ATTENUATION OF MITOCHONDRIAL ZINC IMPORT THROUGH ZnT2 IN TUMORIGINIC BREAST CANCER CELLS RESULTS IN APOPTOSIS

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Statistics indicate that ~200,000 new cases and deaths result from breast cancer annually, yet much about the development and progression of the disease remains unknown. Previous studies link breast cancer with dysregulated zinc (Zn) metabolism at the systemic and cellular level. Our first objective was to characterize the dysregulation of Zn levels and intracellular Zn distribution in human tumorigenic breast cancer cells (T47D). Secondly, we aimed to further explore the relationship between high cellular Zn levels, breast cancer, and the role of the Zn transporter ZnT2. Our results were consistent with data illustrating high Zn concentrations in breast tumor cells compared with normal mammary cells (HC11). Interestingly, our assessment of Zn distribution in breast tumor cells indicated abnormally low endogenous mitochondrial Zn levels. Upon evaluation of ZnT2 protein expression, we found ZnT2 protein abundance to be lower in breast tumor cells compared with normal cells. Similar to normal breast cells, our data indicated ZnT2 localization to the inner mitochondrial membrane in breast tumor cells, indicating that ZnT2 is a mitochondrial Zn importer in tumor cells. The reduction of mitochondrial Zn import through ZnT2 attenuation reduced mitochondrial Zn levels beyond the apoptotic threshold, as indicated by increased Annexin-V staining. Furthermore, ZnT2-attenuation resulted in decreased cellular proliferation and tumorigenicity of breast tumor cells.

In conclusion, our study suggests the reduction of ZnT2 expression results in the depletion of mitochondrial Zn, impeding vital mitochondrial functions and thus inducing cellular destruction through the activation of the apoptotic pathway in breast cancer cells. We believe our results suggest a promising future role for ZnT2 as a potential therapeutic
target for the treatment of breast cancer. ZnT2 presents both a tissue-specific and malignant-specific target, and may prove to be both a safe and effective form of treatment specifically for breast cancer. Our ongoing studies will further develop the potential for ZnT2 as a targeted treatment, in hopes of finding a cure for this deadly cancer that affects so many women.
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Introduction

I. Breast cancer in women
II. Zinc in the body
III. Zinc transporters
IV. ZnT2
V. Breast cancer and zinc
VI. Cellular death
VII. Zinc and apoptosis
VIII. Hypothesis
I. Breast cancer in women

Overview

In the past ten years, breast cancer has taken center stage as the most common type of cancer diagnosed in women in the US. In the year 2008 alone, the American Cancer Society reported 182,460 new cases in women, and 1,990 new cases in men across the nation. In addition, 2008 saw over 40,000 deaths due to breast cancer (1). With so many new cases and deaths every year, finding the underlying factors that lead to the development and progression of the disease has become increasingly important. Advances in molecular biology, such as the capability to manipulate genes, allow scientists to delve deeper than ever before into examining the disease at the cellular level.

Tumor formation is a fundamental step in cancer progression, and is defined as an abnormal mass of tissue formed by the overpopulation of cells in an area. In healthy breast tissue, cellular homeostasis is maintained by an equilibrium between cellular proliferation and cellular destruction via apoptosis. This equilibrium prevents both the atrophy of tissues and the formation of tumors by keeping cell division and death in balance (1). Dysfunctional cellular homeostasis leading to excessive proliferation and cell accumulation results in the formation of a mass, subsequently referred to as a tumor (2).

There are two classes of tumors. Benign breast tumors are not cancerous; the tumors do not grow uninhibitedly and aggressively, are localized only to the breast, and are incapable of metastasis (spreading to other tissues and organs of the body). Benign breast tumors are often removed by surgery and generally do not reappear within the breast (2).
Conversely, malignant tumors are cancerous and more severe than benign tumors. Unlike their counterpart, malignant tumors grow uninhibitedly and are capable of metastasis. The anatomy of the breast makes invasion of nearby lymph nodes by cancer cells a common development in breast cancer. Metastasized breast cancer will invade (via the circulatory or lymphatic system) and damage other tissues and organs through the formation of secondary tumors. The cells comprising secondary tumors are identical to that of the primary tumor in the breast (2).

**Risk Factors**

Certain risk factors and precursors increase the chances of developing breast cancer. As the most important factor, studies have shown that age is positively correlated with increased breast cancer risk. The American Cancer Society reports that 95% of new cases and 97% of deaths reported from 2000-2004 were in women 40 and older, with the median age of diagnosis at 61 (1). As a woman ages, the body’s DNA repair system declines in efficiency (an outcome of accumulated oxidative damage) and therefore results in inhibited mutation repair (3). Mutations that ultimately affect cellular proliferation and apoptosis are hallmarks of cancer. Moreover, the heightened risk of reoccurrence in women who have previously been diagnosed with breast cancer is also attributed to a compromised DNA repair system (2). Surprisingly however, research shows that younger breast cancer patients have a lower five-year relative survival rate than older patients (82% women <45 years, 88% women 65-74 years) (1). Scientists
believe this is a consequence of more aggressive tumors in younger patients that are less responsive to therapies.

Genetics is also an important determinant of breast cancer risk. Woman with immediate family members (mothers, sisters, daughters) afflicted with breast cancer are more likely to also develop cancer. Although there may be an environmental component, this is likely a direct result of inherited genes that likely contain cancer-associated mutations and are passed on from one generation to another (2). Recent studies have identified mutations in specific genes, such as BRCA1 and BRCA2, that are associated with an increased likelihood of developing breast cancer. Up to 10% of breast cancers diagnosed per year are linked to BRCA1 and BRCA2 mutations. BRCA1 and BRCA2 (located on chromosome 17 and 13 respectively) are part of a family of “tumor suppressor genes” (4,5). The two product proteins are different in structure but have similar roles in the breast by being responsible for preventing uncontrollable cell proliferation through DNA repair and transcriptional regulation. Mutations found in BRCA1 and BRCA2 range from simple base-pair changes to exon deletions, but generally result in short and dysfunctional final proteins (4). Women with BRCA1 and BRCA2 mutations are three times more likely to develop breast cancer compared with women with functional proteins (5). ATM, CHEK 2, p53, and PTEN are other genes that, when mutated, are associated with increased risk of breast cancer as well (2).

In addition, studies show that the reproductive and menstrual statuses of women are associated with breast cancer risk. Women who have never been pregnant, women who
first became pregnant after the age of 35, and women with late onset of menopause (>55 years of age) are at a greater risk for developing breast cancer than their counterparts (2). Studies suggest this reflects the length of estrogen exposure as women with prolonged exposure to estrogen are at a greater risk of developing breast cancer (6). This connection is due to the role of estrogen in stimulating breast cell division and activating other hormones that affect breast health. For example, the later a woman begins menopause, the longer she was subject to estrogen as a part of the menstrual cycle, and thus is at increased risk of breast cancer development.

Other factors contributing to risk include race and breast density. Typically, white women are more prone to developing breast cancer than their Latina, Asian, and African American counterparts (2). However, the existence of unique genetic traits that affect racial susceptibility is apparent, as with mutations in the gene BP1 which has a higher prevalence in African-Americans and is linked with increased tumor aggression (7,8). Additionally, denser breast tissue (as opposed to fattier tissue) increases risk as a result of more breast cells undergoing cell division and creating more opportunities for mutations (9).

While risk factors increase a woman’s chances of developing breast cancer, they are not a determinant of whether a person will develop the disease, or how severe it will be. The severity of the cancer is determined upon diagnosis, and classified into 5 stages based on disease progression, particularly tumor size and metastasis status.
**Cancer Stages**

Stage 0 breast carcinomas are localized only to the breast (*in situ*). Tumors are small and subcategorized based on location within the breast. Lobular carcinoma in situ (LCIS) consists of abnormal cancerous cell lining the lobules of the breast, the location of milk production. LCIS cells are normally benign, thus their classification as a “cancer” is controversial (2). Ductal carcinoma in situ (DCIS) consists of abnormal cancerous cells lining the ducts of breast tissue, location of milk secretion. Stage 0 carcinomas are not invasive, yet the presence of LCIS and DCIS tumors increases the likelihood of future malignant breast cancer (2). DCIS can progress into invasive cancer and form tumors; however, LCIS only increases risk by altering the environment of surrounding healthy cells.

Stage I breast cancer represents the early stages of invasive cancer. The breast tumor is <2 cm in diameter but has not spread beyond the breast (2). The American Cancer Society reports a 100% five-year survival rate for women diagnosed with LCIS or DCIS, or stage I breast cancers (1).

Larger tumors (2-5cm) represent stage II breast cancers. In addition, <2 cm tumors that have spread to the lymph nodes behind the breast and tumors >5cm without invasion are also stage II cancers. The spread to lymph nodes is a significant development due to the possibility of further metastasis to other organs and tissues through the lymphatic system (2). Five-year survival rate for stage II patients averages at 87% (1).
Stage III breast cancer is locally advanced cancer and subcategorized into three subcategories (A-C). Stage IIIA tumors are >5 centimeters in diameter and have spread to the lymph nodes behind the breast. Stage IIIB tumors have invaded the chest wall and/or the skin of the breast. Stage IIIB cancers are physically noticeable as “lumps” caused by swelling of surrounding tissue. Stage IIIC tumors are any tumors that have spread to the lymph nodes behind the breast and below the collarbone (2). The survival rate for stage III breast cancer is 60% (1).

Stage IV cancer is distant metastasized cancer, characterized by the spread of cancer cells from the breast to other organs and tissues of the body. Secondary tumor formation has begun with a stage IV diagnosis (2). The five-year survival rate for stage IV breast cancer drops to 20% due to the severe progression of the disease which makes treatment difficult (1).

*Cancer Treatments*

Today there are a variety of treatments utilized for breast cancer. From surgery to innovative gene therapies, treatments are specialized based on cancer stage and patients’ needs. Treatments can be categorized into five different groups based on technique; doctors often utilize a combination of two or more methods for best results. Factors that influence rate of recovery and survival after treatment are highly dependent upon stage of diagnosis and a patient’s individual response to treatments.
A. Surgery

Surgery is the primary method of cancer treatment (2). Lumpectomy or mastectomy surgeries are used with the goal of removing cancerous tissue from the mammary gland. The type of surgery utilized is dependent upon the stage of cancer and its spread in the breast. Lumpectomy surgery results in the removal of the tumor and minimal surrounding healthy tissue to preserve the remainder of the breast. In addition, nearby lymph nodes may be removed as a precautionary measure. Lumpectomies are optimal treatments for early-stage patients whose cancers are small and localized. With more aggressive breast cancers, mastectomies are preferred to lumpectomies. A simple mastectomy involves the removal of the entire cancer-containing breast while a radical mastectomy removes the breast along with surrounding lymph nodes and adjacent chest muscle. Almost always, breast cancer surgery is followed up with radiation treatment to target any residual cancerous tissue remaining in the breast (1,2).

B. Radiation Therapy

X-rays (and other high-energy radiation such as gamma rays) are used to inhibit and kill cancer cells. Radiation therapy (also known as radiotherapy) targets and destroys cells in a pre-determined tumor-containing area. The high-energy radiation used damages genetic material (DNA) in cancer cells beyond repair, inhibiting breast cancer cell division and proliferation (2). Interestingly, although normal cells are affected by radiotherapy, they may recover from the DNA damage and continue to grow and divide normally. This may be due to the higher efficiency of DNA repair enzymes in normal cells. The type of
radiation utilized is dependent upon the diameter and depth of target tumor as some forms of radiation penetrate tissue more effectively than others. Radiotherapy is optimal if the cancer is in the early stages of metastasis since the radiation will reach cells and lymph nodes that surgery cannot (1,2).

