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Metallothionein gene expression in human breast cancer

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Metallothionein Gene Expression in Human Breast Cancer

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Dissertation submitted to the Davis
College of Agriculture, Forestry and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in
Genetics and Developmental Biology

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ABSTRACT

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Metallothioneins (MT) are a family of low molecular weight (6 kDa) cysteine rich proteins that participate in a variety of functions such as detoxification of heavy metals and homeostasis of essential metals. They can also act as scavengers of free radicals. MT-1 and 2 isoforms are ubiquitously expressed, whereas the expression of the third isoform is limited to the neural tissue. In the PC-3 prostate cancer cell line, MT-3 expression has been shown to inhibit cell growth and increase drug resistance. The goal of the present study was to determine if MT-3 overexpression would influence the growth of human breast cancer cell lines. To determine this, the coding sequence of the MT-3 gene was stably transfected into 2 estrogen receptor positive (MCF-7 and T-47D) and 2 estrogen receptor negative cell lines (Hs578T and MDA-MB-231) having no basal expression of MT-3. Cell growth was determined by counting DAPI-stained nuclei, cadmium resistance by the colony formation assay, MT mRNA expression by RT-PCR, and MT protein by immunoblot. It was demonstrated that MCF-7 and Hs578T cells that overexpress the MT-3 gene are growth inhibited compared to untransfected cells. In contrast, T-47D and MDA-MB-231 cells that overexpress MT-3 were not growth inhibited. Stable transfection of the MT-1E gene had no effect on the growth of any of the 4 cell lines. It was also demonstrated that the overexpression of both MT-3 and MT-1E only increased the resistance of MCF-7 cells to Cd^{+2} . In all instances, stable transfection of the MT-3 or MT-1E gene had no effect on the expression of the other MT isoforms. The study shows that MT-3 can influence the growth of some breast cancer cell lines.

DEDICATIONS

I would like to dedicate this work in appreciation to the many excellent teachers that I have had throughout the course of my education. I was blessed to have the greatest family one could ever have. Thanks to my family for being on my side when I need them. I especially want to express my thankfulness to the great people who I have encountered with and become truly friends with. What would my life be without your support? I love you all, “Aaron Lee Smith, Amy Canadee, Barbaros A. Erdoğan, Brian Keith Williams, Chad Aaron Thorpe, Gina Dawn Spadafore Thorpe, Michael King, Mujgân Özkan, Nolan Long, Nurten Beyaz, Paula Lee, Rodney Perry and Stephen Thomas”. Thank you friends. I know you care.

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INTRODUCTION AND REVIEW OF LITERATURE

Breast cancer currently accounts for 20% of all female cancers worldwide and is the most frequent malignancy occurring in women. In Western Europe and North America, one in every eight or nine women will develop the disease. Inheritance of high penetrance susceptibility genes, such as BRCA1 and BRCA2, account for only 5% of breast cancer cases and factors responsible for the other 95% remain obscure [1]. A significant proportion of sporadic breast cancers contain point mutations clustered within exons 5-8 of the P53 gene, and this mutational spectrum suggests the occurrence of xenobiotic-induced mutagenic events in some cases [2].

The only environmental exposure proven to induce breast cancer is ionizing radiation [3]. Total cumulative exposure to estrogen may play a role in breast cancer incidence and is consistent with nulliparity, late age at first pregnancy, early menarch and late menopause as weak factors [4]. However, estrogens probably act as tumor and growth promoters rather than as complete carcinogens but this is not a view that is universally accepted [5]. A correlation between alcohol intake and breast cancer incidence amongst individuals lacking both the glutathionein S-transferase (GST)M1 and GSTT1 genes has been reported [6]. However, different patterns of alcohol intake make it difficult to quantify the importance of this risk factor [7]. In addition, it is also possible that consumption of well-done meats may correlate with an increased risk of breast cancer [8]. So far, classic epidemiological studies have failed to highlight a dominant risk factor that could account for sporadic breast cancer incidence and investigations into the relationship of risk to gene-environment interactions are still in their infancy [9]

There is a sufficient, although not extensive, literature base to support an examination of the possible relationship between Cd^{+2} and metallothionein protein in human breast cancer. A study

from Finland that defines an association between human breast concentrations of Cd^{+2} and breast cancer is very supportive that Cd^{+2} can have a role in the development and progression of breast cancer [10]. There are also several reports that indicate that Cd^{+2} can act like an environmental estrogen. The first of these demonstrated that Cd^{+2} increased the growth rate of MCF-7 cells 5.6 fold but was without effect on MDA-MB-231 cells [11]. The overall conclusion of this study was that Cd^{+2} mimicked the effect of estradiol in the estrogen-responsive MCF-7 cells. In addition to the effects on MCF-7 cell growth, treatment with Cd^{+2} resulted in an increase in the steady state levels of progesterone receptor, pS2, and cathepsin D, and a decrease in the steady state level of estrogen receptor α (ER- α). The changes in the steady state levels of mRNA and protein of these genes were due to changes in transcription as they were blocked by the antiestrogen, ICI-164,384. Transfection assays also demonstrated that the effects of Cd^{+2} were mediated by ER- α . These studies were extended to probe the mechanism by which Cd^{+2} activated ER- α [12]. The result of this study demonstrated that low concentrations of Cd^{+2} activated ER- α through an interaction with the hormone binding domain of the receptor. The interaction of Cd^{+2} with the receptor was shown to involve several amino acids into the hormone binding domain and thereby activate the receptor. It is important to note that in neither study were effects of Cd^{+2} influenced or mimicked by Zn^{+2} .

Since it is highly likely that intracellular bioavailability of Cd^{+2} to ER- α is regulated by the breast MT pool, one can hypothesize that expression of MT may be a major intracellular regulatory determinant of Cd^{+2} stimulation of the ER- α . With the above in mind, it is surprising that the relationship between MT and Cd^{+2} has not been explored in breast cancer.

Metallothioneins:

Metallothioneins (MTs) were first discovered from horse kidney by Morgoshes & Vallee in 1957 and was subsequently purified and characterized by Kägi and Vallee [13]. This discovery marked a field of research focused on the study of a low molecular weight polypeptide superfamily, the metallothioneins. MTs are cysteine rich, low molecular weight (6-7 kDa), nonenzymatic, intracellular proteins ubiquitous in all eukaryotes (often encoded multiple copy genes) as well as some prokaryotes [14, 15]. In the mouse, there are four MT genes (MT-1, 2, 3 and 4) that reside in a 50 kb region on chromosome 8, whereas in the humans in addition to the four genes, numerous isoforms of MT-1 are clustered on chromosome 16q13 (Figure I) [16, 17]. Human MT proteins are encoded by 10 genes: MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3 and MT-4. In addition to these ten functional isoforms, there are seven non-functional isoforms: MT-1C, MT-1D, MT-1I, MT-1J, MT-1K, MT-1L and MT-2B [18]. The MT-1 and MT-2 isoforms, which differ by only a single negative charge, are the most widely expressed isoforms in different tissues and are the best studied (Figure I.)

Figure 1. Location of human metallothionein isoforms with respect to each other on chromosome 8.

Structure:

MT harbors high metal content. Mammalian MTs contain 20 cysteine residues, which are central to the binding of metals. The stoichiometry is such that there are 7 bivalent ions for every 20 cysteines, which form metal thiolate complexes, therefore enabling the MT to bind between 7-10 atoms of metal/mol MT in a two domain structure [18]. The protein has the shape of a dumbbell and envelops the metals that it contains in two separate domains in a manner that effectively shields them from the environment. It is most remarkable that the metals are arranged in a cluster structure unique to biology. In one cluster (N-terminal α -domain), four metal atoms are bound to 11 cysteines, five of which bridge the metals; the other (C-terminal β -domain) has three metal atoms and nine cysteines with three bridges (Figure II) [19]. Zinc is bound extremely tightly (K_D about 10^{-13} M).

Figure 2. Schematic drawing of zinc thiolate clusters in MT based on both x-ray diffraction and NMR spectroscopy data. (Adopted from Edmont H. Fischer and Earl W. Davie, Recent excitement regarding metallothionein, Proc. Natl. Acad. Sci. USA, Vol. 95, pp 3334, 1998.)

Intramolecular metal linkages can stabilize the MT protein secondary structure, therefore, loss of metal can causes structural changes, rendering the polypeptide chain vulnerable to proteolysis [20]. Stability is also influenced by the nature of the metals bound to MT. Predominantly, Zn^{+2} , but sometimes also Cu^{+2} are bound *in vivo* under physiological conditions. However, several less abundant transition metals, such as Cd^{+2} , Bi^{+3} , Pt^{+2} , Ag^{+} and Hg^{+2} also bind eagerly to MT *in vitro*. The binding affinity of different metals for MT varies considerably and has the following order: $\text{Zn}^{+2} < \text{Pb}^{+2} < \text{Cd}^{+2} < \text{Cu}^{+} < \text{Ag}^{+} = \text{Hg}^{+2} = \text{Bi}^{+3}$, therefore making zinc readily displaceable by other metal ions [21].

Function:

Despite the accumulation of detailed information on both the biochemical and molecular aspects of MT structure and expression, its biological role is still not clearly understood more than 40 years after its discovery. The fact that there are multiple copies of MT genes expressed in distinct patterns, and the relatively rapid turnover of the protein suggest that they should have important functions. One of the proposed functions of MT is that MTs play important role in maintaining essential metal homeostasis. Zn^{+2} is a physiologically important metal and the most abundant metal bound to constitutive MT. Zn^{+2} provides essential structural and catalytic functions to a variety of proteins. Zn^{+2} is also crucial in the regulation of gene expression because numerous

transcription factors have “zinc finger motifs” that are maintained by Zn^{+2} . Apo-MT (metallothionein with no metals bound) is a Zn^{+2} acceptor because of the abundance of free sulfhydryl groups and their high affinity for Zn^{+2} . However, the sulfhydryl groups are highly reactive, and Zn^{+2} , although bound with high affinity, can undergo exchange reactions, which allows Zn^{+2} to be transferred from MT to other proteins [22, 23, 24, 25]. The affinity of sulfhydryl groups for Zn^{+2} can also make MT an efficient metal ion scavenger. This implies a possible regulatory role of MT in the activation or inactivation of various molecular effectors. Such a possibility was demonstrated by showing that apo-MT can chelate Zn^{+2} out of the transcription factor IIIA (TFIIIA), a process that inactivates TFIIIA [26]. Therefore it is tempting to speculate that MT might be essential for Zn^{+2} homeostasis by regulating Zn^{+2} absorption, or as a donor of Zn^{+2} to various enzymes and transcription factors during development or protein synthesis.

There is strong evidence that MTs play an important role in protection against metal toxicity. In unicellular eukaryotes, MTs bind copper predominantly [14, 15]. Mutations that prevent MT synthesis confer copper sensitivity, whereas excess expression of MTs confers resistance to copper toxicity [19, 27]. In mice MT is an important protein in the cellular defense against Cd^{+2} toxicity and lethality, but it provides much less protection against the lethality of the other metals such as, Zn^{+2} , Cu^{+} , Fe, Pb^{+2} , Hg^{+2} and As [27]. Cadmium is a ubiquitous and insidious pollutant. A by-product of zinc production by humans and a component of volcanic eruption by nature, the element is chiefly used in the industrial plating process and can be found in products as diverse as solder, artists' pigments, and rechargeable batteries. It is even used to absorb neutrons in the control rods and shielding of nuclear reactors. Depending on the dose, route and duration of

exposure, Cd^{+2} can cause damage to various organs including the lung, liver, kidney, bones, testis and placenta [28, 29, 30, 31, 32, 33]. Acute exposure to Cd^{+2} produces hepatic, pulmonary, and testicular injury, whereas chronic exposure results in renal and bone injury and cancer [34]. The Agency for Toxic Substances and Disease Registry (ATSDR) currently ranks Cd^{+2} 7th on its priority list of hazardous substances [35]. Moreover, various mammalian cell lines that can not synthesize any MT are sensitive to cadmium toxicity whereas mice and the cells that overexpress MT are resistant to Cd^{+2} [36, 37]. In fact, selection for cadmium resistance with mammalian cells results in up to 80-fold amplification of the entire MT-locus [38]. The role of MT in Cd^{+2} disposition has been examined in MT-transgenic mice. In this model, MT does not inhibit intestinal Cd^{2+} absorption, nor does it affect initial Cd^{2+} distribution to various tissues [39, 40]. However, MT decreases Cd^{+2} elimination through the bile and is a major factor for tissue retention of Cd^{+2} [40, 41, 42]. The results of a number of studies with humans environmentally exposed to Cd^{2+} demonstrated that proteinuria is the main renal injury in these subjects [43]. Because Cd^{+2} injures proximal tubules of kidney, an increased excretion of protein into urine is observed.

