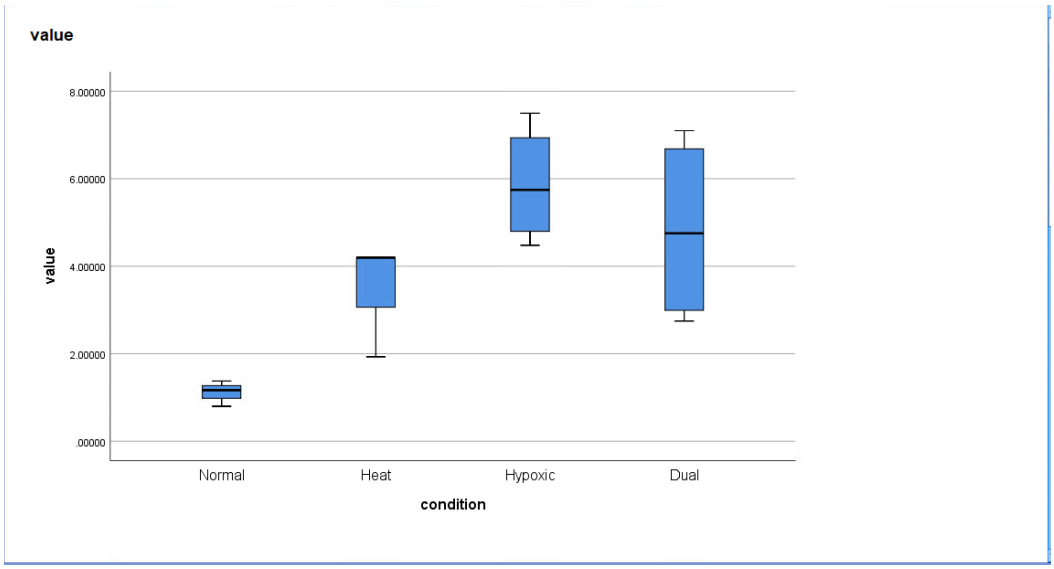


Cell Type / Sample	Concentration (ug/ml)
MB231 NORMAL SET 1	0.61206
MB231 NORMAL SET 2	0.57072
MB468 NORMAL SET 1	0.14727
MB468 NORMAL SET 2	0.38460
MCF7 NORMAL SET 1	2.54176
MCF7 NORMAL SET 2	0.79815
MB231 HEAT SET 1	2.71130
MB231 HEAT SET 2	1.73797
MB468 HEAT SET 1	1.44132
MB468 HEAT SET 2	1.84277
MCF7 HEAT SET 1	4.19536
MCF7 HEAT SET 2	1.92925
MB231 HYPOXIA SET 1	0.79297
MB231 HYPOXIA SET 2	3.42333
MB468 HYPOXIA SET 1	0.23664
MB468 HYPOXIA SET 2	0.18551
MCF7 HYPOXIA SET 1	6.93616
MCF7 HYPOXIA SET 2	4.79523
MB231 DUAL STRESS SET 1	0.95592
MB231 DUAL STRESS SET 2	2.89751
MB468 DUAL STRESS SET 1	0.25829
MB468 DUAL STRESS SET 2	0.44185
MCF7 DUAL STRESS SET 1	6.68212
MCF7 DUAL STRESS SET 2	2.99148