External radiation therapy is administered from outside the body by specialized machines that target radiation to a specified area. External radiation therapy is more common than its counterpart, internal radiation therapy. Internal radiation therapy utilizes small sealed implants (needles, seeds, catheters) containing radioactive substances that are implanted into the breast (2). Upon implantation, contents are gradually released into the tissue. Studies show radiation therapy reduces the recurrence of breast cancer post-surgery by 70%. Radiation treatment is generally administered for 5-7 weeks (1).

C. Hormone Therapy
As mentioned, certain hormone exposures, such as estrogen and progesterone, have been linked with higher incidences of breast cancer. Studies suggest long-term exposure to both hormones promote the development and the progression of breast cancer (6). Hormone therapy manipulates the endocrine system by the addition, subtraction, and/or the blockage of hormones in the body. In specific, hormone therapies used for breast cancer block estrogen and progesterone from reaching cancerous cells. Cells with estrogen and progesterone cell-surface receptors (denoted as ER+ and PR+, respectively) rely on estrogen and progesterone to grow, and therefore will respond to treatment with hormone antagonists (10). Approximately 2 out of 3 breast cancers are estrogen
responsive. Patients with cancers ER- and PR- will not respond to hormone antagonists since they do not rely on estrogen or progesterone for growth and development (2).

There are a handful of approved hormone antagonists currently on the market, the most popular being Tamoxifen®. Tamoxifen® is taken as a daily pill and functions as a competitive inhibitor of estrogen by binding but not activating estrogen receptors (11). Without estrogen, ER+ cancerous cells cannot grow and proliferate. Studies show Tamoxifen® reduces tumor recurrence rate by 41%, and breast cancer death rate by 33% (1). Side effects of Tamoxifen® are similar to ones witnessed with menopause (nausea, hot flashes, irregular menstrual cycle), due to the decreased efficacy of estrogen (2).

Similar to Tamoxifen®, the drug Fluvestrant® affects estrogen delivery. Rather than block receptors, Fluvestrant® completely eliminates estrogen receptors and is often utilized when the body no longer responds to less severe drugs like Tamoxifen®. Due to its irreversible nature, Fluvestrant® is only approved for post-menopausal women (women with low estrogen needs) with advanced breast cancer (12).

Like radiotherapy, hormone therapy is used as an adjuvant treatment post-surgery to prevent the recurrence of cancer. Hormone therapy may be used as the primary form of treatment for invasive cancers to control spread beyond the breast (2). Unfortunately, the side-effects of anti-hormone treatments prevent extended use in some women. Hormone therapy is associated with blood clots, strokes, uterine cancer, and cataracts, although the etiology of these associations is still unclear (2,11,12).
D. Chemotherapy

Similar to anti-hormone therapy, chemotherapy utilizes drugs to destroy cancer cells. Chemotherapy is generally a combination of two or more drugs administered intravenously (into the bloodstream) that target rapidly dividing cells, such as cancer cells. Treatment is useful for metastasized breast cancers due to drug circulation throughout the body (2).

Chemotherapy course duration lasts 6-12 months with allotted recovery periods. These recovery periods are essential due to the severe weakening of the body associated with chemotherapy. Chemotherapy drugs are unable to differentiate between healthy and cancerous cells, but rather primarily affect cells with a rapid division rate. While most organs of the body (liver, kidneys) divide slowly and will not be affected by treatment, some other vital tissues (small intestine, mouth tissue, hair follicles) naturally divide rapidly and will be negatively affected by chemotherapy. Side-effect severity varies with patient and often includes nausea, hair loss, fatigue, and changes in menstrual cycle. Chemotherapy is useful for both adjuvant and primary treatment, depending upon cancer progression (2).

E. Targeted and Gene Therapy Treatment

Alterations in gene and protein expression and function lead to certain characteristic behaviors of cancer cells, such as affinity for tumor formation and hyper-proliferation. The goal of gene therapy is to inhibit and destroy cancer cells by targeting specific
dysregularities (2). For this type of treatment, genes are delivered into a patient’s body and directly or indirectly play a therapeutic role by inhibiting or destroying the cancer. For example, dysfunctional or nonfunctional genes may be replaced with normal genes inserted into the genome that will produce functioning proteins. Moreover, gene regulation may be manipulated to alter protein expression and function within cancer cells to either inhibit cancer progression or result in cell destruction.

A fundamental requirement of targeted therapy is the identification of appropriate carrier vectors. Non-toxic capsules that carry and deliver treatments without having any negative effects on the body are ideal vectors. Viruses are optimal vectors due to their ability to encapsulate nucleic acids and deliver treatment in a pathogenic manner to target cells. Viral vectors are more prevalent in studies and clinical trials of targeted cancer treatments compared with non-viral vectors (72% and 24%, respectively) (13). Vector-gene complexes must meet certain criteria to qualify as gene therapy candidates; vectors must be efficiently transfected with treatment and only cancer cells must be subject to the drugs deleterious effects. Moreover, vector-gene complexes that modulate protein expression must include regulation factors to control the treatment (2,13).

Retroviruses carry double-stranded DNA and therefore are particularly useful for targeted treatment. Double-stranded DNA may be inserted directly into host genome without modification and can utilize host transcription and translation factors. The most common retrovirus used in therapy is derived from the Moloney murine leukemia virus. Other tested vectors include adenoviruses and adeno-associated viruses that characteristically
insert DNA at specific sites on chromosomes that did not previously code for genes. Retroviruses are preferred over adenoviruses due to the latter’s elicitation of immune responses (13).

Non-viral alternatives in use, such as liposome-mediated gene transfer techniques, are less efficient than viral counterparts, but do hold a promising future. Scientists are working on the creation of artificial lipid spheres with hydrophilic centers for use as vectors. The hydrophilic center will house the polar DNA, while the hydrophobic exterior will allow for easy movement across non-polar plasma membranes. Additionally, techniques for binding DNA to molecules with specificity for cell-surface receptors are in testing and may present another method for delivering genetic material into cancerous cells. Once bound to surface receptors, the molecule (along with attached DNA) is engulfed via pinocytosis into cells, whereby DNA insertion can initiate therapeutic treatment. By identifying and targeting surface receptors solely located on breast cancer cells, the method is harmless to healthy cells not expressing the receptor. Scientists hope this and similar treatments may be used to transflect cancer cells with tumor-suppressive genes such as p53 and cyclin dependent kinase (13).

Targeted treatment does not always rely on DNA transfection. Chemicals and molecules may also be transfected into cancer cells for therapeutic treatment (2). Suicide genes are genes capable of converting non-toxic pro-drugs into toxic chemicals. Cancer cells are transfected with suicide genes, or may even already possess abnormal genes that function as suicide genes. When patients are administered a pro-drug the active gene in cancer
cells will convert the pro-drug into a toxin, thus chemically destroying the cells. Theoretically, healthy cells will remain unharmed since they do not have the activated suicide gene. Examples of suicide gene/pro-drug combinations include the Herpes simplex virus thymidine kinase gene with acyclovir as the pro-drug, and fungal cytosine deaminase gene with 5-florocytosine as the pro-drug (13).

Another innovative target therapy is the use of oncolytic viruses. Like suicide genes, oncolytic viruses do not carry genes to cancer cells but rather function to lyse and destroy cancer cells after infection. The adenovirus ONYX-015 is an oncolytic virus that targets cells with p53 mutations (a tumor suppressive gene); non-cancerous cells do not exhibit any mutations in this gene, therefore will not be adversely affected. Other oncolytic virus examples include HSV-1 mutant, Epstein-Barr virus, and Newcastle disease virus (13).

Preliminary research is underway on the proposed idea to introduce a 47th chromosome into the genome of cancer cells. The chromosome would be independent of other chromosomes and carry genetic information that may be transcribed and translated into functional proteins. If the construction of the chromosome is similar enough to host chromosomes, it is speculated that the activation of the immune system may be bypassed by immune cells believing the 47th chromosome to be a self-feature (13).

While targeted and gene therapy for treatment of breast cancer is promising, most methods are still under clinical trial and not approved for widespread medical use. Obtaining an adequate transfection efficiency rate along with target specificity for cancer
cells is essential for successful gene therapy treatments. The identification of a safe and effective targeted therapy will expand and modernize breast cancer treatment.
II. Zinc in the body

Zinc (Zn) is an essential trace mineral and vital to health. The concentration of Zn in the body is second only to iron. The Recommended Daily Allowances for Zn varies with gender and age; males >14 years of age are recommended to intake 11 mg/day, females between 14-18 years of age are recommended 9 mg/day, and females >19 are recommended 8 mg/day. Pregnant women need higher intakes due to fetal requirements necessary for growth and development. Lactating women require +1-2 mg/day extra Zn intake per day to counterbalance Zn secreted into milk. Zn is obtained from a variety of foods. Of all foods, oysters contain the most Zn content per serving (513% Daily Value), however most dietary Zn is obtained through the consumption of red meats (59% DV), poultry (18% DV), and legumes (~11% DV). Dairy products (milk, cheese, yogurt), whole-grain foods, and fortified breakfast cereals are also good sources of Zn. Zn present in plants is not readily absorbed due to binding to phytates that inhibit absorption efficiency (14).

The levels of Zn found in tissue varies depending on specific tissue requirements. Zn concentrations are noticeably higher in muscle, kidney, liver, prostate, and breast cells since these tissues require more Zn for proper function and various biological processes (14). Importantly, Zn is a cofactor for >300 enzymes in 50 different enzyme classes within the body (15), and therefore its presence is a requirement for many different biological processes. Notably, cells of the immune system are dependent upon adequate Zn levels for proper maintenance of function. Neutrophils, natural killer cells, and macrophages all require Zn for division and cytokine production, without which the
immune system is severely compromised. The role of Zn in the immune system makes Zn-deficient patients particularly susceptible to bacterial and viral infections. Without sufficient Zn, the body’s efficiency at destroying foreign antigens is reduced (16).

In addition, the unique role Zn plays in cell division makes Zn fundamental for cellular proliferation and hence growth. All cells of the body require Zn for DNA replication and RNA transcription and translation. The role of Zn in these processes varies from involvement with enzymes that regulate enzymatic rates, to “zinc finger” transcription factors. The vital role of Zn in cellular proliferation makes Zn deficiency detrimental to cellular homeostasis and development. Additionally, without Zn, DNA replication is inhibited and cell division retarded, hindering cell proliferation and cell replacement in organs and tissues. For example, Zn deficiency leads to impaired wound healing resulting in numerous skin lesions. Function of tongue papilla and receptors of the olfactory are compromised as well, leading to reduced sensitivities of taste and smell, respectively. More recent research has found that abnormal Zn concentration is linked to many common diseases such as diabetes, stroke, and a variety of cancers (2,15).