MTs are cysteine-rich molecules. Therefore, it is reasonable to expect that sulfhydryl-rich MTs may function in a manner similar to GSH, wherein MT provides an intracellular nucleophilic sink to “trap” electrophiles, alkalyting agents, and free radicals [44, 45, 46]. The multiple cysteine residues of MT can be oxidized during oxidative stress, and the subsequent release of Zn^{+2} has been proposed to be important in protecting against oxidative damage [47, 48]. However, it has been difficult to demonstrate oxidation of MT *in vivo*. Therefore, there have been controversial reports on the role of MT during oxidative stress.

Gene Regulation:

A variety of stimuli such as metals, hormones, cytokines, a range of other chemicals, inflammation and stress can induce metallothioneins. Some of these stimuli include metals, hormones, cytokines, a range of other chemicals, inflammation, and stress [49]. The most extensively studied of these inducers are metals and glucocorticoids, both being efficient inducers of MT. However, both of these entities display species differences in isoform induction. In mice, both metals and glucocorticoids, equally induce MT-1 and MT-2; in man, metals induce all the MT isoforms whereas glucocorticoids only induce MT-2A and MT-1E [50, 51]. Thus, human MT isoforms are regulated independently of each other whereas in mouse the MT-1 and MT-2 isoforms are co-ordinately regulated. [49, 52]. With the exception of glucocorticoids only metals have been shown thus far to be capable of inducing the human MT-1 isoforms. This demonstrates the apparent simplicity of the human MT-1 promoter region compared with that of MT-2A, which contains several enhancer regions [51]. Moreover, the induction of different isoforms appears to be metal dependent as are the rates, extent and time course of MT mRNA transcript accumulation [52, 53]. Considerable progress has been made in understanding the mechanism by which metals induce MT and the way in which expression of this protein is regulated, even though the regulation of protein expression not yet fully understood [54].

Exposure to heavy metals leads to a significant MT synthesis and this synthesis is thought to be mediated via *cis*-acting DNA sequences or metal responsive elements (MREs) which are present as multiple copies in the promoter region of all the MT genes [55, 56]. These *cis*-acting regions are conserved among many diverse organisms but are not all functionally equivalent [57]. Moreover, variations in the ability of different MREs to mediate metal-activated transcription of

MT genes have been reported [58]. For instance, of the five MREs in the mouse MT-1 promoter, MREd appears to be the strongest enhancer of MT gene transcription [59]. In addition to MREs, glucocorticoid response element (GRE), antioxidant response element (ARE), cyclic AMP responsive elements, TPA-responsive elements and interferon responsive elements have also been defined in a few MT promoters.[60, 61, 62].

Proteins which are thought to be positively acting transcription factors bind with MREs in a metal-dependent manner during metal induction of MT [63]. Following metal exposure, various different proteins from nuclear extracts of cells of both rodent and human origin have been identified as possible regulators of metal mediated gene transcription [60]. These include metal response element binding factor-I (MBF-I), zinc activated protein (ZAP), and zinc regulated factor (ZiRF1). These proteins have different binding affinities for the various MREs. Also, variability in binding activity with different metals has been reported. It is not clear if the binding of these proteins is metal specific [64].

Two proteins identified from nuclear extracts of HeLa cells do affect metal-mediated MT gene transcription. These are MREBP (MRE binding protein) that specifically binds MREs of the human MT-2A gene and MTF-1 (MRE binding transcription factor), constitutively active zinc-sensitive factor. MREBP is thought to inhibit transcription, whereas MTF-1 is thought to have an important role in the control of MT gene expression [56, 61, 65, 66].

Genes encoding human MTF-1 (hMTF-1) and the mouse MTF-1 (mMTF-1) have been cloned. hMTF-1 is a 753 amino acid protein that shares a 93% amino acid sequence identity with

mMTF-1, although it is 78 amino acid longer at the C terminus than MTF-1 [67]. Each contains six zinc fingers of the cys2-his2 type in the DNA binding domain and three different trans-activation domains. The activity of MTF-1 is controlled via complex interdomain interactions and by the first zinc finger, which is saturated with zinc only at higher concentrations than the other zinc finger [68]. Therefore, inactivated MTF-1 with zinc-saturated fingers 2 to 6 can be activated by physiological increases in zinc [69]. Since neither MT-1 nor MT-2 gene were detectable in MTF-1 null cells even after metal exposure, this factor is essential for both constitutive and metal induced MT expression [70].

MTF-1 is mainly activated by zinc. Surprisingly there is evidence suggesting that oxidative stress also activates this transcription factor, resulting in increased binding to MREs [61, 71]. It is possible that oxidation of cellular ligands binding zinc may release metal to activate MTF-1. There is also evidence that hypoxia activates MT gene expression through MREs and that this activation involves MTF-1 [72].

In resting cells, most MTF-1 localizes to cytoplasm from which it is translocated to the nucleus under several different stress situations [73, 74]. The finding that MTF-1 requires elevated concentrations of zinc for strong binding to DNA suggests that MTF-1 is activated by allosteric regulation of DNA binding via binding of metals to the transcription factor itself [65, 70, 75]. Although heavy metals readily induce metallothionein gene transcription in cultured cells, none of them can substitute for zinc in cell-free DNA binding reaction of MTF-1 [70]. The most likely scenario is the replacement of zinc by other heavy metals in cellular and/or extracellular zinc storage proteins, leading to concomitant activation of MTF-1 by the released zinc. In addition,

MTF-1 can be phosphorylated upon metal induction, as a result of the activation of a complex kinase signaling transduction pathway which includes protein kinase C (PKC), phosphoinositol-3 kinase (PI3K), c-Jun N-terminal kinase (JNK) and a tyrosine-specific kinase [76]. (Figure 3.)

Figure 3. Schematic overview of known and inferred activation pathways and downstream functions of the metal transcription factor MTF-1. (Adopted from Peter Lichtlen, Walter Schaffner, The “metal transcription factor” MTF-1: Biological facts and medical implications. Swiss Med Wkly 131: 649, 2001.)

The accumulation of the MT 1/2 protein as detected via immunohistochemistry has different prognostic significance in various human tumors [77]. In tumors such as colonic and bladder cancers, MT 1/2 overexpression is frequently associated with well differentiated and lower histological grade tumors. Whereas in tumors such as ductal breast cancer, cervical carcinoma, endometrial carcinoma and pancreatic carcinoma, MT overexpression appears to be predominantly associated with more aggressive and higher-grade tumors [78, 79]. The expression of MT was also analyzed in normal breast tissue and in variety of benign, pre-invasive, and malignant breast lesions. Normal breast tissue did not stain for MT [80]. The available information on the character and consequences of MT overexpression associated with human cancer is presently too limited to offer complete understanding.

MT protein has been shown to be a useful prognostic and diagnostic marker in a variety of human cancers. First and second isoform of MT protein has been documented to be overexpressed in a sub-set of human breast cancers and that overexpression correlates to poor prognosis. There is a strong evidence that overexpression of metallothionein isoform 3 (MT-3) protein correlates to poor disease outcome in subset of human breast cancers [81]. The goal of this project is to define the role of MT-1/2 and MT-3 protein in cancer progression using a cell culture model and strengthen the potential use of these proteins in diagnosis and prognosis of breast cancer.

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MATERIALS AND METHODS

In this chapter, author will present the materials and methods used in this project. All the cell lines utilized in experiments obtained from American Type of Culture collection. The breast cell lines (MCF-7, MCF-10, T-47D, Hs578T and MDA-MB-231) utilized in the experiments have been used in similar type of applications commonly. Among the five breast cell lines, MCF-10A represent the normal immortalized breast cell line. Rest of the cell lines are well differentiated malignant breast cancer cell lines. Gene expression of Metallothioneins and protein levels were assessed utilizing Reverse Transcriptase Polymerase chain reaction and immunoblot analysis (dot blot). Growth studies were initiated following a 1:10 subculture and cell counts were determined by the automated counting of DAPI- stained nuclei. MT-3 and MT-1E coding sequence were cloned from cultured human proximal tubule cells for the transfection studies.

Cell Culture of MCF-7, T-47D, Hs578-T, MDA-MB-231 and MCF-10A Cell Lines:

The MCF-7, T-47D, Hs578-T, MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD), and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 5 mg/ml glucose and routinely passaged at a 1:10 ratio upon attaining confluence. The MCF-10A cell line was also obtained from the American Type Culture Collection, maintained in 1:1 mixture of Ham's F-12 medium and DMEM supplemented with 5% (v/v) fetal calf serum, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 0.1 µg/ml cholera toxin and routinely passaged at a 1:10 ratio upon attaining confluence. The cells were kept in T75 flasks at a constant temperature of 37⁰ C in a 5% CO₂ : 95% atmosphere. Cells were fed every three days and confluent cultures were sub-

cultured weekly. The medium content of the cells was always replaced with fresh medium 24 hours prior to sub-culturing.

In order to subculture the cells, the culture medium was aspirated and the cells were rinsed with 10 ml of sterile PBS (37⁰ C). Cells were treated with 3 ml of trypsin-EDTA (Gibco-BRL, Cat No. 25300-062), and the flask was placed back into the incubator. The cells were monitored until disassociation from the bottom of the flask. The cell suspension was then transferred into a 15 ml conical tube containing 7 ml of serum containing medium. The cell pellet was obtained upon 7 min centrifugation at 10,000 rpm after removing the supernatant. Following suspension of the pellet in 10 ml of the medium, 1.5 ml of the cell suspension was added into a flask containing 15 ml of culture medium. When the cells were evenly distributed in the flask, the flask was placed in the incubator.

Stable Transfection of the Cells

Transfection of MT-3 Gene into MT-3 Negative Cell Lines:

The MCF-7, T-47D, Hs578-T, MDA-MB-231 and MCF-10A cells were stably transfected with a plasmid construct containing the MT-3 gene coding sequence, blunt-end ligated into the *EcoR* V site of pcDNA 3.1/hygro (+) (Invitrogen, Carsbad, CA). The MT-3 gene was obtained from human proximal tubule (HPT) cell RNA by RT-PCR and the linearization of the plasmid was attained by *Fsp* I restriction enzyme prior to the transfections. All the cell lines were transfected with the MT-3 plasmid construct in the sense direction or the vector without insert using the

EffecteneTM transfection reagent (Qiagen, Valencia, CA). Prior to transfection, the hygromycin B sensitivity curve of the cell lines was determined by adding increasing concentrations of the antibiotic into the medium of the cells in a 12-well plate. The optimal concentration of hygromycin B was aimed to that which gave 100% kill by the end of a week. For all cell lines, 100 µg/ml of hygromycin B produced 100% kill in a week.