Appendix II

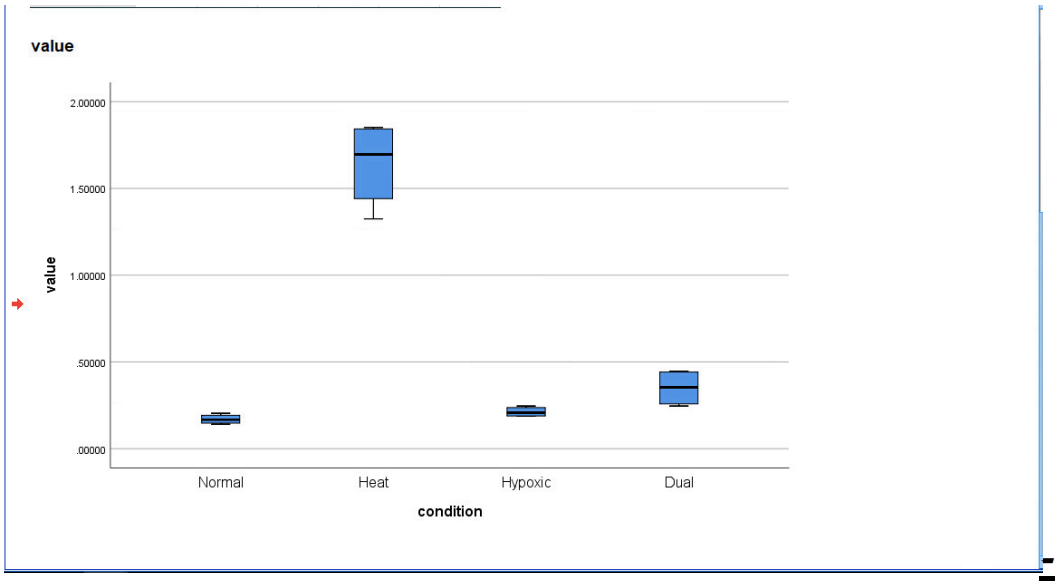
Kruskal-Wallis analysis of quantified miRNA sample concentrations compared for MCF7 breast cancer cells under different stress conditions

The Kruskal-Wallis analysis for MCF7 breast cancer cells reflects a statistically significant difference ($p < 0.05$) in median miRNA concentrations for comparison of cells under normal, heat stress, hypoxic stress, and dual stress conditions following quantification ($H = 8.990$, df 3, $p = 0.029$). Analysis was performed in SPSS statistical software.



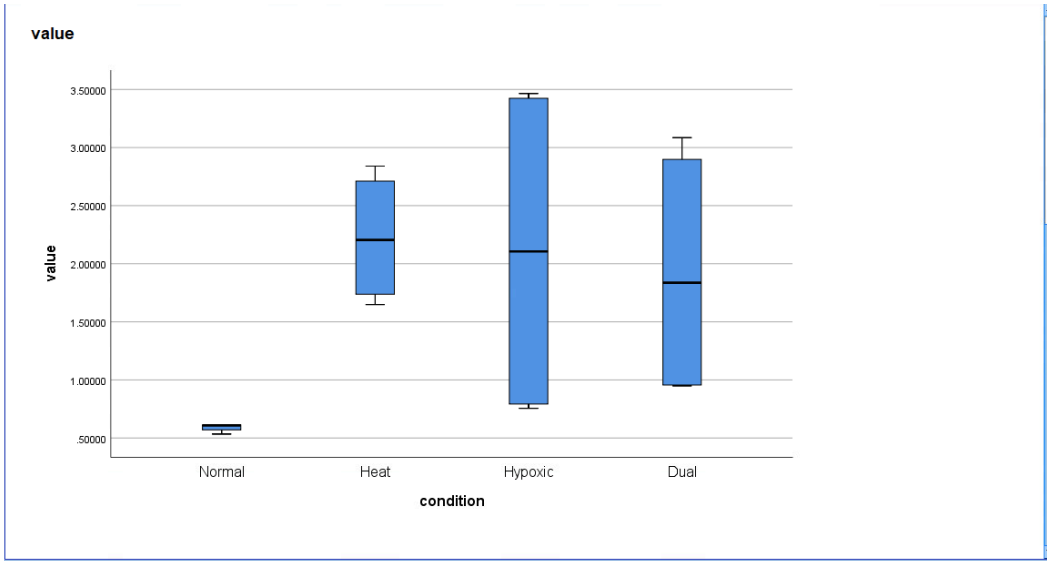
Kruskal-Wallis analysis of quantified miRNA sample concentrations compared for MB468 breast cancer cells under different stress conditions.

The Kruskal-Wallis analysis for MB468 breast cancer cells reflects a statistically significant difference ($p < 0.01$) in median miRNA concentrations for comparison of cells under normal, heat stress, hypoxic stress, and dual stress conditions following quantification, ($H = 13.125$, df 3, $p = 0.004$). Analysis was performed in SPSS statistical software.



Kruskal-Wallis analysis of quantified miRNA sample concentrations compared for MB231 breast cancer cells under different stress conditions.

The Kruskal-Wallis analysis for MB231 breast cancer cells reflects a statistically significant difference ($p < 0.05$) in median miRNA concentrations for comparison of cells under normal, heat stress, hypoxic stress, and dual stress conditions following quantification, ($H = 8.493$, df 3, $p = 0.037$). Analysis was performed in SPSS statistical software.



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