Zn is present in all cells. Zn is considered “free” when it exists in its ionic form and high concentrations of free Zn are toxic to cells. To prevent toxicity, Zn is bound to intracellular molecules. One family of such molecules is collectively known as metallothioneins (MT). MTs are cystein-rich proteins that bind Zn (and other metals such as cadmium and copper) within the cytosol, and in turn are responsible for regulating free intracellular Zn concentration and detoxifying Zn exposure. The expression of MT
proteins is proportional to intracellular Zn concentration; influx of Zn into cells will trigger MT transcription and Zn efflux will result in MT degradation (17).

However not all Zn is cytosolic. Various intracellular compartments function as Zn pools and store or utilize labile Zn. Some pools exhibit stable Zn concentrations suggesting little or no flux of Zn. In such instances, Zn is typically bound to metaloenzymes and functions as coenzymes for metabolic activity. The nucleus is a prime example of a pool with little variation in Zn concentration; a homeostatic environment is necessary for DNA and RNA synthesis and excessive changes in nuclear Zn levels would disturb these processes. Other intracellular Zn pools are labile and serve to handle fluxes of Zn into and out of the cell to provide for optimal cellular function and/or prevent toxicity. Pools such as mitochondria and vesicles are sensitive to minute changes in Zn levels and will uptake or release Zn during supplementation or deprivation based on cellular needs (18). These pools maintain intracellular Zn homeostasis and keep cellular Zn concentrations within an optimal threshold. The importance of Zn homeostasis is mirrored by vast numbers of Zn transporter proteins responsible for the tight regulation of Zn movement into, out of, or within the cell (19).
III. Zinc transporters

Two families of Zn transporters in the body have opposing action on Zn flux. The Zip family (SLC39A) is composed of 14 varying human sequences encoding Zip1-Zip14 and the ZnT family (SLC30A) includes 10 sequences and encodes ZnT1-ZnT10 (19). The mechanism by which Zn transporters function remains unknown, but preliminary studies suggest facilitated diffusion or symporter activity may be utilized to move Zn across membranes (20). The history of Zn metabolism and transporter research is short (~10 years), but science is beginning to realize the great importance of these biological processes and the mechanisms by which they function.

Zip proteins are responsible for the movement of Zn into cells, increasing intracellular Zn concentrations. The 14 identified transporters responsible for influx are subdivided into 4 categories: Zip subfamily I (Zip9), Zip subfamily II (Zip1, Zip2, Zip3) guf A subfamily (Zip11), and LIV-1 subfamily (Zip4, Zip5, Zip6, Zip7, Zip8, Zip10, Zip12, Zip13, Zip14) (20). Bioinformatic research predicts that Zip proteins have 8 transmembrane domains. To present date, most research has focused on the LIV-1 subfamily consisting of 9 of the 14 Zip proteins. LIV-1 family proteins are typically localized to the plasma membrane and import Zn from extracellular pools. In addition, studies indicate Zip transporters are also located on intracellular membranes such as that of the mitochondria and vesicles (19). Although located intracellularly, these Zip proteins also increase intracellular Zn levels by transporting Zn out of compartmentalized Zn pools into the cytoplasm (20).
Studies indicate that while certain Zip proteins are found ubiquitously throughout the body, others are tissue-specific. Zip6, Zip7, Zip8, Zip10, Zip13, and Zip14 aid in Zn transport in all tissues. Specifically, Zip6, Zip10, and Zip14 are generally localized to the plasma membrane in cells, Zip7 is found on the endoplasmic reticulum (ER) and Golgi apparatus, while Zip8 is found solely on vesicular membranes (19,20). Conversely, tissue-restricted Zip proteins are only expressed in certain tissues where they are required for certain processes or functions. For instance, Zip1 has been localized to the plasma membrane of only small intestine and pancreatic cells (20). The localization of Zip4 on the plasma membrane of stomach and small intestine cells is essential for Zn absorption from the diet (20). Zip5 is found on the basolateral membrane of polarized cells of the liver, spleen, and colon (19). Zip12 has only been detected in the brain, lung, testis, and retina of the eyes (19,20). While tissue and membrane localizations differ, all Zip transporters move Zn in a pattern that increases intracellular Zn concentrations.

To counterbalance the function of Zip proteins, ZnT proteins move Zn in a manner that reduces cytoplasmic Zn levels. Although less is known about ZnT family transporters, they are equally important in the maintenance of Zn homeostasis. ZnT family proteins reduce intracellular Zn by sequestering Zn out of cells or into intracellular compartments (mitochondria, vesicles, ER, etc.) (19). Most ZnT proteins are predicted to have 6 transmembrane domains, with the exception of ZnT5 that has 12 transmembrane domains. Studies of bacterial and yeast homologues of ZnT proteins suggest H+/Zn\(^{2+}\) exchange system may be utilized to transport Zn through these proteins (21).
ZnT1, the first Zn transporter identified, is also the most studied. Although ZnT1 is ubiquitous, it is only highly expressed in the small intestine and placenta. Immunofluorescence illustrates ZnT1 localization to plasma membranes and intracellular compartments. Interestingly, non-functioning ZnT1 results in fetal death, highlighting the importance of this transporter (19,21). Similarity, ZnT4 expression appears ubiquitous with particularly high expression found in the brain, mammary gland, small intestine, placenta and mast cells of the immune system (21).

Similar to certain Zip proteins, ZnT transporter expression may be restricted to certain tissues. For example, ZnT3 protein is found in the brain on synaptic vesicles (21). ZnT5 is found abundantly in cells of the pancreas and small intestine with traces in the heart, brain, and liver. Interestingly, ZnT5 has been found complexed with ZnT6, also in the small intestine, liver, and brain (21). Studies show the presence of ZnT7 in the retina, spleen, and lungs, primarily on the membrane of the Golgi apparatus while ZnT8 expression is unique to insulin secretory vesicles of pancreatic cells (21).

The Zn transporter ZnT2 is also a tissue-restricted Zn transporter and only found in the mammary, prostate, and pancreas (21). All three tissues display higher-than-normal levels of Zn, making ZnT2 an interesting candidate to study when considering Zn transport in breast cancer (21).
IV. ZnT2

ZnT2 (official gene name SLC30A2) is a ZnT family protein whose gene is encoded on chromosome 1 in the human genome. As a part of the ZnT family, ZnT2 functions to reduce intracellular Zn levels by either transporting Zn out of cells or sequestering Zn into intracellular compartments. Our previous studies show that two different sized ZnT2 proteins (A and B) are produced via alternative splicing (22); isoform A consists of 8 exons totaling 383 amino acids, while the B variant has 7 exons totaling 334 amino acids (23). Notably, ZnT2 is not a ubiquitously expressed Zn transporter with studies indicating expression is restricted to a select number of tissues. The mammary gland, prostate, and pancreas all express ZnT2, and similarly all have high Zn requirements (21,23). Restricted distribution illustrates secretion into unique biological fluids such as milk, seminal fluid, and pancreatic fluid by the mammary, prostate, and pancreas, respectively.

Interestingly, previous studies from the Kelleher Lab have associated mutations in ZnT2 with dysfunctional Zn transport leading to transient neonatal Zn deficiency (24). Transient neonatal Zn deficiency is a disease caused by insufficient delivery of Zn in milk to a nursing infant from the lactating mother. This illustrates not only the vital role of ZnT2 in the mammary gland, but also documents a role for ZnT2 in the vesicular transport of Zn out of the cell for delivery into milk (24).

Determining differences in localization and function in Zn transport of the two transcripts is underway, with evidence suggesting that the different isoforms are localized to different membranes (23). Importantly, recent studies on ZnT2 document localization of
ZnT2 to the inner mitochondrial membrane in addition to vesicular membranes (19,21,22,23). Localization to the mitochondrial membrane suggests a role as a mitochondrial Zn importer where the protein sequesters Zn from the cytosol into mitochondria. As a result, we predict that ZnT2 plays a critical role in the regulation of mitochondrial Zn homeostasis and thus function.
V. Breast cancer and zinc

Zn homeostasis is critical for proper tissue and organ function. Abnormal Zn trafficking has many consequences (immune system dysfunction, impaired wound healing, etc. (14)) and has recently also been associated with several cancers, such as breast (25), prostate (26) and pancreatic (26). Studies on different cancers illustrate dysfunctional Zn metabolism, although a consensus on the involvement of Zn with cancer development and progression has yet to be reached. Interestingly, depending on cancer type, Zn levels may be high or low (25,26). Dysregulated Zn metabolism is proposed to have severe consequences in relation to growth and apoptosis, both features that are abnormal in cancer.

Recent studies from breast biopsies indicate that breast cancer tissue exhibits higher-than-normal Zn concentrations. Studies show that although cellular levels are high, breast cancer patients tend to have low serum Zn levels (27,28). It is important to note that the dysregulation of Zn metabolism present in breast cancer does not imply Zn supplementation or deficiency is a direct cause of breast cancer. Rather, these studies suggest that dysregulated Zn distribution is a consequence of the disease. As a result, scientists believe that Zn transporters may be practical biomarkers for breast cancer diagnosis and prognosis (29).

Movement of Zn is regulated by Zip and ZnT transporters and expression of both families is highly responsive to changes in intracellular Zn levels (19,20). Dysregulated Zn metabolism in malignant breast cells may result from the modification of Zn transporter
expression and possibly localization. Changes in Zn transporter expression and localization may be responsible for or an adaptation to (or both) the hyper-accumulative Zn metabolism of breast cancer cells.

Although studies on breast cancer and Zn are limited, there has been much progress in the past decade. In fact, studies have already associated specific alterations in Zip family proteins to cancer prognosis. For example, preliminary studies indicate higher Zip7 and Zip14 expression in breast tissue is associated with more aggressive tumors, suggesting roles for these Zn transporters in metastasis (28). Moreover, heightened Zip7 and Zip8 expression are linked to breast cancers that prove to be increasingly resistant to commonly used hormone therapies such as Tamoxifen® and Fluvestrant® (29). In addition, Zip10 over-expression is characteristic of invasive and already-metastasized breast tumor cells; attenuation of the Zip10 transporter significantly reduces cancer aggressiveness, suggesting possible future roles for Zn transporters in breast cancer therapy (30). The same study indicates that Zn levels measured after Zip10 attenuation were lower compared with non-Zip10-attenuated cells, suggesting “normalization” of cellular Zn levels is associated with cancer inhibition (30). Unlike Zip proteins which are associated with more aggressive cancers, enhanced Zip6 mRNA and protein expression is associated with smaller, less aggressive breast tumors. Notably, Zip6 is estrogen responsive and expression is also linked with ER+ breast cancers that are more treatable (27).
As the research suggests, quantitative analysis of Zn transporter expression may eventually be a useful tool in determining breast cancer prognosis. Much is unknown about the relationship between Zn status and cancer, yet the field remains promising for exploring the role of Zn dysregulation in breast cancer.
VI. Cellular death

Cell death occurs through two distinct processes, necrosis and apoptosis. Necrosis is the premature unnatural death of cells. Necrosis may be initiated by a variety of factors that alter cellular homeostasis beyond repair (31). Factors that may induce necrosis include tissue injury, bacterial infections, toxins, and some cancers that disrupt the internal or external cell environment. Necrotic death is rapid and destructive, often negatively affecting surrounding cells by the release of internal fluids and/or toxins. In particular, release of lysosomal enzymes severely damages nearby cells, often resulting in inflammation and pain as the body’s immune system responds to the chaotic death (32).