The cells were seeded at a 50 % confluence in a 6-well plate (9.6 cm²/well) the day before transfection. The transfection protocol for one well of 6-well plate is as follows. One µg of the plasmid was diluted in 10 µl sterile water to give 0.1 µg/µl DNA. With the DNA condensation buffer, buffer EC, the total volume was brought up to 150 µl. One to 8 DNA:Enhancer (w:v) ratio was previously determined to be optimum for particular cell lines. After addition of 8 µl of Enhancer, the mixture was vortexed for 2 seconds and incubated at room temperature for 5 minutes. Twenty five µl of Effectene reagent was added to the DNA-Enhancer mixture. Following vortexing for 10 seconds, the mixture was incubated for 10 minutes at room temperature to allow the lipid complex formation. Then, 1.5 ml media (with the serum) was added to the tube containing the transfection complex. The complex was added on to the cells that were previously washed twice with PBS. The plate was then gently swirled and placed into an incubator for overnight incubation. The following morning, transfection complexes were removed and cells were passaged at ratios of 1:10, 1:20, 1:40 and 1:80 in an appropriate selective media to allow for clone formation. In approximately two weeks, clones expressing the hygromycin B resistance gene started forming. As the clones reached a size visible to the naked eye, cloning rings were utilized to isolate and transfer cells to one well of a 12-well plate for further expansions. Finally, the clones were transferred to 6-well plates from which they were

harvested for RNA, protein and preserved in liquid nitrogen for future studies. Throughout the expansion process, the cell medium contained 100 µg/ml hygromycin B to keep the cells under selective pressure. Clones expressing the MT-3 gene and vector only were chosen by RT-PCR using insert primers that recognize the 3' and 5' ends of the MT-3 insertion point on pcDNA3.1 hygro (+) plasmid. The product of the PCR reaction is 395 bp in size.

Transfection of MT-1E Gene into MT-1E Negative Cell Lines:

The MCF-7 and T-47D cell lines were stably transfected with a plasmid construct containing MT-1E gene coding sequence, blunt-end ligated into *EcoR* V site of pcDNA 3.1/hygro (+) (Invitrogen, Carsbad, CA). This vector has a cytomegalovirus mediated-early promoter upstream of the multiple cloning site and a hygromycin B resistance gene driven by an SV40 early promoter. The MT-1E gene was obtained from human proximal tubule (HPT) cell RNA by RT-PCR. The cell lines were transfected with the MT-1E plasmid construct in the sense direction or the vector without insert using the EffecteneTM transfection reagent (Qiagen, Valencia, CA). The plasmid DNA construct was linearized by *Fsp* I prior to transfection. The MCF-7 and T-47D cell lines were transfected with the MT-1E plasmid construct in the sense direction or the vector without insert using the EffecteneTM transfection reagent (Qiagen, Valencia, CA). Prior to transfection, the hygromycin B sensitivity of the cell lines was determined by adding increasing concentrations of the antibiotic into medium of the cells in a 12-well plate. The optimal concentration of the hygromycin B was aimed to be that which gave 100% kill by the end of a week. For all the cell lines, 100 µg/ml of hygromycine obtained 100% kill in a week.

The cells were seeded at a 50 % confluence in a 6-well plate (9,6 cm²/well) the day before transfection. The transfection protocol for one well of 6-well plate is as follows. One µg of the plasmid was diluted in 10 µl sterile water to give 0.1µg/µl DNA. With the DNA condensation buffer, buffer EC, the total volume was brought up to 150 µl. One to 8 DNA : Enhancer (w:v) ratio was previously determined to work the best for each cell line. After addition of 8 µl of Enhancer, the mixture was vortexed for 2 seconds and incubated at room temperature for 5 minutes. Twenty five µl of Effectene reagent was added to the DNA-Enhancer mixture. Following vortexing for 10 seconds, the mixture was incubated for 10 minutes at room temperature to allow the lipid complex formation. Then, 1.5 ml media (can contain serum) was added to the tube containing the transfection complex. The complex was added on to the cells that were previously washed twice with PBS. The plate then was gently swirled and placed into an incubator for an overnight incubation. The following morning, transfection complexes were removed and the cells were passaged at ratios of 1:10, 1:20, 1:40 and 1:80 in an appropriate selective media to allow for clone formation. In approximately two weeks, clones expressing the hygromycin B resistance gene started forming. As the clones reached to a size visible to the naked eye, cloning rings were utilized to isolate and transfer cells to one well of a 12-well plate for further expansions. Finally, the clones were transferred to 6-well plates from which they were harvested for RNA, protein and preservation in liquid nitrogen for future studies. Throughout the expansion process, the cell medium contained 100 µg/ml hygromycin B to keep the cells under selective pressure. Clones expressing the MT-E gene and vector only were chosen by RT-PCR using insert primers that recognize the 3' and 5' ends of the MT-1E insertion point on pcDNA3.1 hygro (+) plasmid. The product of the PCR reaction is 354 bp in size.

Isolation of mRNA from Cells

The total RNA was isolated from MCF-7, T-47D, Hs578-T, MDA-MB-232 and MCF-10A using the TRI-Reagent[®] protocol (Molecular Research Center, Inc. Cincinnati, OH, USA). Following removal of media, cells were washed with PBS and 0.5 µl of TRI-Reagent was applied to the cells (per well of 6-well plate). Cells were incubated at room temperature for about 10 minutes to allow complete disassociation of nucleo-protein complex. Then the content of the well was transferred to an RNase free microfuge tube. The cell lysates were stored at -70⁰ C.

In order to isolate RNA, samples were thawed at room temperature and 50 µl of BCP reagent (Bicinchoninic Acid) was added to each tube. The tubes were vortexed vigorously for 20 seconds and incubated at room temperature for 15 minutes. Then tubes were centrifuged at 4⁰ C for 15 minutes at 14,000 rpm, and the colorless upper aqueous phase was transferred to new RNase free microfuge tubes. In order to precipitate the RNA, 250 µl of isopropanol was added. The tubes then were placed into a centrifuge for 15 minutes at 14,000 rpm. The supernatants were removed and the RNA pellets were washed twice in 1 ml of 75% ethanol. After the second ethanol wash, the pellets were dried at room temperature for 45 minutes. Finally, the fully dried RNA pellets were eluted in 30 µl of RNase free water and stored at -70⁰ C.

The RNA samples were quantitated by adding 3µl of stock RNA sample into 97 µl of phosphate buffer saline in a microfuge tube. Then the samples were analyzed in a spectrophotometer and the absorbance was read at 260 and 280 nm. The RNA concentrations were calculated by multiplying the absorbance at 260 nm by 40 µg/ml and by the volume of the sample (100µl), and

dividing it by the volume of the RNA sample (3 μ l). The working RNA solutions were prepared at a concentration of 0.167 μ g/ μ l and stored at -70⁰ C for RT-PCR analysis.

Isolation of Protein from Cells

The cell media was removed and the cells were rinsed with PBS twice. After removal of PBS, 0.5 ml of 10 mM Tris-Cl (pH 8.0) supplemented by dithiothreitol (DDT) (final concentration of 1mM) was added to the cells. The cells were scraped from the bottom of the plate and transferred to 1.5 ml microfuge tubes. Then the cells were lysed by two cycles of freezing and thawing in liquid nitrogen and 37⁰ C water bath. Finally, the cells were centrifuged at 14,000 rpm for 20 minutes and the supernatants were stored at -70⁰ C.

RNA Isolation from Breast Tissue Specimens

Total RNA extracted from human breast tissue obtained from two cases of elective reduction surgeries. The tissue source was anonymous, no clinical data was recorded, and the samples were obtained following completion of diagnostic protocols. The tissue specimens were dissected free from attached fat prior to the isolation of total RNA based on the different textures and colors of the tissue types. Fatty tissue was identified as gelatinous and yellow while the ductal tissue was identified as firm and tan. Immediately after dissection, 50-100 mg of breast ductal tissue samples were transferred to RNAase free eppendorf tubes and placed in liquid nitrogen. Frozen tissue specimens then were ground to a powder using a pestal under liquid nitrogen. Total RNA was isolated from the powdered tissue according to protocol supplied with the TRI

ReagentTM (Molecular Research In., Cincinnati, OH). TRI ReagentTM (1 ml) was added to 50-100 mg powdered breast tissue and vortexed to allow homogenization. After the phase separation at room temperature for 15 min, the samples were centrifuged at 14,000 rpm for 20 min at 4⁰ C. RNAs were precipitated from the aqueous phase with 1.25 ml isopropanol. Following centrifugation at 14, 000 rpm for 15 min at 4⁰ C, liquid was decanted, leaving the RNA pellet. The pellet was washed three times with 1ml of 75 % ethanol and air-dried at room temperature for 45 min. Finally, the fully-dried RNA pellets were eluted in 30 µl of RNase free water and stored at -70⁰ C.

The RNA samples were quantitated by adding 3µl of stock RNA sample into 97 µl of phosphate buffer saline in a microfuge tube. Then the samples were analyzed in a spectrophotometer and the absorbance was read at 260 and 280 nm. The RNA concentrations were calculated by multiplying the absorbance at 260 nm by 40 µg/ml and by the volume of the sample (100µl), and dividing it by the volume of the RNA sample (3µl). The working RNA solutions were prepared at a concentration of 0.167 µg/µl and stored at -70⁰ C for RT-PCR analysis.

Protein Isolation from Breast Tissue Specimens

Total protein was extracted from human breast tissue obtained from two cases of elective reduction surgeries. The tissue source was anonymous, no clinical data was recorded, and the samples were obtained following completion of diagnostic protocols. Frozen tissue specimens were ground to a powder using a pestal under liquid nitrogen. Then, powdered tissue samples were homogenized in 1 ml of 10 mM Tris-Cl (pH 8.0) supplemented by dithiothreitol (DDT)

(final concentration of 1mM). Following homogenization, samples were lysed by two cycles of freezing and thawing in liquid nitrogen and 37⁰ C water bath. Finally, the specimens were centrifuged at 14,000 rpm for 20 minutes and the supernatants were stored at -70⁰ C.

Protein Determination

Bio-Rad Protein Assay (Bio-Rad, Hercules, CA USA) was used to determine protein concentrations for each of the samples. Ten µg/µl of bovine serum albumin (BSA, Fisher Scientific, Fair Lawn, NJ, USA. Cat No. BP1605-100) was used to make standard solutions containing 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 µg BSA per 10 µl. First two wells of a 96-well plate were assigned as blanks (10 ul of water and 5 ul of 10mM Tris-Cl buffer) and standards containing 10 µl of each standard solution and 5 µl of Tris-Cl buffer were loaded in subsequent wells in duplicates. Rest of the empty wells were assigned for samples. Five µl of protein samples were mixed with 10 µl of deionized water and loaded in triplicates. Before adding Bio-Rad protein reagent into wells, the agent was filtered through a paper filter using an air vacuum and diluted with water at a ratio of 1:5. Two hundred µl of this dilution was added to each well of 96 well using a multichanel pipette aid. After incubation at room temperature for 5 minutes, the plate was scanned by Dynatech MR5000 plate reader (Dynatech Inc., Guernsey Channel Islands). Protein concentration for each sample was calculated by the regression of standard optical densities.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All RT-PCR reactions were run using 0.5 µg total RNA from samples. Master mixes were prepared using PCR Core Kits (Perkin-Elmer-Cetus, Foster City, CA, USA, Part No. N808-0017) by mixing 5mM MgCl₂, 1x PCR Buffer II, 1mM dATP, 1mM dCTP, 1mM dGTP, 1mM dTTP, 2.5 mM random hexamers, 1U/µl RNase inhibitor and 2.5 U/µl MuLV reverse transcriptase in a microfuge tube. Aliquots of 1.5 µl (0.25 µg) of total RNA samples were placed in sterile PCR reaction tubes on ice and thoroughly mixed master mix solution was added (8.5 µl) to RNA containing reaction tubes. In addition to the samples, every RT-PCR reaction contained two negative controls. One of the control reaction tubes included all the RT-PCR reagents but Reverse Transcriptase, and the second control tube did not include RNA sample. The missing amounts of Reverse transcriptase or RNA were replaced by Millie Q water. All the reaction tubes were transferred to a PCR rack and placed in a DNA thermocycler (Perkin –Elmer GeneAmp PCR System 9600). The reverse transcription steps were as follows: a single cycle of 25⁰ C for 10 minutes, 42⁰ C for 20 minutes, 99⁰ C for 5 minutes. The reaction tubes were taken out from the thermocycler to proceed to the PCR step. At this point it is possible to store tubes at – 20⁰ C overnight.