In contrast, apoptosis is the natural death of cells through gene-programmed mechanisms that result in cellular self-destruction (31). Apoptosis can be characterized into two distinct phases that ultimately result in death: biochemical signaling and morphological changes (32). Cells will undergo apoptosis for a variety of reasons. Both intrinsic and extrinsic signals can initiate apoptogenesis including cellular damage (damage to DNA, membranes, mitochondria), cellular stress (deficiency of nutrients, toxic chemicals, lack of ATP, etc.), and infection with viral pathogens. Moreover, the immune system and surrounding cells are capable of activating apoptogenesis as well (31). Most cells of the body undergo programmed cell death when they have aged and room needs to be made for younger, more efficient cells (32). Ultimately through apoptosis, cell shrinkage and division results in multiple individual cellular fractions. These fractions encapsulate intracellular fluids ensuring contents do not spill out into the extracellular space and therefore avoiding any immune system response. The release of chemical signals during
apoptosis promotes the movement of phagocytic cells to the area so that fragments are cleanly removed (31).

Billions of cells undergo apoptosis in the body daily, highlighting the importance of proper apoptotic regulation. In a healthy environment, the rate of cell synthesis and programmed death is at an equilibrium (2). Imbalances pose severe consequences; if more cells undergo apoptogenesis than the opposing rate of proliferation, tissues will undergo atrophy. Common causes of atrophy include malnourishment and poor circulation of nutrients, both of which will elicit stress responses by previously healthy cells, and ultimately apoptosis. In contrast, if the rate of cell proliferation is greater than destruction by apoptosis, the result will be an over-population of cells often resulting in masses known as tumors. Moreover, cancers are characterized by mutated cells that are anti-apoptotic by nature, resulting in high proliferation rates along with little-to-no cellular death (2).

In particular, mitochondria play a uniquely important role in apoptogenesis (31,32). It has been speculated that mitochondria channel signals from interactions with activated apoptotic regulatory proteins that either promote or inhibit apoptosis (such as Bax or Bel-X family proteins, respectively) (33,34,35,36). Moreover, internal changes in mitochondrial homeostasis can also trigger apoptogenesis. If mitochondria experience loss of membrane potential, altered cellular oxidation-reduction, or diminished electron transport chain function, apoptosis may be activated (33). Pro-apoptotic signals often result in the loss of membrane potential and integrity, resulting in pore-formation. These
pore-like channels are known as MAC channels (mitochondrial apoptosis-induced channels) and hinder cellular energy-production by elimination of energy gradients normally a result of a tightly regulated membrane potential (34). Moreover, opened pores allow for the release of apoptosis caspase activators such as cytochrome c. Originally a heme protein part of the electron transport chain, cytochrome c is a soluble protein and will enter the intracellular space of cells where it cleaves procaspase proteins resulting in active caspases, and ultimately a full caspase cascade (34).

Activated caspases bring on cellular destruction through a variety of morphological changes. Breakdown of the cytoskeleton results in cells losing shape and structure. Once the cell begins to shrink, the cytoplasm becomes densely packed with organelles (32). Simultaneously within the nucleus, chromatin condensation and karyorrhexis (fragmentation of DNA) results in the destruction of genetic material and the nuclear envelope (32). As a final step, cells are fragmented into small, contained divisions referred to as apoptotic bodies. Previously released signals attracting phagocytes result in the phagocytosis of the apoptotic bodies. The final result is the complete elimination and removal of all cell components from the tissue (31,32).
**VII. Zinc and apoptosis**

Recently, scientists have found an interesting link between Zn and apoptosis. Studies indicate that cellular Zn homeostasis is critical for cell health, and dysregulation of Zn metabolism may be linked apoptotic dysregulation (37). To date, no other trace minerals exhibit such a clear relationship, highlighting the importance of understanding Zn metabolism, and apoptotic homeostasis.

Many scientists attribute this unique relationship to the distinct involvement of Zn in nucleic acid synthesis. The term “zinc finger” refers to protein domains that recruit and utilize Zn ions to stabilize their folds. Zinc figures are often replication or transcription factors found bound to DNA or RNA, or other small protein molecules (38). Thus, without Zn, replication and transcription would be inhibited, possibly resulting in apoptosis. Moreover, some studies suggest the accumulation of cellular Zn directly affects the mitochondrial integrity, and excess Zn will result in mitochondrial pore-formation, degradation, and activation of apoptosis (36).

Although the exact relationship between Zn and apoptosis is currently not well understood, studies show that both Zn supplementation and deprivation will initiate apoptosis (39,40). Zn deficiency has been shown to induce apoptosis in vitro using Zn chelation agents (40). Similarly, Zn supplementation produces identical results in vitro. Notably, cell type and concentration of supplementation will determine if cell death is by apoptosis or necrosis (39,41,42). Results of these studies can be partially explained by the diverse role of Zn in cellular functions. Importantly, Zn has a vital role as a cofactor
for many biological processes (14). Without Zn to fulfill that role, many enzymes are not activated and certain processes become hindered, including DNA and RNA synthesis, which rely on zinc finger regulation. Conversely, high levels may induce apoptosis due to Zn cytotoxicity, considering the +2 charge ionic Zn carries. In addition, supplementation of Zn has been shown to increase the levels of intracellular Bax (pro-apoptotic cytosolic protein) and promote its movement to the mitochondria (36), where it induces pore formation.

The degree of Zn deficiency or toxicity determines whether cell death is by necrosis or apoptosis. Different tissues optimally function in response to different levels of cellular Zn. For instance, mammary and prostate tissues transport higher amounts of Zn, therefore may have a higher tolerance for and unique regulation of intracellular Zn pools. Concomitantly, studies suggest the existence of tissue-specific apoptotic thresholds. Zn concentrations within this apoptotic threshold are levels at which cells remain viable. Beyond the apoptotic threshold, whether as a result of Zn deprivation or supplementation, are Zn levels at which cells can no longer function, and thus will initiate cell death. Although apoptotic death is controlled and more efficient, if cells are severely damaged, they are unable to produce ATP that is required for the initial steps of apoptosis, thus necrotic death may predominate (39,41,42).
VIII. Hypothesis

Based on the above background, we hypothesize that the Zn transporter ZnT2 may play a critical role in breast tumor cell Zn metabolism. Considering that Zn metabolism is dysregulated in breast cancer, we hypothesize that the role and/or function of ZnT2 may be altered in breast cancer as well.

The main objectives of our study were 1) to characterize the cellular dysregulation in Zn metabolism in malignant breast cells; and 2) to determine the role of the Zn transporter ZnT2 in breast tumor cell Zn metabolism.
Materials and Methods
I. Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

II. Cell culture of the human T47D cell line

Human tumorigenic mammary carcinoma cells (T47D) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in growth medium of RPMI-1640 supplemented with 1% penicillin/streptomycin antibiotic, 0.2% sodium bicarbonate, sodium pyruvate (1mM), insulin (5µg/ml), and 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a 5% CO₂ humid atmosphere.

III. Cell culture of the mouse HC11 cell line

Mouse normal mammary epithelial cells (HC11) were a gift from Dr. Jeffery Rosen (Baylor College of Medicine; Houston, TX) and used with permission of Dr. Bernd Groner (Institute for Biomedical Research, Frankfurt, Germany). Cells were maintained in growth medium RPMI-1640 supplemented with insulin (5µg/ml), epidermal growth factor (EGF, 10ng/ml), 0.2% sodium bicarbonate, gentamycin (50µg/ml), and 10% FBS. Cells were cultured at 37°C in a 5% CO₂ humid atmosphere.

IV. Cell culture of the human HME cell line

Human normal mammary epithelial cells (HME) were obtained from Cell Applications Inc. (San Diego, CA). Cells were cultured in pituitary extract-supplemented Medium 171 (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂ humid atmosphere.
V. ICP-AA mineral analysis

To measure Zn concentration in HC11, HME, and T47D cells, cells were rinsed with 1X Phosphate Buffered Saline (PBS, 3.2mM Na₂HPO₄, 0.5mM KH₂PO₄, 1.3mM KCl, 135mM NaCl, pH 7.2-7.4) + 1mM EDTA to remove loosely bound Zn. Cells were scraped into 1X PBS (1ml) and centrifuged at 2000xg for 10 minutes. Pellets were resuspended in 69% ultra-pure nitric acid and digested overnight. ICP-AA was used to measure Zn concentration. Mitochondrial Zn concentrations were determined by analyzing isolated mitoplast samples (see Mitoplast Isolation). Mitoplasts were resuspended in 69% ultra-pure nitric acid, digested overnight, and analyzed by ICP-AA.

VI. Fluorometric evaluation of Zn distribution

To quantify Zn levels in various intracellular Zn pools, T47D cells were cultured for 48 hours in 96-well plates (6.25x10⁵ cells/well). At ~60% confluency, cells were rinsed 2X with 1X PBS and incubated in FluoZin-3 dye (2µM) or RhodZin-3 dye (1µM) diluted in Opti-MEM medium (Invitrogen) for 1 hour at 37ºC. Dye was removed and cells rinsed 2X with 1X PBS, followed by a 1X PBS wash with gentle shaking for 20 minutes (shielded from light). Cells were incubated in 1X PBS (50µl) and fluorometric measurements taken on FLUOstar OPTIMA (BMG Labtech, Durham, NC) and analyzed using Prism Graph Pad v.5 (Berkeley, CA).
VII. Mitoplast isolation

Cultured cells were rinsed with 1X PBS followed by a rinse with MB buffer (210mM Hepes, 10mM mannitol, 70mM sucrose, 1mM EDTA, pH 7.5). Cells were scraped into ice-cold MB buffer (1ml) and homogenized using a glass homogenizer 30X on ice. Samples were centrifuged at 2,000xg for 5 minutes and the resulting supernatant aspirated. Remaining crude mitochondria pellets were resuspended in MB buffer and recentrifuged at 12000xg for 12 minutes (repeated twice) to achieve optimal purification. Crude mitochondria pellets were lysed with cold water on ice for 20 minutes to isolate mitoplasts (mitochondria devoid of outer membrane). Samples were centrifuged at 15000xg for 15 minutes to collect mitoplasts and resulting pellets resuspended in 1X PBS. Protein concentrations were determined by Bradford assay. Remainder contaminating proteins were digested in 1X PBS (1ml) with proteinase K (Biolabs, Ipswich, MA) in an equal enzyme:protein ratio for 20 minutes on ice. Enzymatic digestion was inactivated with phenylmethylsulphonyl fluoride (PMSF, 20mmol/L) while incubated on ice for 20 minutes. Resulting mitoplast samples were centrifuged at 15000xg for 15 minutes to pellet purified product.

VIII. Production of antibodies to ZnT2

Peptides, predicted from the published mRNA sequence ZnT2 (GKFNFHTMTIQIESYSEDMKSCQECQGPSE, Genemed Synthesis, South San Francisco, CA) was synthesized with an additional cysteine residue for conjugation to keyhole limpet hemocyanin (KLH) at the C-terminal end. The sequence was verified by amino acid analysis and mass spectroscopy. KLH-conjugated peptides were injected into
New Zealand White rabbits (1mg peptide/rabbit) for polyclonal antibody production. Antibody specificity was verified by peptide competition analysis.

**IX. Immunoblotting**

To isolate total membrane protein from cells, cultured cells were collected into homogenization buffer (1ml/well, 20mM HEPES pH 7.4, 250mM sucrose, 1mM EDTA, with protease inhibitor). Cell suspensions were sonicated for 10 seconds 3X to homogenize samples. Cells were centrifuged at 500xg for 5 minutes and the post-nuclear supernatant centrifuged at 100,000xg for 20 minutes. Resulting pellets were resuspended in homogenization buffer and protein concentration determined by Bradford assay. Laemmli Sample Buffer (Bio-Rad, Hercules, CA) with DTT (100 mmol/L) was added to cell suspensions and mixture boiled at 95°C for 5 minutes. Proteins were cooled and loaded onto 10% SDS-PAGE gel and separated via gel electrophoresis (200V, 1 hour) followed by transfer to nitrocellulose (100V, 1 hour, GE Healthcare, Piscataway, NJ). To prevent unspecific binding, membranes were submerged in a 5% milk solution in 1X Phosphate Buffered Saline-Tween (PBST) for 1 hour. Primary antibodies were used to probe for proteins in question, followed by incubation in appropriate secondary detection antibody (all dilutions in PBST, 1 hour). Immunoblots visualized using chemiluminescence (SuperSignal Femto, Pierce) via exposure to autoradiography film.

To control for protein loading, membranes were stripped with Restore Western Blot Stripping Buffer (ThermoFisher Scientific, Waltham, MA) and reprobed with antibodies as indicated.
X. Attenuation of ZnT2

T47D cells (5x10⁶) suspended in antibiotic-free medium were centrifuged at 1000xg for 10 minutes to pellet cells. Resulting supernatant was aspirated and pellets resuspended in Nucleofector Solution V (100µl, Nucleofector Kit V, Lonza, Gaithersburg, MD). Human-ZnT2 siRNA (SLC30A2) was added to cells (200pmol/5x10⁶ cells) and resulting solutions transferred to Amaxa cuvettes for transfection using Amaxa technology (Program X-005, Lonza). Following transfection, antibiotic-free medium (500µl) was added into the cuvettes and solutions transferred to appropriate dishes for culture.

XI. 4x-MRE Luciferase assay

All reagents were obtained from the Dual-Luciferase Assay kit (Promega, Madison, WI). The 4x-MRE (metal-responsive element) pGL3-luciferase reporter was kindly provided Dr. Colin Duckett (University of Michigan Medical School, Ann Arbor, MI). Large-scale plasmid purification carried out using the Plasmid Midi Kit. Cytoplasmic Zn movement resulting from ZnT2 attenuation was determined by luciferase fluorescence. Cells were transfected with 4x-MRE luciferase (0.8µg/transfection) and TK renilla (0.5µg/transfection) at time of gene attenuation. The fluorescent tag on 4x-MRE fluoresces when bound to metallothionein, with TK renilla used as a protein control. After incubation time of 48 hours, treated cells were rinsed with 1X PBS and collected by incubation in 1X Passive Lysis Buffer (PLB, 100µl/well) for 15 minutes. PLB lysates (20µl) were combined with LAR II (100µl) and gently mixed. Luciferase activity was measured using a luminometer device (TD20/20, Turner Designs, Sunnyvale, CA),
followed by the addition of Stop & Glo Reagent (100µl) to measure TK renilla activity. Luminescence units calculated as the ratio of firefly luciferase:renilla activity.

**XII. Annexin-V staining**

Annexin-V is a fluorescent dye that binds to externalized phosphatidylserine on the plasma membrane and is used as a marker for early apoptosis. Treated cells were plated on glass slides in a 24-well plates and incubated in antibiotic-free medium for 20 hours. Cells were rinsed with 1X PBS and incubated in Annexin-V Incubation Reagent (200µl/well, Trevigen, Gaithersburg, MD) for 15 minutes (shielded from light). Following staining, cells were washed 2X with 1X PBS (2min/wash). Glass cover slips were mounted onto glass slides with ProLong Gold (Molecular Probes, Carlsbad, CA) and visualized using Olympus BX50WI, with UPlanApo 100X oil lens N.A. 1.35 and images saved as RGB .jpeg files to maintain image quality.

**XIII. Trypan blue exclusion**

Cell viability following ZnT2 attenuation was measured using the trypan blue exclusion method. Attenuated cells were trypsinized for collection followed by the addition of antibiotic-free medium to neutralize the reaction and collected cells centrifuged at 1000rpm for 10 minutes. Resulting pellets were resuspended in culture medium and aliquots (5/well) mixed with trypan blue, a stain that incorporates into non-viable cells. Cell counts of viable and non-viable cells were taken to quantify results.
XIV. Soft agar cellular proliferation and tumorigenicity assessment

The effect of ZnT2 attenuation on cell viability and tumorigenicity was determined by soft agar cell and colony counts. Treated cells were embedded into a 0.7% Agarose/1.0% Agar gelatin matrix in a 6-well dish. Cells and colonies (defined as >3 clustered cells) were counted (10 planes/well/day) 1 and 5 days after treatment.

XV. Statistical analysis

Quantitative results are presented as a mean ± standard deviation. Statistical comparisons were done using Student’s t-test (Prism, Graph Pad, Berkeley, CA). A significant difference was determined by a value of p<0.05 and a strong trend determined by a value of p<0.10.
Results
**I. Zinc concentration in tumor cells is higher than in non-tumor cells.**

Previous studies show that Zn levels are higher in human breast tumor cells compared with non-cancerous cells. We speculate that the dysregulated Zn metabolism in cancer cells leads to distinct physiological differences compared with normal cells. To verify those findings, we analyzed Zn concentrations in human breast tumor cells (T47D) using ICP- AA to determine if we found the same anomaly in Zn levels. Human breast cancer cells (T47D) were compared with normal human epithelial cells (HME) and normal mouse mammary epithelial cells (HC11). Results demonstrated that breast tumor cells (T47D) possessed significantly higher (p<0.05) Zn concentration than both normal mouse (HC11) and normal human (HME) mammary cells (Figure 1). Results suggest that breast tumor cells have a dysregulated Zn metabolism and hyper-accumulate Zn.

**II. Zinc concentration in tumor cell vesicles is higher than non-tumor cell vesicles.**

**Zinc concentration in tumor cell mitochondria is lower than in non-tumor cell mitochondria.**

Zn homeostasis is essential for the maintenance of overall cell homeostasis. Since ICP- AA illustrated that Zn levels in breast tumor cells (T47D) are higher than that of normal human (HME) and normal mouse (HC11) mammary cells, we found it key to examine vesicular and mitochondrial Zn pools to determine where Zn is accumulated in these Zn-enriched cells.

To assess vesicular storage of Zn in breast tumor cells (T47D) compared with normal mouse cell (HC11), FluoZin-3 fluorescent dye was utilized due to its specificity for
vesicularized labile Zn. Detected fluorescence was quantified and results indicated that breast tumor cells (T47D) possessed significantly more (p<0.05) labile Zn in vesicles compared with normal mammary cells (HC11)(Figure 2A). Data suggest that hyper-accumulative breast tumor cells accumulate excess Zn in vesicles, and in turn likely prevent Zn cytotoxicity by removing Zn from the cytosol.

In normal cells, elevated or depleted levels of Zn trigger intrinsic apoptosis through the mitochondria, thus Zn homeostasis in mitochondria is critical. To evaluate the concentration of Zn in the mitochondria, isolated mitoplasts (mitochondria without the outer membrane) from normal mammary (HC11) and breast tumor (T47D) cells were digested in nitric acid and subjected to ICP- AA. Results illustrated that breast tumor cells (T47D) had significantly less (p<0.05) mitochondrial Zn levels compared with normal mammary cells (HC11)(Figure 2B). Results suggest no relationship between the hyper-accumulation of Zn and Zn levels in the mitochondria. Interestingly, although mitochondrial Zn levels were low, they did not seem to be affecting cellular viability.

**III. ZnT2 expression is lower in breast tumor cells compared with normal mammary cells.**

Zn homeostasis and Zn movement are maintained by regulatory Zn transporters. There are many transporters involved in the regulation of Zn movement within a cell, and all have characteristic localization and function. The ZnT-family Zn transporter ZnT2 was of particular interest in our study due to its tissue-specific expression and localization to
both mitochondrial and vesicular membranes. As a ZnT protein, ZnT2 transports Zn in a manner that reduces cytoplasmic Zn levels.

To compare the expression of ZnT2 in breast tumor cells (T47D) with normal mammary cells (HC11), crude membranes were resolved by SDS-PAGE, and immunoblotted for ZnT2. Results indicated that breast tumor cells (T47D) do express ZnT2. In addition, results illustrated that both cell types expressed a ~42kDa protein (Figure 3). Interestingly, results indicated ZnT2 protein abundance was ~10 fold lower in tumorigenic breast cells (T47D) compared with normal mammary cells (HC11).

**IV. ZnT2 is localized to the mitochondria in breast tumor cells.**

We previously had determined that ZnT2 is localized to the mitochondria in normal mammary cells (HC11) where the protein functions as a mitochondrial Zn importer. To assess the localization of ZnT2 in breast cancer cells, we examined the expression of ZnT2 on the tightly regulated inner mitochondrial membrane of breast tumor cells (T47D). Isolated mitoplasts were resolved by SDS-PAGE, and immunoblotted for ZnT2. Results showed expression of the protein ZnT2 on the inner mitochondrial membrane of breast tumor cells (T47D)(Figure 4). Localizing ZnT2 to the mitochondrial membrane suggests that ZnT2 may function as a mitochondrial Zn importer, thus possibly playing a key role in mitochondrial apoptotic-regulation.
V. Attenuation of ZnT2 reduces ZnT2 protein abundance in total extracts at 12 and 48 hours, and in the mitochondria.

To verify the functionality of ZnT2 in breast tumor cells (T47D), gene-attenuation was utilized to reduce ZnT2 protein expression. ZnT2 was attenuated by siRNA transfection for 12-hours (Figure 5A) or 48 hours (Figure 5B). Cells were collected and total extracts resolved by SDS-PAGE, and immunoblotted for ZnT2. Our results indicated the successful reduction in ZnT2 protein expression at 12 and 48 hours (Figure 5A, 5B). In addition, to verify that attenuation of ZnT2 could be achieved in the mitochondria, ZnT2 in breast tumor cells (T47D) was attenuated by siRNA treatment for 48 hours. Following treatment, mitoplasts were isolated and resolved by SDS-PAGE, and immunoblotted for ZnT2. Results indicated the successful attenuation of ZnT2 in the mitochondria.

VI. Attenuation of ZnT2 reduces mitochondria Zn pools but increases cytoplasmic and vesicular pools in breast tumor cells.

With the successful attenuation of ZnT2 in breast tumor cells (T47D), in particular from the inner mitochondrial membrane, it was essential to assess the changes on Zn distribution as a result of reduced ZnT2 protein expression. Three separate Zn pools were assessed: mitochondrial, vesicular and cytoplasmic.

ZnT2 was attenuated for 48 hours in a 96-well plate. Using RhodZin-3 (fluorescent dye that binds to Zn in mitochondria), mitochondrial Zn pools were quantified. The attenuation of ZnT2 in breast tumor cells (T47D) resulted in a significant decrease (p<0.05) in mitochondrial Zn (Figure 6A). This suggests that ZnT2 is a functional
mitochondrial Zn importer. As a result, our data suggest that reduced ZnT2 protein expression in breast tumor cells (T47D) is associated with low mitochondrial Zn import.