PCR master mixes were prepared with 2 mM MgCl₂, 1x PCR Buffer II, upper and lower primers (See appendix B), 1.25 U AmpliTaq® DNA polymerase and Millie Q water to bring the volume of each reaction up to 40 µl. The MgCl₂, PCR Buffer II and AmpliTaq® were provided by Perkin-Elmer GeneAmp RNA PCR Core Kit. The master mix tube was vortexed and placed on ice before combining it with sample from transcription step. The PCR reaction steps were as

follows: DNA sample was denatured at 95⁰ C for 2 minutes and successive cycles of temperature change took place between annealing and extension at 68⁰ C and denaturation at 95⁰ C. The PCR reactions were samples at 25, 30, and 35 cycles for the metallothionein genes. Aliquots of 15 µl PCR reactions were taken at these specific cycles and refrigerated for electrophoresis analysis. One and a half µl of 10x loading buffer was mixed with 15 µl of PCR product and electrophoresed on an ethidium bromide stained 2% agarose gel.

Agarose Gel Electrophoresis

Two percent agarose gels were prepared by combining 2 grams of PE-Xpress GeneAmp® Agarose (Perkin- Elmer, Cat No. 930-2774) with 100 ml of Gel Mix Running Mate 0.5X TBE Buffer (Gibco-BRL, Cat No. 15546-013). The mixture was heated in microwave for approximately 3 minutes until the agarose was completely in solution. After the agarose cools to manageable temperatures, 2.5 µl of 10mg/ml EtBr was added to 100 ml of agarose solution. After swirling several times, the agarose solution was poured into a gel mould containing combs. The agarose was allowed to solidify at room temperature for 1 hour. Before loading 10 µl of PCR products, the comb was carefully removed and the agarose was transferred to an electrophoresis apparatus filled with 0.5X TBE running buffer. The first lane was loaded with 10 µl of Hi-Lo™ DNA markers and the following wells with 10 µl of PCR products. The gel was run at 150 amps for about 1.5 hours, and visualized on a UV tray. The digital images of the gel were obtained using KODAK Professional DCS 420 digital camera interfaced with Adobe Photoshop™ software. Finally, the intensity of the RT-PCR product bands were quantitated as integrated optical density

(IODs) using Kontron KS 400 image analysis software (Carl Zeiss Vision, Thornwood, NY, USA).

Dot Blots:

An immuno-blot analysis, dot blot, technique was utilized to determine the MT ½ or MT-3 protein levels. For MT-1 and -2 protein determination, standards were prepared using protein extracted from a Metallothionein Null immortalized fibroblast cell line, MT 1/2 standard protein (Sigma Chemical Co., St Louis, Mo, USA) and PBS. Ninety six well plates were utilized to load the samples. Four rows out of 12 were dedicated for standards and the standard range was determined based on MT protein levels measured by preliminary observations. For MT-1 and -2 protein, standards ranged from 0 to 10 ng MT protein (0, 1, 2, 4, 6, 8, 9, 10 ng MT). Standards were prepared as follows; 1.5 times the desired amount of purified rabbit liver Cd/Zn metallothionein-1 protein (as a standard) (Sigma Chemical Co., St Louis, MO, USA) were loaded in four alternating rows, with each standard well containing 1.5 µg of protein extract from the MT Null cell line. The standard wells were brought to a volume of 75 µl with PBS. Then the samples were loaded into wells in triplets (1.5 µg total protein) and the volume of the wells was brought up to 75 µl with 1 X PBS. Using a multichannel pipetman, 75 µl of 3% glutaraldehyde was added to each well, and the contents were thoroughly mixed by pipetting up and down several times. The plate was kept at room temperature for 30 minutes while the blot apparatus was being prepared.

MT-3 dot blot preparations were slightly different from MT-1 and 2 blot preparations. For MT-3 protein determination, standards were prepared using a synthetic peptide of 8 unique amino acid conjugated to bovine serum albumin (Sigma Chemical Co., St Louis, MO), 3 µg of BSA and 1 X PBS. Ninety six well plates were utilized to load the samples. Four rows out of 12 were dedicated for standards and the standard range was determined based on MT-3 protein levels measured by preliminary observations. For MT-3 protein determination, standards ranged from 0 to 0.3 ng MT protein (0, 0.01, 0.03, 0.06, 0.12, 0.18, 0.24, 0.3 ng MT-3). Standards were prepared as follows; 1.5 times the desired amount of MT-3 cross linked peptide (as a standard) (Sigma Chemical Co., St Louis, MO, USA) were loaded in four alternating rows, with each standard well containing 3 µg of BSA. The standard wells were brought to a volume of 75 µl with 1 X PBS. Then the samples were loaded into wells in triplets (3 µg total protein) and the volume of the wells was brought up to 75 µl with 1 X PBS. Using a multichannel pipetman, 75 µl of 3% glutaraldehyde was added to each well, and the contents were thoroughly mixed by pipetting up and down several times. The plate was kept in room temperature for 30 minutes while the blot apparatus was being prepared.

For each blot, an 8 cm x 12 cm piece of Sequi-Blot™ PVDF membrane (Bio-Rad, Cat No.162-0182) was cut and soaked in methanol for 5 minutes and submerged in 1 X PBS for 15 minutes. The dot blot apparatus was assembled and the lower chamber was filled with 1 X PBS using 50 ml syringe. The syringe remained attached to the apparatus and the PVDF membrane was carefully placed over the rubber adppter which contains 96 holes to allow the drainage. The top piece of the apparatus was screwed in tightly and the wells were filled with 600 ml of 1 X PBS. In order to obtain gravity dependent drainage, 50 ml of 1 X PBS was sucked through and then

slowly withdrawn from apparatus. The wells were filled up with 1 X PBS again and allowed complete drainage of 1 X PBS from the wells after the syringe was removed. Excess amount of 1 X PBS was collected in a beaker. After making sure all the PBS had soaked through the membrane, 100 µl of each sample were transferred from the 96-well plate to the dot blot apparatus using a multichannel pipetman. After the samples drained completely, each well was rinsed twice with 600 µl of 1 X PBS. The apparatus was dismantled after all the 1 X PBS drained from the top piece. Finally, the membrane was removed and soaked in 1 X PBS at 4⁰ C overnight.

The following day, the membrane was blocked in 10 % (w/v) milk solution made in 1 X PBS for an hour. During blocking, the membrane was placed on a shaker to agitate it constantly. After an hour on shaker, blocking solution was discarded and the membrane was rinsed with 1 X PBS three times quickly. The membrane then rinsed with 1 X PBS three times for 15 minutes. Then the membrane was incubated with primary antibody (see appendix) for an hour on a shaker. Following 3 quick successive 1 X PBS rinses, the membrane was again rinsed three times 15 minute long with 1 X PBS. The membrane then was incubated with secondary antibody for an hour and quick rinsed three times with 1 X PBS. After two 15 minute 1 X PBS rinses, the membrane was rinsed with alkaline phosphatase buffer for 15 minutes. Finally, the colorimetric detection of the alkaline phosphatase conjugated secondary antibodies was performed using an alkaline phosphatase Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, USA). Ten drops of each reagent 1, 2 and 3 were added in succession to 25 ml of 100 mM Tris-HCl (pH 8.2) and the solution was poured on the membrane. Standard detection time was usually two or three minutes. Then, the membrane was rinsed immediately in tap water as soon as the control wells

became visible, and left to air dry on a piece of Whatmann paper overnight. Digital image of the membrane was generated using Epson® Expression 63 color scanner and Adobe® Photoshop® software. The intensity of the metallothionein protein dots were quantitated as integrated optical densities (IODs) using Kontron KS 400 image analysis software. The amount of MT protein in each dote was determined by comparing the IODs of each sample to that of the standard curve.

Metallothionein Gene Characterization and Protein Expression by Exposure of MCF-10A Cells to CdCl₂ for 48 hours (Short Term) and 16 days (Long Term):

The MCF-10A cells were exposed to CdCl₂ in order to characterize metallothionein gene and protein expression. For 48 hours of CdCl₂ exposure, enough number of T-75 flasks of MCF-10A cells were cultured until all the flasks reached confluence. Cells were fed the day before seeding into 6-well plates. All the 6-well plates were fed every three days with 2 ml of media until the cells reached confluence. Twenty four hours before the exposure of CdCl₂, the cells were fed with fresh media.

On the day cells reached confluence, 7, 15 and 30 µM CdCl₂ containing media were prepared and added to the cells. The MCF-10A cells in 6-well plates were harvested at 0, 4, 8, 12, 24, 36 and 48 hour time points for RNA, protein and toxicity determination. The cells were harvested in triplets for each time point and concentrations.

For 16 days of CdCl₂ exposure, enough number of T-75 flasks of MCF-10A cells were cultured until all the flasks reached confluence. Cells were fed the day before seeding them into 6-well

plates. All the 6-well plates were fed with 2 ml of media every three days until the cells reached to confluence. Twenty four hours before the exposure of CdCl₂, the cells were fed with fresh media.

On the day cells reached confluence, 3, 7, 15 and 30 µM CdCl₂ containing media were prepared and added to the cells. The MCF-10A cells in 6-well plates were harvested at 0, 1, 2, 4, 7, 10, 13 and 16 day time points for RNA, protein and toxicity determination. The cells were harvested in triplets for each time point and concentration. Cells were fed with fresh media 24 hour before harvests at each time point.

Colony Formation Assay:

The cell lines MCF-7, T-47D, Hs578-T and MDA-MB-231 were maintained in T75 flasks and fed the day before setting up the colony formation assay. Confluent flasks were trypsinized using 3 ml of trypsin-EDTA (Bibco-BRL, Cat No. 25300-062), and the flasks were placed into an incubator until the cells disassociated. Then the cells were washed and transferred from flasks to 15 ml tubes using 10 ml of 1 X PBS. The tubes were centrifuged for 7 minutes at 10, 000 rpm, after which, the supernatant was removed and thoroughly resuspended in 10 ml of media. Aliquots of 1 ml of the cell suspension were aliquoted out and placed into several conical tubes containing media until dilution of 100 cell/well was achieved. The cells were counted by a hemocytometer to make sure dilutions contained desired number of cells per ml of media. Then the cells were seeded into 6-well plates in triplets in a manner that each well contained 100 cells. The plates were incubated overnight at 37⁰ C to allow cells to attach to the bottom of the plates.

The following day, the cells were given various concentrations of CdCl₂ ranging from 0 μM to 10 μM. During 3 weeks, the cells were fed every 3 days with various concentrations of CdCl₂ until they formed colonies visible to the naked eye. After 3 weeks, the plates were taken out from the incubator, fixed using 70 % EtOH for 10 minutes, and stained with crystal violet. Plates were left at room temperature overnight to dry. Colonies containing at least 150 cells were counted. The number of colonies formed at each concentration of CdCl₂ was determined averaging triplet wells.

Growth Curves:

To determine if MT-3 or MT-1E overexpression had any effect on the growth of MCF-7, T-47D, Hs578-T and MDA-MB-231 cells, growth rates were determined for untransfected wild-type cells, blank vector transfected cells and three clones of MT-3 or MT-1E transfected clones. This was accomplished by sub-culturing the cells at a 1:10 ratio and monitoring the growth of the cells by the automated counting of DAPI-stained nuclei until the cells reached confluence.

The cells were maintained in T75 flasks and fed every three days until they reached confluence. Twenty four hours prior to setting up the growth experiment, cells were fed with fresh media. The following day, cells were trypsinized and the cell suspensions were prepared in 10 ml of media. After a series of dilutions, aliquots were taken from the cell suspensions and counted using a hemocytometer. For all cell lines, 2 ml of cell suspensions containing 50,000 cells/ml were seeded into 6-well plates in triplets with MT-3 or MT-1E clones, vector alone clones and non transfected-parent cells. The plates were harvested daily until cells reached confluence.