To examine cytoplasmic Zn pools, a 4xMRE (metal responsive element) promoter reporter construct was transfected into breast tumor cells (T47D). Measurement of luciferase activity was quantified. Results showed that attenuation of ZnT2 resulted in increased in cytosolic Zn levels compared with ZnT2-expressing breast tumor cells (T47D) (Figure 6B). Moreover, using FluoZin-3 (a fluorescent dye specific for vesicularized labile Zn), our results indicated a significant increase (p<0.05) in vesicularized Zn in ZnT2-attenuated breast tumor cells (T47D) compared with non-ZnT2 attenuated cells (Figure 6C).

Our data suggest that the attenuation of mitochondrial ZnT2 and subsequent decrease in mitochondrial Zn import results in the redistribution of Zn from the mitochondria into the cytoplasm and vesicles.

**VII. Attenuation of ZnT2 results in the apoptotic death of breast tumor cells**

Previous studies suggest that increases or decreases in Zn pools may affect cellular viability. Apoptosis is mediated by the mitochondria, thus changes in mitochondrial Zn levels may induce programmed cell death. Our results showed that breast tumor cells exhibited endogenously low mitochondrial Zn levels, and that the attenuation of mitochondrial Zn import through ZnT2 reduced mitochondrial Zn levels further. To determine if altering cellular Zn pools by the attenuation of ZnT2 affects cellular
viability, Annexin-V (fluorescent stain that binds to externalized phosphatidylserine on the plasma membrane) was used to detect apoptotic cells. Externalization of phosphatidylserine is an early apoptotic marker. Results showed greater fluorescence (more Annexin-V binding) in ZnT2-attenuated cells compared with ZnT2-expressing cells, illustrating that the attenuation of mitochondrial Zn import or the increase in cytoplasmic Zn induces apoptosis in breast tumor cells (T47D) (Figure 7). Data suggest that although breast tumor cells are anti-apoptotic by nature, the modulation of mitochondrial Zn levels by the attenuation of mitochondrial Zn import reduces Zn levels beyond viable levels, and results in cell death.

**VIII. Attenuation of ZnT2 reduces cellular viability in breast tumor cells.**

To assess whether detected apoptogenesis in breast tumor cells as a result of ZnT2-attenuation was significant, cell viability was assessed by a trypan blue exclusion method. Trypan blue (a stain incorporated solely by dead cells) was used to differentiate between viable and non-viable cells in ZnT2-attenuated and ZnT2-expressing cells. The attenuation of ZnT2 in breast tumor cells resulted in a significant (p<0.05) decrease in cellular viability compared with ZnT2-expressing cells (Figure 8). These data suggest that the manipulation of ZnT2 expression and cellular Zn pools leads to cellular death.
**IX. Attenuation of ZnT2 reduces cellular proliferation and tumorigenicity in breast tumor cells**

Cellular hyper-proliferation and the formation of tumors are hallmarks of cancer, including breast cancer. To assess the effect of ZnT2-attenuation on proliferation and tumorigenicity, breast tumor cells (T47D) were treated with siRNA to reduce ZnT2 expression and embedded onto a soft agar matrix, allowing for the free movement and growth of cells. One and five days after treatment, cell number and tumor counts were taken. The attenuation of ZnT2 reduced cellular proliferation in breast tumor cells (Figure 9). In addition, attenuation of ZnT2 reduced the formation of tumors. These data suggest that the manipulation of ZnT2 and cellular Zn pools not only results in the death of tumor cells, but also minimizes the key cancer traits of hyper-proliferation and tumorigenicity.
Discussion
Previous studies indicate that Zn metabolism is dysregulated in breast tumor cells and that tumor biopsies show elevated Zn levels in breast cancer tissue (27,28). Our ICP-AA analysis of Zn concentration in breast tumor cells were in agreement with these findings by demonstrating significantly higher Zn levels in cultured breast tumor cells compared with normal breast cells. These findings beg the question, where is the Zn that is a result of hyper-accumulation located in these breast tumor cells? We approached this question by examining two different cellular compartments that are subject to labile Zn fluctuations (18): vesicular Zn pools, and mitochondrial Zn pools. We utilized Zn-specific fluorophores to detect and quantify relative differences in mitochondrial and vesicular Zn concentrations between breast tumor cells and normal cells. Our findings indicated that vesicular Zn levels were higher in breast cancer cells when compared with Zn levels in non-cancerous cells. Conversely, mitochondrial Zn levels in breast tumor cells were lower with respect to normal cells. Our data illustrate an interesting relationship between Zn hyper-accumulation in breast cancer cells and intracellular Zn distribution to mitochondrial and vesicular Zn pools. Similar to our results, previous studies in other cancer cell types documented increased sequestration of Zn into intracellular vesicles (43). It has been suggested that Zn sequestration in vesicles may serve as a protective mechanism given that sequestration into vesicles is associated with a decrease in cytotoxicity (43) as high cytosolic Zn levels will result in massive cell death (44). Interestingly, the same study suggests a possible signaling mechanism between the plasma membrane and Zn transporters located on vesicular membranes that may promote the transport of Zn into vesicles, maintaining cytosolic Zn levels at viable levels (44). Cytosolic Zn levels that are too high or low are toxic to the cell, therefore measures are
taken to prevent this toxicity. We speculate breast tumor cells may be utilizing the same or a similar protective mechanism to adjust to the hyper-accumulation of Zn by sequestering Zn from the cytosol into vesicular compartments, thus resulting in an abnormal vesicular Zn distribution pattern not witnessed in normal breast cells. The adaptation to high Zn levels by sequestration into vesicles by breast cancer cells may allow for continued proliferation and tumor formation.

In contrast, Zn levels in the mitochondria were not elevated. In fact, although breast cancer cells hyper-accumulate Zn, mitochondrial Zn levels were maintained at a concentration that was significantly lower than that found in normal breast cells. The ability of malignant breast cells to maintain such low Zn levels in the mitochondria despite higher-than-normal overall Zn concentration is another indicator of the convoluted Zn metabolism observed in tumor cells. Interestingly, studies in malignant prostate cells (cells characterized by the hypo-accumulation of Zn as opposed to the hyper-accumulative status of breast cancer cells) suggest that diminished accumulation of Zn in mitochondria may inhibit apoptogenesis (36). Although the exact mechanism is unclear, these studies suggest that Zn possesses tumor-suppressive characteristics (36). While studies indicate low and high cellular Zn levels in prostate and breast cancer, respectively, both display low mitochondrial levels (27,28,45). Thus, we speculate that the similar mitochondrial patterns of Zn in cancer cells may be responsible for inhibited apoptosis, and in turn promotes the cell proliferation and tumor formation distinctive of cancer. This finding presents not only a key novel finding in the study of breast cancer
and its characteristics, but implicates the regulation of mitochondrial Zn level as a critical area for further exploration as it may be associated with malignancy.

The low mitochondrial Zn level begs the question of functional relevance. It has been postulated that pools including the mitochondria are more susceptible to extreme fluctuations in Zn concentrations (18,21). Such extremes will interfere with essential functions of the mitochondria, such as cellular respiration and energy production. Importantly, mitochondria play a vital role in the regulation of apoptosis, in particular intrinsic apoptosis (37,38,39,42). Stress to the mitochondria has been shown to elicit an apoptogenic response and result in cell death. While concentrations vary for different tissues in different environments, in general, exceedingly high levels of Zn will induce apoptosis, even in cancer cells that are characteristically anti-apoptotic (39). Similar results are witnessed in breast cancer cells that have been treated with chelating agents, mimicking Zn-deprivation conditions (40). To our knowledge, no studies have focused on the specific depletion of mitochondrial Zn and its relation to apoptosis.

Understanding how and why individual Zn pools are abnormal in breast tumor cells may be key to understanding the etiology behind breast cancer. The movement of Zn within cells is the role of two distinct families of Zn transporters. While Zip family proteins transport Zn into the cytoplasm, ZnT proteins function to transport Zn out of the cytoplasm, into organelles or outside the cell (19,20,21). Previous studies in breast cancer cells have shown that the expression of certain Zip proteins is altered in cancer cells when compared with normal cells (27,28). Interestingly, Zip protein levels are increased
in breast tumor cells (29,30). This may provide a mechanistic explanation for the hyper-accumulation of Zn, considering the role of Zip proteins in increasing intracellular Zn levels. In contrast, no studies on the relationship between ZnT family proteins and breast cancer exist. We speculate that the unusual Zn distribution observed in the mitochondria of breast tumor cells may be the result of abnormal Zn transporter expression or function: either a decrease in Zn import or an increase in Zn export from this organelle.

A key finding from previous research in the Kelleher Lab in normal mammary cells indicates the presence of ZnT2 on the inner mitochondrial membrane which functions as a mitochondrial Zn importer (22). Considering the present results indicating abnormally low mitochondrial Zn levels in breast tumor cells, the next key question is whether or not ZnT2 expression and/or localization is altered in breast tumor cells, and, if so, if alterations linked to the observed abnormal Zn distribution are the mitochondria. To address the former part of the question, we first compared ZnT2 protein expression in normal and breast tumor cells through immunoblotting. A similar method was used to test for the expression of ZnT2 on breast tumor mitoplasts (mitochondria devoid of the outer membrane). We found that ZnT2 is expressed in breast tumor cells, but our findings indicated that protein expression in the tumor cells is ~10 fold lower than that of normal cells. Similar to normal breast cells however, ZnT2 is expressed on the inner mitochondrial membrane of breast tumor cells. We confirmed the functionality of ZnT2 in breast tumor cells by reducing expression using small interfering RNA (siRNA) and evaluating changes in Zn distribution. Immunoblot assays verified the successful reduction of ZnT2 protein in total extracts as well as isolated mitoplasts. FluoZin-3 and
RhodZin-3, fluorescent dyes specific for vesicularized and mitochondrial Zn, respectively, were used to quantify subcellular Zn pools. In addition, cytosolic Zn levels were examined by the use of 4xMRE (metal responsive element) Zn biosensor. Our findings indicated that the attenuation of Zn transport by a reduction in ZnT2 expression resulted in the lowering of mitochondrial Zn levels, while cytosolic and vesicular levels were increased. The ability to lower ZnT2 expression in mitochondria and change Zn distribution verify our hypothesis of ZnT2 as mitochondrial Zn importer in breast tumor cells, similar to its role in normal cells.

Based on our localization of ZnT2 to the inner mitochondrial membrane in breast tumor cells, our data suggest that the low levels of mitochondrial Zn are a direct effect of reduced ZnT2 expression on the organelle. Without adequate ZnT2 to import Zn and maintain optimal Zn levels, breast tumor cells cannot maintain regular levels of mitochondrial Zn, resulting in the observed dysregulated low Zn concentrations in breast tumor cells. However, our interpretation is based upon the assumption that lower-than-normal detected ZnT2 expression in breast tumor cells is mirrored by lower ZnT2 protein expression on the inner mitochondrial membrane. It is possible that while breast cancer cells may have low ZnT2 abundance, expression on the mitochondria may still be normal, or even higher than normal. To validate our speculation, further studies must examine relative ZnT2 protein abundance on the inner mitochondrial membranes of breast tumor compared with normal mammary cells. Importantly, future detailed studies must assess the functionality of all ZnT proteins detected on mitochondrial membranes to ensure that the proteins expressed are capable of Zn transport. Such studies will provide
more detailed and informative information on the role of ZnT mediated Zn transport in mitochondria.

Although literature indicates that ZnT2 may be localized to vesicular membranes (24), vesicular Zn levels did not decrease from the attenuation of ZnT2 expression as was witnessed in the mitochondria. In fact as stated above, both cytosolic and vesicular Zn pools were increased. While it may be interpreted that ZnT2 is not localized to vesicular membranes, we speculate that the results suggest there are other transporters responsible for this occurrence. We believe it unlikely that ZnT2 is not localized to vesicular membranes due to strong evidence suggesting otherwise, particularly the role of ZnT2 in the secretion of Zn in normal mammary cell (24). Another transporter that may be involved in vesicular Zn import is ZnT4 which is found on vesicular membranes in mammary cells. Moreover, Zn exported from the mitochondria might be taken up by intracellular compartments we did not examine in this study such as the Golgi apparatus by the transporter ZnT7 (21). We interpret the increase in cytosolic Zn pools as a secondary effect of decreased mitochondrial Zn; less Zn transported into the mitochondria from the cytosol results in increased cytosolic Zn. Subsequently, increased cytosolic Zn may drive vesicular Zn transporters to upregulate Zn import to protect the cell from Zn cytotoxicity (43,44). Interestingly, recent studies suggest that vesicular compartments are metabolically active and alterations in Zn levels may affect biological processes. Studies on both Zip3 and Zip8 illustrate the biologically active role of vesicles, and the effects of Zn fluctuations upon them. Zip8 is expressed on lysosomal membranes in T cells. Upon T cell activation, Zip8 increases in abundance, decreasing vesicular Zn.
levels, suggesting T cell regulation through vesicular Zn regulation (46). Moreover, ZnT8 studies in pancreatic beta cells suggest vesicular Zn levels plays an important role in the modulation of various hormone secretions, such as insulin by the pancreas (47). Interesting and informative future studies might explore effects of elevated vesicular Zn levels in breast tumor cells compared with normal cells, and how modulating these levels affect various cellular function.

It is speculated that cells have a threshold for mitochondrial Zn concentrations, alterations above and below which will induce apoptosis. Breast cancer cells are able to survive in spite of low mitochondrial Zn levels (suggesting Zn levels are within the threshold); however, they may in turn be more susceptible to further, even minute, reductions in mitochondrial Zn reducing Zn levels beyond the threshold. Therefore, the next obvious question for our study asks, “Do endogenously low levels of mitochondrial Zn make breast tumor cells sensitive to further Zn reductions in from the mitochondria?” Considering the role of mitochondria in apoptosis, we approached this question by first decreasing mitochondrial Zn levels, and assessing changes in apoptosis. Since ZnT2 attenuation significantly decreases mitochondrial Zn levels, we used Annexin-V fluorescent dye as a detector for early apoptosis. Results indicated that the induction of apoptogenesis in breast tumor cells increased after the decrease of mitochondrial Zn by ZnT2-attenuation. Importantly, results from ZnT2-attenuation in normal mammary cells (data not shown) indicated ZnT2-attenuation does not induce cell death in normal mammary cells. Our findings suggest the dysregulated Zn metabolism of breast cancer cells, in particular low endogenous mitochondrial Zn levels, leads to marked increases in
cellular death when mitochondrial Zn levels are further compromised. In context of the apoptotic threshold, the attenuation of mitochondrial Zn import lowers mitochondrial Zn levels beyond the threshold, resulting in cell death. We speculate the observed results to be a cause of Zn depletion from the mitochondria, inhibiting vital functions such as energy production, cell proliferation, and genomic stability, all of which are essential roles of healthy mitochondria (48). Literature shows that Zn also has anti-oxidative effects in the mitochondria and actively reduces oxidative damage; Zn depletion may lead to overwhelming oxidative damage (48). By inhibiting Zn import through the attenuation of ZnT2, mitochondria do not have adequate Zn to support processes, whether through its antioxidant properties or role as cofactors (16), and resulting stress signals activate the apoptotic cascade.

An alternative interpretation of the mechanisms behind the induction of apoptosis may in fact lie in the discussed increase in cytosolic Zn. Numerous studies point high levels of cytosolic Zn accountable for increased incidence of apoptotic death (39,40). An interesting study hypothesizes a unique interaction between cytosolic Zn and the mitochondria that results in pore-formation, and the subsequent release of cytochrome c and caspase cascade activation (31), ultimately resulting in massive cell death. Our results indicate both decreased mitochondrial Zn levels and increased cytosolic Zn levels, and thus more studies are necessary to pinpoint the exact etiology behind the activation of apoptosis. Nevertheless, the ability to modulate Zn distribution and induce apoptosis in breast tumor cells is a novel and fascinating outcome in view of the anti-apoptotic nature of cancer cells.
A very important characteristic of cancer cells is their tendency to form tumors. Development of tumors in mammary tissue from the hyper-proliferation of cells resulting in localized over-population (2). By attenuating mitochondrial Zn import, we induced apoptosis in characteristically anti-apoptotic cells. The observed apoptotic cellular death of breast cancer cells begs the question, how does a decrease in mitochondrial Zn pools affect distinct cancer traits such as hyper-proliferation and tumor formation? We used trypan blue exclusion staining to evaluate the affects of ZnT2 attenuation on cell viability. Our results demonstrated that by decreasing mitochondrial Zn levels by ZnT2 attenuation, we significantly reduced the viability of breast cancer cells. In addition, our results showed that both proliferation and tumorigenicity—fundamental characteristics of cancer cells—were negatively affected by the attenuation of mitochondrial Zn import.

We speculate the attenuation of ZnT2 reduces the efficiency of mitochondria, compromising energy production and in turn, energy availability for cellular processes. In specific, vital ATP-dependent processes including DNA replication, repair, and recombination are severely hindered without energy (49). As a result, the process of proliferation, a heavily energy-dependent procedure, is quickly scaled down. Traditionally hyper-proliferative breast cancer cells are no longer able to divide and multiply at previous rates. Moreover, we speculate the production of non-essential proteins, such as the cell-surface adhesion molecules, may be reduced when energy availability is compromised, resulting in the observed decrease in tumorigenicity. ALCAM (activated leukocyte cell adhesion molecule) and E-cadherin are cell-surface adhesion molecule that play major roles in the maintenance of interactions between
epithelial cells. Reduced expression or suppression of these adhesion molecules in breast cancer is associated with migration away from the primary tumor (50,51). It should be noted that many studies correlate reduced cell-surface adhesion molecules with metastasis of breast tumor cells and increased aggression of the cancer. However, since our results exhibit apoptosis and reduced cellular proliferation in combination with decreased tumorigenicity, we speculate metastasis to not be the case. We interpret our data to suggest that decreased tumor formation is a result of both a reduction in cell-surface adhesion molecules as well as a secondary effect of a reduced proliferation rate. The ability to decrease the prevalence of two characteristic breast cancer traits holds a promising future for further research in breast cancer.

In summary, findings from this study provide insight into the dysregulated Zn metabolism found in breast cancer. Not only do our findings validate previous research identifying elevated Zn levels in cancer cells, but our present study also identified key alterations in Zn distribution patterns of vesicular and mitochondrial Zn pools. Importantly, we show that cancer cells display low endogenous mitochondrial Zn levels at a concentration significantly lower than that of normal mammary cells. Furthermore, mitochondrial Zn levels may be modulated by the attenuation of the Zn transporter, ZnT2, resulting in lowered Zn levels and the subsequent activation of apoptosis. The attenuation of ZnT2 also reduces the proliferation and tumorigenicity rates of breast cancer cells. As a result, we believe our study illustrates the potential for ZnT2 as a new and innovative candidate for the targeted treatment of breast cancer. Our future studies on
ZnT2 will focus on its therapeutic possibilities and other future implications in relation to breast cancer.
Future Implications
The findings from this study suggest ZnT2 may hold a promising future in breast cancer treatment. Currently, there are four primary methods of treatment used by breast cancer patients, however a treatment that is both safe, effective, and with little or no side effects has yet to be discovered.

Surgery is the most commonly used method to treat breast cancer (2). Surgeons use invasive measures to physically remove tumors from the breast. However, the major setback of surgery for cancer treatment is that it rarely removes all cancerous cells from the breast; cells that have migrated away from the primary tumor into the rest of the breast tissue or nearby lymph nodes cannot be reached unless a full mastectomy is performed, along with the removal of surrounding lymph nodes (2). As a result, many women undergo adjuvant therapies in combination with surgery to target isolated cancer cells, particularly if doctors suspect the cancer has metastasized (1). Moreover, the process of surgery itself is dangerous and recovery may last for extended periods of time.

Radiation therapy utilizes high-energy radiation to target cancerous cells and inhibit or destroy them within the breast (2). While radiation therapy is generally less invasive than surgery, it faces the same problems in efficiency. In particular, it is difficult to target cells within a dense tumor with radiation rays, allowing tumors to proliferate and grow from the untreated centers. Also, radiation therapy is ineffective if the cancer has metastasized beyond the breast since radiation treatment is normally targeted to the tumor containing tissue (1,2).
Unlike surgery and radiation therapy, hormone therapy is a whole-body treatment. Hormone therapies utilized for breast cancer often modulate the availability of vital hormones, such as estrogen and progesterone (10). While tamoxifen is the most commonly used hormone treatment for breast cancer, it is neither breast nor cancer specific and will act upon any cells in the body with cell-surface estrogen receptors (11). Consequently, hormone therapies are generally accompanied with a high frequency of side-effects. Moreover, studies show that women show increased immunity to the effects of hormone therapy drugs after several treatments, decreasing drug efficiency (12).

Similarly, chemotherapy for breast cancer patients is also useful for when breast cancers are highly aggressive or have metastasized. Intravenously injected drugs move through the body through the bloodstream and are specialized for targeting and destroying rapidly dividing cells (2). While chemotherapy is successful as destroying cancer cells that are characteristically hyper-proliferative, treatments also have adverse effects on healthy tissues that are composed of naturally rapid-dividing cells, such as those of the small intestine and hair follicles. As a result, breast cancer patients undergoing chemotherapy generally experience severe weakening of the entire body (2).

We believe the results from this study in combination with previous and ongoing studies on ZnT2 and breast cancer indicate that ZnT2 is a good potential candidate for the targeted therapy of breast cancer. An optimal targeted therapy for breast cancer will not only be highly effective, but also only negatively influence cancer cells (13). We believe our data suggests a role for ZnT2 that satisfies both criteria.
Firstly and most importantly, unlike most Zn transporter proteins that are ubiquitous in tissues, we know ZnT2 protein expression is restricted to tissues that have high Zn trafficking patterns. In particular, the breast, prostate, and pancreas all exhibit expression of ZnT2, and are all known to have high Zn requirements (19). The unique localization of ZnT2 presents a tissue-specific target; for breast cancer, meaning that treatment can be targeted almost exclusively to breast tissue. Secondly, our data indicates the deleterious effects of attenuation of ZnT2 is only exhibited in malignant cells. Subject to the same attenuation procedure, normal healthy breast cells did not experience a decrease in viability. It is the dysregulated Zn metabolism in breast tumor cells, characterized by the hyper-accumulation of Zn, compared with normal breast cells that explains this phenomenon; the abnormal Zn distribution in breast tumor cells (particularly low endogenous mitochondrial Zn levels) lead to cell death when mitochondrial Zn import is inhibited. Normal breast cells do not display low endogenous mitochondrial Zn levels, and thus are able to adapt to a slight decrease of mitochondrial Zn levels. As a result, we believe the utilization of ZnT2 will allow for the therapeutic treatment of exclusively malignant breast cells, leaving all other cells of the body and healthy breast cells unaffected. A tissue and malignancy specific model is a novel and innovative new approach to cancer therapy and may reinvent breast cancer treatment as a whole.