Depending upon the cell line, it took 8 to 11 days for cells to reach confluence. Cells harvested daily were rinsed in 1X PBS and fixed with 70 % ethanol. Two ml of 1 X PBS was placed in each well and cells were stained with 10 µl of DAPI dilactate (10mg/100ml in H₂O). An automated-counting of DAPI-stained nuclei was performed using Kontron Image Analysis Software. All the experimental groups were run in triplets.

Crystal Violet Staining:

Preparation of crystal violet solution is presented in Appendix C. Following 3 weeks of colony growth, plates were ready for crystal violet staining. Media were aspirated from the plates and the colonies were rinsed with 1 x PBS twice before fixation with 70 % ethanol. Colonies were fixed with 70 % ethanol for about 7 minutes and rinsed once again with 1 x PBS solution. Each well was treated with 0.5 ml of crystal violet solution for a minute. Then the wells were rinsed thoroughly with 2 ml of 1 x PBS twice. After 30 minutes of air-drying at room temperature, colonies visible to naked eye were counted independently at least three times per well.

Laser Captured Microdissection (LCM):

The Laser Captured Microdissection System, The PixCell™ LCM (Arcturus Engineering, Mountain View, CA) was used for microdissection protocol. Five µm thick sections were cut from formalin-fixed, paraffin embedded tissue blocks of normal human breast obtained from the pathology archives and mounted on plain glass slides. The slides were stained with hematoxylin and eosin. Total RNA was extracted from samples using the micro RNA isolation kit (Stratagene, La Jolla, CA, Catalog No: 200344).

**STABLE TRANSFECTION AND OVEREXPRESSION OF METALLOTHIONEIN
ISOFORM 3 INHIBITS THE GROWTH OF MCF-7 AND Hs578T CELLS
BUT NOT THAT OF T-47D OR MDA-MB-231 CELLS.**

INTRODUCTION

Third isoform of the metallothionein gene family (MT-3) is overexpressed in a subset of human breast cancers and that overexpression correlates with poor disease prognosis [1]. Using a similar retrospective analysis of paraffin embedded archival samples, it has also been shown that MT-3 is highly expressed in all human bladder cancers examined to date [2] and has a variable expression in prostate cancers compared to control tissue [3]. MT-3 is a member of the MT gene family which, in the human, is composed of at least 10 active genes and 7 pseudogenes [4,5,6,7]. All family members share the property of being low molecular weight (6 kDa), intracellular proteins that have a very high conserved number of cysteine residues that function to allow the efficient binding of transition metals [8]. There are several features that distinguish MT-3 from the other MT family members. The MT-1 and MT-2 isoforms exhibit a ubiquitous pattern of tissue expression and are highly inducible by any number of stimuli, where as, MT-3 exhibits neither of these characteristics [9,10,11]. The major structural difference between MT-3 and all other MT isoforms is that the MT-3 isoform possesses 7 additional amino acids that are not present in any other member of the MT gene family, a 6 amino acid C-terminal sequence and a Thr in the N-terminal region [6,9,12]. Of interest in the current study is that the MT-3 protein has been shown to inhibit the survival and neurite outgrowth of cultured rat cortical neurons when added exogenously to such cultures [9,13] and to have a growth inhibitory activity when expression is indirectly elevated in cultures of rat C-6 glial cells [14]. The growth inhibitory activity in the neural cells is unique to MT-3 and is not duplicated by the other members of the MT gene family [12,15]. This non-duplication of function occurs despite a 63-69% identity in amino acid sequence among MT-3 and the other 9 active human MT isoforms [12]. A recent study has shown that stable transfection of PC-3 prostate cancer cells with MT-3 results in growth inhibition, extending the growth inhibitory action of MT-3 beyond the neural system [11]. Transfection of the PC-3 cells with the MT-1E isoform had no effect on cell growth, confirming that the growth inhibitory activity was also MT-3 specific in prostate cancer cells. In

contrast, the resistance of the PC-3 cells to cadmium was enhanced by both the MT-3 and MT-1E isoforms. The goal of the present study was to determine if MT-3 overexpression would influence the growth of human breast cancer cell lines. To determine this, the coding sequence of the MT-3 gene was stably transfected into 2 estrogen receptor positive (MCF-7 and T-47D) and 2 estrogen receptor negative cell lines (Hs578T and MDA-MB-231) having no basal expression of MT-3.

RESULTS

Stable Transfection and Expression of MT-3 in MCF-7, T-47D, Hs578T and MDA-MB-231 Cells:

It was determined using RT-PCR at a total RNA input of 1 µg and 40 reaction cycles that there was no basal expression of MT-3 mRNA in the MCF-7, T-47D, Hs578T or MDA-MB-231 cell lines (Fig 4 A,C,E,G). Immuno-blot analysis of the corresponding protein sample from these 4 cell lines also demonstrated no detectable MT-3 protein (Table 1). The coding sequence of the MT-3 gene was obtained from human proximal tubule cell RNA by RT-PCR, blunt end ligated into the *EcoR* V site of pcDNA3.1/Hygro(+), and linearized by *Fsp* I prior to transfection of the respective cell lines. All four cell lines were transfected with the MT-3 plasmid construct in the sense direction or with the blank vector by application of the Effectene protocol. Following selection in hygromycin B-containing growth medium, 5 clones from each cell line were selected for further characterization. Each of the 5 clones from each cell line overexpressed MT-3 mRNA when compared to wild type cells or cells containing the blank pcDNA3.1 vector (Fig 4 A,C,E,G). The MT-3 mRNA was first detected from each of the 5 overexpressing clones of each cell line between 20 and 22 cycles of PCR with cDNA derived from 500 ng total RNA inputs. This level of expression was similar to that of the glyceraldehyde 3-phosphate dehydrogenase gene. It was also demonstrated that the transfection protocol had no effect on the expression on

the glyceraldehyde 3-phosphate dehydrogenase housekeeping gene (Fig 4 B,D,F,H). The MT-3 protein was not detected in the 4 cell lines transfected with the blank vector, but was detectable in the clones of each cell line of the MT-3 transfectants (Table 1).

Figure 4. MT-3 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene expression in transfected MCF-7, T-47D, Hs578T and MDA MB 231 cells. *RT-PCR was used to determine the expression of MT-3 and G3PDH mRNA. The reactions were sampled at 30, 35 and 40 cycles of PCR for the MT-3 transfected clones and the control cells, and the reaction products were visualized on ethidium bromide (EtBr) stained 2% agarose gels. Shown in A, C, E, G are the MT-3 RT-PCR products at 30 cycles of PCR for the five independent MT-3 clones, 40 cycles of PCR for the two clones containing blank vector and the two samples of nontransfected MCF-7, T-47D, Hs578T and MDA MB 231 cells. Shown in B, D, F, H are the G3PDH products at 30 cycles for five independent MT-3 clones, two clones with blank vector and two samples of nontransfected MCF-7, T-47D, Hs578T and MDA MB 231 cells.*

Table 1. MT-3 and MT-1/2 Protein Expression in MT-3 and MT 1E Transfected Breast Cancer Cell Lines.

Cell Line	MT-3 transfectants	MT-3 protein (ng/ug protein)	MT-1E transfectants	MT-1/2 [†] (ng/ug protein)
MCF-7	MT-3 clone 1	8.25 (± 1.43)	MT-1E clone 1	11.45 (± 0.77)
	MT-3 clone 2	7.72 (± 1.27)	MT-1E clone 2	11.32 (± 0.57)
	MT-3 clone 3	6.39 (± 0.94)	MT-1E clone 3	10.12 (± 0.52)
	Vector only	0	Vector only	5.60 (± 0.28)
	Parent	0	Parent	5.10 (± 0.72)
T-47D	MT-3 clone 1	8.26 (± 0.84)	MT-1E clone 1	11.31 (± 1.11)
	MT-3 clone 2	5.61 (± 0.89)	MT-1E clone 2	10.24 (± 0.91)
	MT-3 clone 3	5.45 (± 0.37)	MT-1E clone 3	9.21 (± 0.71)
	Vector only	0	Vector only	6.41 (± 0.54)
	Parent	0	Parent	5.32 (± 0.41)
MDA-MB-231	MT-3 clone 1	4.93 (± 0.24)	*	
	MT-3 clone 2	2.82 (± 0.60)	*	
	MT-3 clone 3	2.82 (± 0.50)	*	
	Vector only	0	*	
	Parent	0	Parent	5.31 (± 0.71)
Hs578T	MT-3 clone 1	8.31 (± 1.81)	*	
	MT-3 clone 2	9.16 (± 1.47)	*	
	Vector only	0	*	
	Parent	0	Parent	4.43 (± 0.33)

[†] MT-1/2 denotes the fact that the E 9 antibody recognizes both the MT-1 and MT-2 proteins.

* Cells were not transfected with the MT-1E gene since these cell lines have a basal expression of the MT-1E gene.

Stable Transfection and Expression of MT-1E in MCF-7 and T-47D Cells:

It has been shown previously that the Hs578T and MDA-MB-231 cell lines express mRNA for the MT-1E isoform, while the MCF-7 and T-47D cell lines have no expression of the MT-1E gene [16]. For this reason, the MT-1E gene was transfected only into the MCF-7 and T-47D cell lines. It was confirmed using RT-PCR that both MCF-7 and T-47D cell lines did not express MT-1E mRNA following 40 cycles of PCR at an input of 1µg total RNA (Fig 5 A). However, immuno-blot analysis of corresponding triplicate protein samples from the wild-type MCF-7 and T-47D cell lines demonstrated that these cell lines did have MT-1 and MT-2 protein (Table 1). This finding was expected since the E9 antibody used for MT-1 and MT-2 protein determinations cannot distinguish between the MT-1 and MT-2 isoforms, and the MT-2A and MT-1X genes have been shown previously to be expressed in both the MCF-7 and T-47D cell lines [16]. The amount of MT-1/2 protein found was consistent with the previous findings in these cell lines [16]. The coding sequence of the MT-1E gene was also obtained from human proximal tubule cell RNA by RT-PCR, blunt end ligated into the *EcoR* V site of pcDNA3.1/Hygro(+), and the resulting construct linearized by *Fsp* I prior to transfection of the MCF-7 and T-47D cells. An identical protocol was used to transfect the cells with the MT-1E plasmid. Similarly, it was demonstrated that each of 5 independent clones from each cell line overexpressed MT-1E mRNA when compared to wild-type cells or cells containing the blank pcDNA3.1 vector (Fig 5 A,C). The MT-1E mRNA was first detected from each of the respective 5 overexpressing clones at 22 cycles of PCR and 500 ng total RNA inputs. This level of expression was similar to that of the glyceraldehyde 3-phosphate dehydrogenase gene and expression of this housekeeping gene was also similar among all the respective clones (Fig 5 B,D). The expression of the MT-1/2 protein was significantly elevated in both the MCF-7 and T-47D cells transfected with the MT-1E gene compared to the wild type and blank vector controls (Table 1).

Figure 5. MT-1E and G3PDH gene expression in MCF-7 and T-47D cells stably transfected with MT-1E cDNA. RT-PCR was used to confirm expression of MT-1E and G3PDH mRNA. The reactions were sampled at 30, 35 and 40 cycles of PCR for the MT-1E transfected clones and the control cells, and the reaction products were visualized on ethidium bromide (EtBr) stained 2% agarose gels. Shown in (A, C) are the MT-1E RT-PCR products at 30 cycles PCR for five independent MT-1E clones, 40 cycles of PCR for the two clones with blank vector and two samples of nontransfected MCF-7 and T-47D cells. Shown in (B, D) are the G3PDH products at 30 cycles for five independent MT-1E clones, two clones with blank vector and two samples of nontransfected MCF-7 and T-47D cells.