Importantly, developing mechanism of action and delivery system is a key consideration in developing a therapeutic treatment. In our near-future studies, we will conduct a large chemical screen in order to identify small molecule compounds (SMCs) that function to
attenuate the expression of ZnT2 and induce apoptosis and cause the death of breast tumor cells similar to our use of siRNA in our study model. Using SMCs is a more cost-effective approach than the use of siRNA, and the identification of one or more compounds will allow for the optimization of treatment. Identified SMCs will be tested against a variety of breast cancer strains to evaluate the efficiency of the treatment against cancers with different characteristics, including tumor, non-tumor, invasive, and non-invasive strains. In the future, we hope to take our in vitro studies in vivo. Our proposed method of delivery involves the use of calcium phosphate nanoparticles. These non-toxic particles are inert within the body and able to encapsulate SMCs of choice. Once administered into the body, the calcium phosphate nanoparticles will gradually release the contents. We speculate the effect of the drug will only be witnessed in malignant breast tumor cells since those cells are the only ones that respond adversely to the attenuation of ZnT2. As a result of treatment, breast cancer cells will display decreased proliferation, tumorigenicity, and will undergo apoptosis ultimately resulting in the destruction of the cancer.

Breast cancer affects more than 200,000 women every year, and numbers are not going down (1). While current treatments have increased the chances of survival, they are plagued with inefficiency and unavoidable side-effects. Our novel approach hopes to bypass these side-effects through an effective means of destroying breast cancer cells, regardless of progression or metastasis status. We believe results from our current study provide a promising future for ZnT2 as a therapeutic target for breast cancer. In our
future studies we hope expand our knowledge of ZnT2 and breast cancer etiology, in the hopes of uncovering a cure for this deadly disease.
Figures and Legends
Figure I: Zinc concentration in tumor cells is higher than in non-tumor cells.

Zn concentrations are higher in human breast tumor cells (T47D) compared with mouse normal mammary cells (HC11) and normal human breast cells (HME). Zn concentration was measured using ICP-AA. Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. Results show a significantly higher (p<0.05) Zn concentration in breast tumor cells (T47D, n=9) compared with normal mouse cells (HC11, n=5). Furthermore, breast tumor cells (T47D) have a significantly higher (p<0.05) Zn concentration than normal human cells (HME, n=8). Data represent mean Zn concentrations as µg Zn/mg protein ± SD with sample size (n) as indicated.
Figure II: Zinc concentration in tumor cell vesicles is higher than non-tumor cell vesicles (A). Zinc concentration in tumor cell mitochondria is lower than in non-tumor cell mitochondria (B).

(A) Zn concentration in vesicles of breast tumor cells (T47D) is significantly higher (p<0.05) compared with normal mammary cells (HC11). Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. FluoZin-3 dye (specific for labile vesicularized Zn) was used to assess endogenous labile Zn pools in breast tumor cells (T47D, n=6) and normal mammary cells (HC11, n=5). Data represent mean fluorescence (arbitrary units)/µg protein ± SD, with sample size (n) as indicated.

(B) Zn concentration in mitoplasts isolated from breast tumor cells (T47D, n=2) is significantly lower (p<0.05) than in mitoplasts isolated from normal mammary cells (HC1, n=5). Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. Mitoplasts were isolated and rigorously digested in proteinase K to eliminate residual protein. Zn levels were analyzed by ICP-AA. Data represent mean Zn concentrations as µg Zn/mg protein ± SD, with sample size (n) as indicated.
Figure III: ZnT2 expression is lower in breast tumor cells compared with normal mammary cells.

Expression of ZnT2 in breast tumor cells (T47D) is lower compared with normal mammary cells (HC11). Representative immunoblot of total membrane fractions (25µg protein/well) probed for ZnT2 using ZnT2 antibody (1:1000) and detected with anti-rabbit IgG-HRP (1:30,000). The membrane was stripped and reprobed for B-actin (1:30,000) and detected with anti-mouse IgG-HRP (1:30,000) to control for protein loading.
Figure IV: ZnT2 is localized to the mitochondria in breast tumor cells.

ZnT2 is found on the inner membrane of the mitochondria in human tumor cells (T47D). Representative immunoblot of ZnT2 total membrane fractions and human tumor cell (T47D) mitoplast fractions probed for ZnT2 using ZnT2 antibody (1:1000) and detected with anti-rabbit IgG-HRP (1:30,000).
Figure V: Attenuation of ZnT2 reduces ZnT2 protein abundance in total extracts at 12 hours (A), 48 hours (B), and in mitochondria (C).

(A, B) Attenuation of ZnT2 using h-ZnT2 siRNA (200pmol) for 12 hours (A) or 48 hours (B) reduce ZnT2 expression in total membrane extracts of breast tumor cells (T47D). Representative immunoblot of ZnT2 in crude membrane and ZnT2-attenuated fractions, probed using ZnT2 antibody (1:1000) and detected with anti-rabbit IgG-HRP. Blot was stripped and reprobed for B-actin with B-actin antibody (1:1000) and detected with anti-mouse IgG-HRP (1:30,000) to control for protein loading.

(C) Attenuation of ZnT2 reduces protein expression in the mitochondria. Representative immunoblot of ZnT2 in isolated mitoplasts for ZnT2-expressive and ZnT2-attenuated fractions. Blot was stripped and reprobed for COX IV with antibody (1:1000) and detected using anti-mouse IgG-HRP (1:30,000) to control for protein loading.
Figure VI: Attenuation of ZnT2 reduces mitochondria Zn pools (A) but increases cytoplasmic (B) and vesicular pools (C) in breast tumor cells.

(A) Attenuation of ZnT2 significantly reduces (p<0.05) mitochondrial Zn pools within breast tumor cells (T47D). Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. ZnT2-attenuated cells (n=6) displayed lower Zn levels compared with ZnT2-expressive cells (n=7). Representative image of labile Zn in the mitochondria assessed by fluorometric analysis detected with RhodZin-3 dye (specific for Zn in the mitochondria). ZnT2 attenuated cells were treated with siRNA (200pmol) for 48 hours. Data represent mean fluorescence (arbitrary units)/µg protein ± SD, with sample size (n) as indicated.

(B) Attenuation of ZnT2 in breast tumor cells (T47D) shows a strong increase (p=0.078) in cytoplasmic Zn compared with ZnT2-expressive cells after a 48 hour period. A strong trend is demonstrated at p<0.10 using Student’s t-test and signified by “^” symbol. Zn in the cytoplasm measured using 4xMRE transcript with attached fluorescence tag that binds cytoplasmic metallothionein. Luciferase fluorescence measurements represent mean luminescence (arbitrary units)/µg protein ± SD, sample size=6 wells/genotype.

(C) Attenuation of ZnT2 significantly increases (p<0.05) vesicular Zn pools within breast tumor cells (T47D). Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. ZnT2-attenuated T47D cells (n=8) display increased vesicularized Zn compared with the ZnT2-expressive cells (n=8). Representative image of labile Zn in vesicles assessed by fluorometric analysis using FluoZin-3 dye (specific for vesicularized Zn). ZnT2 attenuated cells were treated with siRNA (200pmol) for 48 hours. Data represent mean fluorescence (arbitrary units)/µg protein ± SD, with sample size (n) as indicated.
ZnT2 attenuation results in the apoptotic death of breast tumor cells (T47D). Representative figure of laser confocal microscopy images of Annexin-V stained T47D cells. ZnT2 attenuated cells were treated with siRNA (200pm) 20 hours prior to imaging. Annexin-V stain (green fluorescence) binds to cells in the preliminary stages of apoptosis. Annexin-V staining done with sample size=3/genotype.
Figure VIII. Attenuation of ZnT2 reduces cellular viability in breast tumor cells.

Attenuation of ZnT2 in breast tumor cells (T47D) significantly reduced (p<0.05) cell viability. Significance demonstrated at p<0.05 using Student’s t-test and signified by “*” symbol. Cells were plated (5x10^6 cells/well) and treated with siRNA (200pmol) for 48 hours. Data was obtained through trypan blue exclusion method using trypan blue dye which incorporates solely into non-viable cells. Counts (5/well) of viable and non-viable cells were taken and data converted to percent of viable cells out of total cells.
Attenuation of ZnT2 in breast tumor cells (T47D) results in decreased cellular proliferation and tumorigenicity. Cells were treated with siRNA (200pmol) and embedded into a soft agar matrix and given antibiotic-free medium daily. Cell counts (10 planes/well) were taken for total cells and colonies (defined as a cluster of 3 or more cells) 1 and 5 days after treatment. Data from day 5 represented as percent change from day 1, with sample size=3/genotype.
References


EDUCATION
Aug 2006 – May 2010: Penn State University, University Park, PA
• Major: Science BS, Life Science Option, The Eberly College of Science
• Minors: Nutritional Sciences, History

RECOGNITIONS
The Schreyer Honors College student scholar, Penn State University
• Penn State University honors program with 4-year scholarship
• Senior thesis requirement, “Attenuation of mitochondrial Zn import through ZnT2 in tumorigenic breast cancer cells results in apoptosis.”

1st Place in Health and Life Sciences, Penn State Undergraduate Exhibition 2009
• Annual university-wide competition showcasing novel undergraduate research

Summer Discovery Grant recipient 2008
• Grant awarded to conduct original independent research

Ruth L. Pike Scholarship recipient 2008 – 2009
• Awarded to students displaying academic excellence and potential in scientific research

Travel Grant Award recipient, Spring 2009
• Grant awarded by Dept. of Undergraduate Education, Schreyer Honors College, College of Health and Human development, Dept. of Nutritional Sciences

Dean’s List student, all semesters
• Academic excellence recognition for obtaining semester GPA of >3.5

EXPERIENCE
Undergraduate Research Assistant, Kelleher Laboratory, Penn State University
• Aug 2007 – Dec 2009, conducting laboratory research on breast cancer and zinc
• Comfortable with range of techniques including immunoblotting, fluorometry, cell culture, PCR, confocal imagery

Pharmacy Technician, Rite Aid, State College, PA
• July 2009 – present, working along side a certified pharmacist at a retail pharmacy to dispense and distribute medications to patients

Teaching Assistant, Penn State University
• Aug 2008 – Dec 2008, courses include Nutrition 100 and 251
• Helped students with coursework and assisted in grading assignments

Intern, Pennsylvania Higher Education Assistance Agency (PHEAA)
• Jan 2007 – Aug 2007, human resources and customer service internship

OTHER
State College Area School District Summer ESL program volunteer, Jun-Jul 2009
• Worked alongside a teacher to help ESL students with their reading and writing abilities

Experimental Biology 2009 conference, New Orleans, LA
• Presented poster of original research for the American Society of Nutrition (ASN) and American Physiological Society (APS)