Effect of Stable Transfection of MT-3 and MT-1E on the Expression on other Isoforms of the MT Gene Family:

Experiments were also performed to determine if the overexpression of either the MT-3 or MT-1E genes had any influence on the basal levels of expression of the other 9 active MT genes in the 4 cell lines. In the case of MT-3, this was determined by isolating total RNA from one MT-3 transfected clone and one blank vector control from each of the 4 cell lines and determining the basal expression of the MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A and MT-4 genes by RT-PCR. It was demonstrated using 40 cycles of PCR and a total RNA input of 1 µg that there was no expression of the MT-1A, MT-1B, MT-1F, MT-1G, MT-1H, and MT-4 genes in any of the MT-3 transfectants or vector controls of all 4 cell lines (data not shown). This finding that these genes were not expressed was in agreement with the expression patterns noted previously for these MT genes in the 4 parental cell lines prior to transfection [16]. In agreement with the previous study, it was also found that the MCF-7 and T-47D cell lines had no expression of MT-1E, where as, the Hs578T and MDA-MB-231 cell lines did express the MT-1E gene (data not shown). For the MCF-7 and T-47D cell lines, it was demonstrated that the levels of expression of the MT-2A and MT-1X genes were similar among the MT-3 transfectants and the blank vector controls (Fig 6). Likewise, for the Hs578T and MDA-MB-231 the levels of expression of the MT-2A, MT-1X and MT-1E genes were similar between the MT-3 transfectant and vector controls (Fig 6). In all cases, the level of the MT-1/2 protein was also similar among the MT-3 transfectants and the blank vector controls (data not shown). In the case of MT-1E, total RNA was isolated from a MT-1E transfected clone and a blank vector control from the MCF-7 and T-47D cell lines and the expression of the MT-1A, MT-1B, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3 and MT-4 genes determined by RT-PCR. It was demonstrated at 40 cycles of PCR and total RNA inputs of 1µg that there was no expression of the MT-1A, MT-1B, MT-1F, MT-1G, MT-1H, MT-3 and MT-4 genes in either the MT-1E transfectants or blank vector controls (data not shown). The levels of expression of the MT-2A and MT-1X genes

were similar between the MT-1E transfectants and vector controls for each cell line (Fig 6). The MT-3 protein was not detected in either the MT-1E transfectants or the blank vector controls (data not shown).

Figure 6. The effect of stable transfection of MT-3 and MT-1E on the expression of other isoforms of the MT gene family. Expression of MT-2A, 1E and 1X was assessed by using RT-PCR with isoform specific primers on total RNA from MT-3 or MT-1E transfected clones and clones with blank vector. The reactions were sampled at 25, 30 and 35 cycles, and the products were visualized on 2% ethidium bromide stained gels.

Effect of Stable Transfection of MT-3 and MT-1E on MCF-7, T-47D, Hs578T and MDA-MB-231 Cell Growth:

To determine if MT-3 overexpression had an effect on the growth of the 4 transfected cell lines, growth rates were determined for untransfected wild-type cells, a blank vector transfectant, and three clones of MT-3 transfectants for each cell line. This was accomplished by subculturing the cells at a 1:10 ratio and monitoring the growth of the cells by the automated counting of DAPI-stained nuclei until the cells reached saturation density. The results demonstrated that MCF-7 and Hs578T cells stably transfected to overexpress the MT-3 gene had a markedly reduced growth rate compared to both untransfected cells or cells containing the blank vector control (Fig 7 A,B). This marked reduction in growth was confirmed for three independent MT-3 overexpressing clones of the MCF-7 and two independent clones of Hs578T cell lines. The mean doubling time of the three independent clones of MCF-7 cells overexpressing MT-3 was 61.0 ± 2.9 hrs compared to a doubling time of 38.5 ± 2.9 hr in control MCF-7 cells and blank vector transfectants (significantly different at $p < 0.001$). The mean doubling time of the two independent clones of Hs578T cells overexpressing MT-3 was 97.3 ± 1.5 hrs compared to a doubling time of 71.6 ± 1.8 hrs in control Hs578T cells and blank vector transfectants (significantly different at $p < 0.001$).

Figure 7 A, B. The effect of MT-3 on MCF-7, Hs578T cell growth. *The cells were seeded at 1:10 ratio and the cell numbers were determined at indicated time points by automated counting of DAPI-stained nuclei. Shown in panel A, B, is effect of MT-3 overexpression on MCF-7 and Hs578T cell growth.*

In contrast, it was shown that the growth of the T-47D or MDA-MB-231 cells was unaffected by the stable transfection and expression of the MT-3 gene (Fig 7 C, D). The mean doubling time of the three independent clones of T-47D cells overexpressing MT-3 was 47.7 ± 1.4 hrs compared to a doubling time of 43.4 ± 0.3 hrs in control T-47D cells and blank vector transfectants ($p > 0.05$). The mean doubling time of the three independent clones of MDA-MB-231 cells overexpressing MT-3 was 28.7 ± 1.3 hrs compared to a doubling time of 26.7 ± 0.4 hrs in control cells and blank vector transfectants ($p > 0.05$).

Figure 7 C, D. The effect of MT-3 overexpression on T-47D, and MDA MB 231 cell growth. The cells were seeded at 1:10 ratio and the cell numbers were determined at indicated time points by automated counting of DAPI-stained nuclei. Shown in panel C and D is effect of MT-3 overexpression on T-47D and MBA-MB-431 cell growth.

An identical protocol was utilized to determine if MT-1E overexpression had an effect on the growth of either the MCF-7 or T-47D cell lines. The results demonstrated that stable transfection of the cells with the MT-1E gene had no significant effect on the growth rate of the cells when compared to either control cells or blank vector controls (Fig 7 E,F). The mean doubling time of the three independent clones of MCF-7 cells overexpressing MT-1E was 34.7 ± 1.7 hrs compared to a doubling time of 36.5 ± 0.1 hrs in control cells and blank vector transfectants ($p > 0.05$). The mean doubling time of the three independent clones of T-47D cells overexpressing MT-1E was 31.3 ± 0.5 hrs compared to a doubling time of 31.6 ± 0.4 hrs in control cells and blank vector transfectants ($p > 0.01$).

Figure 7 E,F. The effect of MT-1E overexpression on MCF-7, T-47D cell growth. *The cells were seeded at 1:10 ratio and the cell numbers were determined at indicated time points by automated counting of DAPI-stained nuclei. Shown in panel E and F is the effect of MT-1E overexpression on MCF-7 and T-47D cell growth.*

Effect of Stable Transfection of MT-3 and MT-1E on Resistance of MCF-7, T-47D, Hs578T and MDA-MB-231 Cells to Cadmium:

Identical clones to those used above in the growth studies were used to determine if the overexpression of MT-3 or MT-1E would increase the resistance of the cells to cadmium. For MT-3, LD₅₀ values were determined for each of the MT-3 expressing and non-expressing clones when the cells were exposed to cadmium. The results of this analysis demonstrated that the overexpression of MT-3 increased the resistance of only the MCF-7 cell line to the toxic effects of cadmium (Fig. 8). The data are presented as the calculated LD₅₀ ± the Wald confidence interval for the MT-3 non-expressing and expressing clones, respectively. Identical studies for the MCF-7 (Fig. 8) and T-47D cells (data not shown) overexpressing the MT-1E gene demonstrated that overexpression only increased the resistance of the MCF-7 cells to the toxic effects of cadmium.

Figure 8. The effect of MT-3 and MT-1E overexpression on Cd⁺² resistance. LD₅₀ values were measured by colony formation assay for all four breast cancer cell lines for MT-3 clones (■)

), MT-1E clone (□), vector alone (▲) and nontransfected wild-type (●) cells. In all cases, the error bars represent the Wald confidence interval at the 95% confidence level, calculated for each side of the LD₅₀ determination.

DISCUSSION

MT-3 was originally proposed as a brain-specific metallothionein with limited expression outside the neural system [6]. A limited expression pattern of MT-3 in normal human tissues does appear to be the norm for this protein, with the only major exception noted to date being that of the human kidney, where moderate to high levels of expression were found in most of the various renal epithelial cell types [10]. An interest in how MT-3 expression might affect the cancer cell was motivated by the observation that, while MT-3 was not expressed in normal bladder urothelium or breast epithelium, it was overexpressed in all bladder cancers and in selected breast cancers [1,2]. In breast cancer, expression appeared to correlate with ductal *in situ* carcinomas having a poor outcome [2]. In prostate cancer, MT-3 was shown to have variable expression ranging from none to occasional instances of moderate levels in normal prostate epithelium, and to have high levels of overexpression in some cancers and PIN lesions and to be absent in others [3]. The finding that MT-3 was overexpressed in paraffin-embedded diagnostic specimens from patients with bladder, prostate and breast cancer, motivated an examination of what effect MT-3 overexpression would have in cell lines derived from these cancers. This was first approached in the PC-3 prostate cancer cell line when it was demonstrated that of the LNCaP, Du-145 and PC-3 cell lines, only the PC-3 cells expressed no detectable MT-3 mRNA and protein, making them a suitable recipient for stable transfection of an MT-3 overexpression vector [3]. The results of this study demonstrated that PC-3 prostate cancer cells that overexpress MT-3 have a reduced growth rate and increased chemotherapeutic drug resistance compared to cells containing a blank vector [11]. An identical transfection protocol for introduction of MT-1E gene was employed to show that the growth reduction was

specific to the MT-3 isoform while chemotherapeutic drug resistance was a general feature of cells transfected with either MT-1E or MT-3. These results served as the first demonstration that the growth inhibitory activity of MT-3 noted in the neural system could extend to cell types of epithelial origin. The initial goals of the present study were to determine if MT-3 expression was growth inhibitory for breast cancer cells and if such inhibition might be a general feature of breast cancer cell lines.

The present study demonstrated that MT-3 expression can inhibit the growth of breast cancer cell lines as evidenced by the marked growth inhibition stable transfection and overexpression of MT-3 elicited in the MCF-7 and Hs578T cell lines. However, it was also demonstrated that growth reduction was not a general property of MT-3 expression in all breast cancer cell lines since identical stable transfection and overexpression of MT-3 had no effect on the growth of the T-47D and MDA-MB-231 cell lines. That these findings were specific for MT-3 and not a general feature of MT overexpression was strongly indicated by the finding that the overexpression of the MT-1E isoform gene had no effect on the growth of both cell lines. The MT-3 gene has not been widely studied outside the neural system and there exists no established mechanism to explain why MT-3 overexpression is growth inhibitory in the neural system or in certain cancer cell lines. An examination of the genetic backgrounds of the 4 cell lines is not very helpful in assigning mechanism since the 4 cell lines are very diverse and contain a variety of both common and specific genetic deletions and mutations that accumulate in cell lines derived from advanced cancers that have been cultured for many years. One interesting feature was that the growth inhibition mediated by MT-3 did not correlate with the estrogen receptor status of the cells, with 1 ER positive (MCF-7) and 1 ER negative (Hs578T) cell line demonstrating growth inhibition. This finding that MT-3 mediated growth inhibition is independent of ER status agrees with earlier findings that expression of immunoreactive MT-3 in archival specimens for breast cancer patients was also independent of this marker [1]. The ER status of a breast cancer was originally used to predict response to endocrine ablative therapy for

patients with advanced breast cancer, but ER is now more widely used to select patients with early breast cancer likely to respond to the antiestrogen, tamoxifen [18]. In a meta-analysis of over 37,000 women, ER-positive patients were 7 times less likely to develop recurrent disease than ER-negative patients following at least 5 years of adjuvant tamoxifen treatment [19, 20]. The finding that both MT-3 expression and its effect on cell growth are independent variables from ER status can potentially provide an additional prognostic marker to further stratify the ER positive sub-group of breast cancers.

The effects of MT-3 on both cell growth and its role as a potential marker for breast cancer prognosis appear to be unique properties that are distinct from the MT-1 and MT-2 members of this gene family. The effect on cell growth is confined to the MT-3 isoform as evidenced by the fact that MT-1E has no effect in both prostate or breast cancer cell lines. Evidence that MT-3 might have a role in predicting tumor prognosis also appears specific for the MT-3 isoform. An antibody (E9) that recognizes both the MT-1 and MT-2 isoform proteins has been employed in many studies to show that co-overexpression of these two isoforms correlates to breast cancers having an aggressive course [21-28]. However, in contrast to MT-3, which appears independent of ER status, expression of MT-1 and MT-2 does not increase the prognostic power provided by ER status alone. In addition to the limited tissue distribution [6], the limited number of inducing agents [15], and growth inhibitory activity [9], there is also other evidence that MT-3 has distinct functional properties in the brain compared to the MT-1 and MT-2 isoforms. Despite an approximate 70% sequence identity with MT-1/MT-2, there is recent evidence that the β -domain of MT-3 may have different functional properties than the corresponding β -domain of the MT-1 and MT-2 isoforms [29]. Studies of CdMT-3 using ^{113}Cd -NMR have shown that the β -cluster region of MT-3, in contrast to MT-1/MT-2, is conformationally dynamic [30,31]. A similar conclusion also drawn when the N-terminal region of MT-3 was not resolved by high resolution NMR of CdMT-3 while the C-terminal part of MT-3 was shown to fold into the four metal cluster similar to the α domain of MT-1 and MT-2 [32]. These results, together with the recent

analysis of CdMT-3 using ESI-TOF-MS (Ettan Electrospray time-of-flight mass spectrometry), suggest that the β -domain of MT-3 is adapted for noncooperative binding of variable concentrations of Zn^{+2} or Cd^{+2} ions and exposes high metal exchange potential. This suggests that one of the specific functions of MT-3 in the brain could be buffering of highly fluctuating concentrations of zinc and transfer of zinc into zinc-requiring vesicles. These finding suggest that breast cancer cells that overexpress MT-3 could have alterations in Zn-requiring proteins such as p53, which requires Zn^{+2} to maintain an active protein confirmation.

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**POST-TRANSCRIPTIONAL RESTRICTION OF METALLOTHIONEIN ISOFORM-3
(MT-3) EXPRESSION IN CULTURES DERIVED FROM HUMAN BREAST
EPITHELIAL CELLS**

INTRODUCTION

The third isoform of metallothionein (MT-3) is encoded by a single-gene member of the MT gene family which, in the human, is composed of at least 10 active and 7 pseudogenes [1,2,3,4]. All family members share the property of being low molecular weight (6 kDa), intracellular proteins that have a very high number of conserved cysteine residues that function to allow the efficient binding of transition metals [5]. The MT-1 and MT-2 members of this gene family have been extensively studied and are believed to serve an important role in the homeostasis of essential metals such as Zn^{+2} or Cu^{+2} during growth and development as well as in the detoxification of heavy metals such as Cd^{+2} and Hg^{+2} ; rendering the MTs important mediators and attenuators of heavy metal-induced toxicity, particularly hepato- and nephrotoxicity [5,6,7,8,9,10]. The MT-1 and MT-2 isoforms exhibit a ubiquitous pattern of tissue expression and are highly inducible by any number of stimuli [5,6,9,10]. The MT-3 isoform, on the other hand, has received much less attention. MT-3 has a limited tissue distribution, being initially identified as a brain-specific MT, and is not induced by many of the stimuli known to be inducers of MT-1 and MT-2 isoforms [1,11,12]. Although possessing extensive homology with the MT-1 and MT-2 isoforms, there is a small, but significant structural difference between MT-3 and all other MT isoforms. The MT-3 isoform possesses 7 additional amino acids; a 6 amino acid C-terminal sequence and a Thr in the N-terminal region [1,11,13]. The MT-3 protein has been shown to inhibit the survival and neurite outgrowth of cultured rat cortical neurons when added exogenously to such cultures [11,14] and to have a growth-inhibitory activity when expression is indirectly elevated in cultures of rat C-6 glial cells [13]. The growth inhibitory activity in the neural cells is unique to MT-3 and is not duplicated by the other members of the MT gene family [12,13]. This non-duplication of function occurs despite a 63-69%

identity in amino acid sequence among MT-3 and the other 9 active human MT isoforms [12].

In addition to expression in the neural system, it has been demonstrated that MT-3 is overexpressed in a subset of human breast cancers and that overexpression correlates with poor disease prognosis [15]. Using a similar retrospective analysis of paraffin embedded archival samples, it has also been shown that MT-3 is highly expressed in all human bladder cancers examined to date [16] and to have a variable expression in prostate cancers compared to control tissue [17]. It was also shown in the above studies that MT-3 mRNA and protein was not expressed in normal breast and bladder epithelium and only weakly expressed in normal prostate epithelial cells. Recent studies have also shown that the growth inhibitory activity of MT-3 extends beyond the neural system. The stable transfection of PC-3 prostate cancer cells with MT-3 resulted in markedly reduced cell growth, while transfection with the MT-1E isoform had no effect on cell growth [18]. In breast cancer cell lines, the stable transfection and overexpression of MT-3 was shown to inhibit the growth of the MCF-7 and Hs578T cells, but to have no effect on the growth of the T-47D and MDA-MB-231 cell lines [19]. The overexpression of MT-1E in the MCF-7 and T-47D cells had no effect on cell growth. The goal of the present study was to determine the effect that overexpression of MT-3 would have on the immortal, but non-tumorigenic, MCF-10A human breast epithelial cell line.

RESULTS

Stable Transfection and Expression of MT-3 mRNA in MCF-10A Cells: It was determined using RT-PCR at a total RNA input of 1 µg and 40 reaction cycles that there was no basal expression of MT-3 mRNA in the MCF-10A cell line (Fig 9 A). The coding sequence of the MT-3 gene was obtained from human proximal tubule cell RNA by RT-PCR, blunt end ligated into the *EcoR* V site of pcDNA3.1/Hygro(+), and linearized by *Fsp* I prior to transfection of the respective cell lines. The MCF-10A cell line was transfected with the MT-3 plasmid construct in the sense direction or with the blank vector using the Effectene protocol. Following selection in hygromycin B-containing growth medium, 5 clones of MCF-10A cells were selected for further characterization. It was demonstrated that each of the 5 clones overexpressed MT-3 mRNA when compared to wild type cells or cells containing the blank pcDNA3.1 vector (Fig 9 A). The MT-3 mRNA was first detected from each of the 5 overexpressing clones of each cell line between 20 and 22 cycles of PCR and 500 ng total RNA inputs. This level of expression was similar to that of the glyceraldehyde 3-phosphate dehydrogenase gene. It was also demonstrated that the transfection protocol had no effect on the expression on the glyceraldehyde 3-phosphate dehydrogenase housekeeping gene (Fig 9 B).

Figure 9: MT-3 and glyceraldehydes 3-phosphate dehydrogenase (G3PDH) gene expression in transfected MCF-10A cells. *RT-PCR was used to determine the expression of MT-3 and G3PDH mRNA. The reactions were sampled at 25, 30 and 35 cycles of PCR for the MT-3 transfected clones and the control cells, and the reaction products were visualized ethidium bromide- stained 2% gels. (A) is the MT-3 RT-PCR products at 30 cycles of PCR for the five independent MT-3 clones using 500 ng total RNA, and 40 cycles of PCR for the two clones containing blank vector and the two samples of non-transfected MCF-10A cells using 1 μ g of total RNA. (B) is the G3PDH products at 30 cycles for five independent MT-3 clones, two clones with blank vector and two samples of non-transfected MCF-10A cells.*

The relative level of MT-3 mRNA expression in the MT-3 transfected MCF-10A cells was similar to that obtained in previous studies conducted with identical methods, in which the MT-3 sequence was transfected into the MCF-7, T-47D, Hs578T or MDA-MB-231 breast cancer cell lines [19] and the PC-3 prostate cancer cell line [18] that did not express MT-3 mRNA or protein (Fig 10 A).

Failure of MCF-10A Cells Stably Transfected with MT-3 to Express MT-3 Protein: It was also determined that there was no basal expression of MT-3 protein in the MCF-10A cell line or in the blank vector control, with the limit of detection in cell lysates being between 0.1 - 0.5 ng MT-3/ μ g total cell protein (Fig 10 B). An analysis of MT-3 protein expression in the above 5 clones of MCF-10A cells transfected with the MT-3 sequence also showed very low levels of MT-3 protein, with all 5 clones producing MT-3 near the detection limit of the assay (Fig 10 B). That this was not an artifact of the assay system or the cloning procedure was suggested by the finding that the identical MT-3 sequence transfected into MCF-7, T-47D, Hs578T or MDA-MB-231 breast cancer cell lines [19] and the PC-3 prostate cancer cell line [18] by using identical methodology yielded elevated levels of MT-3 protein expression (Fig 10 B).

Figure 10: The MT-3 mRNA and protein levels in MCF-10A cells. (A) is the relative levels of MT-3 mRNA expression demonstrated by RT-PCR (B) is the levels of MT-3 protein expression as determined by immuno-blot analysis in five independent clones of MT-3 transfected MCF-10A cells, non-transfected MCF-10A cells and a vector only clone. The MT-3 protein levels were also determined in MT-3 transfected MCF-7, T-47D, MDA-MB-231, Hs578T breast cancer cell lines and a PC-3 prostate cancer cell line. Non-transfected MCF-10A cells and vector only transfected MCF-

10A cells showed non-detectable levels of MT-3 protein. Error bars represent the standard errors.

Effect of Cd^{+2} on MT-3 Protein Expression in MCF-10A Cells: Since Cd^{+2} is bound by all members of the MT protein family with high affinity and is an inducer of MT-1 and MT-2 gene expression in most mammalian cell systems, studies were designed to determine what effect Cd^{+2} exposure would have on the accumulation of MT-3 protein in the MT-3 transfected MCF-10A cells.

The expression of MT-3 and the MT-1/2 proteins was determined on confluent MT-3 transfected MCF-10A cells and blank vector controls when exposed to lethal (15 and 30 μM) and sub-lethal (3 and 7 μM) amounts of Cd^{+2} for a 16 day period (Fig 11). There was no difference in toxicity to Cd^{+2} among the MCF-10A parental cell line, the MCF-10A cells transfected with the blank vector control, or the MCF-10A cells stably transfected with the MT-3 sequence (Fig 11). Similarly, there was no difference in Cd^{+2} toxicity among the five MT-3 transfected MCF-10A clones (data not shown).

Figure 11: Viability of MT-3 transfected MCF-10A cells exposed to CdCl_2 . MT-3 transfected MCF-10A cells were exposed to 0 μM (◆), 3 μM (□), 7 μM (▲), 15 μM (○) and 30 μM (■) of CdCl_2 for 16 day of extended time course. Cell viability was determined by automated counting of

DAPI-stained nuclei and all determinations were in triplicate. Values shown are the percentage of the mean cell numbers at each time point divided by the mean cell numbers of the control cells for each triplicate determination.

Exposure of the MT-3 transfected MCF-10A cells to 3 μM Cd^{+2} resulted in no significant increase in the level of the MT-3 protein over that of MCF-10A cells containing the blank vector (Fig 12 A). In contrast, this identical exposure resulted in a significant increase in the level of the MT-1/2 protein in the MT-3 transfectants and the blank vector controls (Fig 12 A). The finding that Cd^{+2} exposure resulted in no increase in MT-3 protein expression in the MT-3 transfected cells and controls was confirmed at increasing concentrations of Cd^{+2} , including those eliciting rapid cell death (Fig 12 B, C, D). In all instances, the MT-1/2 protein level was shown to be significantly increased by Cd^{+2} exposure of the cells under identical experimental conditions (Fig 12 B, C, D).

Figure 12: MT-3 and MT 1/2 protein levels. *MT-3 and MT1/2 protein levels were determined by immuno-blot analysis in MT-3 transfected and vector only transfected MCF-10A cells after exposure to (A) 3 μ M, (B) 7 μ M, (C) 15 μ M and (D) 30 μ M of CdCl₂. MT- 1/2 protein was only determined on days 0,1,2,10 and 16 for the blank vector control.*

To check for an effect of cell proliferation on MT-3 protein accumulation, the MT-3 transfected MCF-10A cells were also exposed continuously to 2, 4, and 6 μ M of CdCl₂ for 5 passages at a 1:10 subculture ratio. An analysis of MT-3 protein expression at the end of 5 passages demonstrated that the MT-3 transfected MCF-10A cells had accumulated no MT-3 protein, with levels near the limit of assay detection (Fig 13).

Figure 13: Analysis of MT-3 protein accumulation in MCF-10A cells after 5 passages. *MT-3 protein levels were determined in MT-3 transfected MCF-10A cells after maintaining them for 5 passages.*

DISCUSSION

It has been demonstrated that MT-3 is overexpressed in a subset of human breast cancers and that overexpression is associated with poor disease prognosis [15]. As part of this study, an analysis of MT-3 mRNA expression in total RNA isolated from breast tissues obtained following elective surgical reductions demonstrated that no MT-3 mRNA was expressed in the normal human breast. This would indicate that at some point along the transformation pathway, the breast epithelial cell gains the ability to express MT-3 mRNA and protein in selected cases of human breast cancer. The finding that MT-3 was overexpressed in some ductal carcinoma *in situ* (DCIS) lesions provides evidence that such overexpression might be early in the pathway leading to progression. To begin to understand the consequences of MT-3 overexpression on the breast epithelial cell *in situ*, the present study was designed to determine the effect that MT-3 expression would have on a cell culture model of the breast epithelial cell. The cell culture model chosen for use was the immortal MCF-10A human breast epithelial cell line that retains contact inhibition of growth and does not form colonies in soft agar or tumors in nude mice. The initial studies using this cell line demonstrated that the MCF-10A cells had no expression of MT-3 mRNA, a finding in agreement with that found for MT-3 mRNA expression in the *in situ* human breast. The finding that MCF-10A cells had no basal expression of MT-3 mRNA allowed an experimental strategy to be employed that involved the stable transfection and overexpression of the MT-3 gene into this cell line using widely employed and established methodology.

The stable transfection of MT-3 into the MCF-10A cell line did not result in the expected overexpression of MT-3 mRNA and protein. Rather, it was demonstrated that stable transfection

resulted only in the expected overexpression of MT-3 mRNA, but that there was no corresponding expression of the MT-3 protein. There is strong evidence that the lack of MT-3 protein expression was a legitimate finding and not due to a failure of the transfection technology. The most important evidence is that the identical vector has been used successfully for the stable transfection of the MT-3 sequence into the MCF-7, T-47D, Hs578T and MDA-MB-231 breast cancer cell lines [19], and the PC-3 prostate cancer cell line [18]. As detailed in the results, all 5 of these cell lines produced comparable amounts of MT-3 mRNA and increased amounts of the MT-3 protein. The stable transfection of MT-3 into these cell lines resulted in marked growth inhibition for the MCF-7, Hs578T and PC-3 cell lines; suggesting that the transfected MT-3 protein retained its growth inhibitory bioactivity first noted in the neural system [1,11,14]. The identical vector system has also been utilized successfully to stably transfect the HK-2 cell line, an immortalized non-tumorigenic cell culture model of the human renal proximal tubule [22]. In this study, the MT-3 stable transfectants were shown to express both MT-3 mRNA and protein, and to regain the differentiated property of vectorial active ion transport. The MT-3 protein has also been shown to be expressed *in situ* in many of the epithelial cell types of the human kidney including those of the proximal tubule [23]. In a current study using an immortalized, non-tumorigenic, cell culture model of human urothelium (UROtsa cells), stable transfection with MT-3 has produced comparable findings to those found using the MCF-10A cell line [D. Sens, personal communication]. Stable transfection of MT-3 into the UROtsa cell line was shown to result in MT-3 mRNA expression similar to that noted above in the MCF-10A cells, but to result in no expression of the MT-3 protein. Similar to the human breast, MT-3 has been shown to be overexpressed in bladder cancer, but to have no expression in the normal human bladder urothelium [16]. Thus, the present study shows that MT-3 protein expression is restricted in the MCF-10A cells stably transfected with the MT-3 coding sequence.

One reason that the MCF-10A cells stably transfected with the MT-3 coding sequence would restrict the expression of the MT-3 protein would be if such expression would be lethal or prevent cell proliferation. Since stable transfection requires the clonal growth of the antibiotic resistant, transfected cells from the original population, there is an obvious selection bias for cells that can survive and proliferate in culture. In this regard, it is not unusual for attempts at stable transfection to end with false positive colonies that have adapted to survive the selection procedure and that do not express the transfected gene sequence. What is surprising in the present study, is that the stably transfected MCF-10A cells express MT-3 mRNA and not protein; and that malignant breast cancer cell lines show no such restriction, being able to express both MT-3 mRNA and protein under identical transfection conditions. While there presently is no mechanistic explanation for these findings or what might stabilize the expression of the MT-3 protein, one possible mechanism was removed from consideration. It is well-documented from studies in rodents, and in cell lines derived from rodent and human resources, that the MT-1 and MT-2 isoforms are stimulated by a wide variety of factors and are primarily regulated at the level of transcription [24]. However, there is recent evidence in rodents following treatment with Cu^{+2} and Cd^{+2} which shows that the MT-1 and MT-2 protein levels can be regulated at the level of translation and that protein level often has no clear relationship with the level of mRNA [25,26]. In these studies high tissue levels of MT-1 and MT-2 mRNA expression were found to be unaccompanied by MT protein accumulation until the cells were exposed to either Cu^{+2} and Cd^{+2} , at which time there was a substantial accumulation of MT protein without additional mRNA expression. An explanation for this finding was that metal saturation of the MT protein stabilizes the MT protein against degradation. In the present study, the treatment of the MT-3 transfected MCF-10A cells with both lethal and sub-lethal concentrations of Cd^{+2} had no effect on MT-3 protein accumulation, even though this exposure did result in greatly

elevated levels of the MT-1 and MT-2 proteins. This effectively rules out the possibility that MT-3 protein is not found in MCF-10A cells due to there being insufficient metal to saturate the MT-3 protein and thus prevent it from being rapidly degraded due to a lack of metal saturation. The finding that MT-1 and MT-2 protein was induced by Cd^{+2} treatment also provides evidence that the MCF-10A cells are able to accumulate Cd^{+2} from the external environment.

There exists only one study which has analyzed matched samples from cancer patients for both MT-3 mRNA and protein expression. In this study, a retrospective analysis of MT-3 mRNA and protein expression was performed on archival paraffin-embedded specimens of bladder cancer [16]. Immunohistochemistry was used to semi-quantify the MT-3 protein which it was found to be unexpressed in the normal bladder and to have low intensity staining in low-grade bladder cancers, moderate staining in high grade bladder cancers, and very intense staining in *carcinoma in situ* (CIS), with QuickScore values of 1.29, 2.07 and 2.43 for the cancers, respectively. An analysis of MT-3 mRNA on total RNA extracted from paraffin-embedded sections of the identical cancer specimens demonstrated that MT-3 mRNA was present in all the cancers at the same relative levels of expression when compared to the β -actin housekeeping gene. While both techniques can be subject to substantial technical errors, the data provide initial evidence that post-transcriptional regulation of MT-3 expression might also occur in *in situ* cancers. This would suggest that MT-3 expression could be a two-step process during progression, the first being the overexpression of MT-3 mRNA which could be followed later by the overexpression of the MT-3 protein.

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**HIGH CONSTITUTIVE EXPRESSION OF METALLOTHIONEIN ISOFORM 1 AND 2
mRNA, BUT NOT PROTEIN, IN NORMAL HUMAN BREAST EPITHELIAL CELLS
AND THE EFFECT OF CADMIUM EXPOSURE ON IMMORTALIZED HUMAN
BREAST CELL LINE, MCF-10A**

INTRODUCTION

In addition to their role in metal toxicology, an involvement of MT-1 and MT-2 in the development of human breast cancer is suggested by numerous studies which show a correlation between MT-1/2 protein expression and breast cancer prognosis and progression. In an immunohistochemical analysis of MT-1/2 expression in 86 cases of routinely fixed and paraffin-embedded primary breast carcinomas, MT over-expression was found in the invasive components of 7 of 32 pT1 and 17 of 28 pT2 invasive ductal carcinomas, whereas all 26 invasive lobular carcinomas gave weak or negative results [1]. Fourteen of 17 pT2 and 2 of 7 pT1 invasive ductal carcinomas with MT over-expression developed metastases during follow-up with poor prognostic outcome. In contrast, only 3 of 11 pT2 and none of the 25 pT1 cases without MT over-expression had a poor clinical course. This difference was found to be highly significant. This initial study concluded that MT over-expression is associated with a significantly poorer prognosis, particularly in pT2 invasive ductal breast carcinomas. These findings were confirmed and extended in a study employing 79 breast carcinomas [2]. In this study, a statistically significant association was found between MT immunostaining and histological grade as well as nuclear grade. An inverse relationship between MT staining and estrogen receptor content of tumors was also demonstrated. A statistically significant association was demonstrated between moderate and strong MT immunostaining and decreased overall survival and shorter disease-free survival. MT immunostaining was also predictive of a worse prognosis in the subgroup of lymph node-negative and estrogen receptor-negative patients. These studies were further confirmed and augmented to include an analysis showing that MT staining was also predictive of a poor prognosis in lymph node positive patients [3].

The expression of MT was also analyzed in normal breast tissue and in a variety of benign, pre-invasive, and malignant breast lesions [4]. It was confirmed in this study that normal breast tissues did not stain for MT. Also, of particular significance was the finding that in 12/24 ductal *in situ* carcinomas and in 9/20 invasive ductal carcinomas there was MT over-expression; suggesting that the *in situ* components within invasive ductal carcinomas generally reflected the MT status of their invasive counterpart. It was concluded that breast carcinoma cases with MT over-expression arise from lesions which also demonstrate MT over-expression and that MT over-expression in carcinomas is a genuine feature of the tumor cells and not simply related to endogenous or exogenous factors known to induce MT synthesis. This concept is in agreement with studies by Douglas-Jones and coworkers [5] who analyzed MT expression in duct carcinoma *in situ* and found MT over-expression to arise from early lesions which also over-express MT. Two other recent studies confirm and reinforce these findings [6, 7]. A recent large study, with multiple prognostic and proliferative markers in addition to MT, concludes that MT-1/2 immunoreactivity has potential for use as a prognostic marker for breast cancer [8].

To effectively study the role of MT-1 and MT-2 expression in the development and progression of breast cancer requires a cell culture model representative of the normal human breast epithelial cell. The initial goal of the present study was to determine if the immortalized human breast epithelial cell line, MCF-10A, could serve as an effective model to study the regulation of metallothionein expression in the human breast epithelial cell.

RESULTS

Basal Expression of MT-1 and MT-2 mRNA and Protein in MCF-10A Cells: Total RNA and protein was isolated from confluent MCF-10A cells and used to determine the basal expression of MT-1/2 protein and the MT isoform-specific mRNAs using RT-PCR as described previously [9, 10, 11]. Based on a total RNA input of 500 ng and 40 cycles of PCR there was no detectable expression of mRNA representing the MT-1A, MT-1B, MT-1F, MT-1G, or MT-1H genes in MCF-10A cells (Fig 14). In contrast, mRNAs representing the MT-1E, MT-1X, MT-2A and g3pdh genes were expressed in MCF-10A cells based on identical total RNA inputs following 30, 22, 25 and 30 cycles of PCR, respectively (Fig 14).

Figure 14. RT-PCR assay showing of MT-1A, -1B, -1E, -1F, -1G, -1H, -1X, -2A and glyceraldehyde 3-phosphate dehydrogenase (g3pdh) mRNA in MCF-10A cells. *No expression was detected at 40 cycles of PCR in reactions containing 500 ng of total RNA for MT-1A, -1B, -1F, -1G and -1H. In contrast, MT-1E, -1X, -2A and g3pdh mRNAs were detected at 30, 22, 25 and 30 cycles of PCR, respectively. The last two lanes representing the negative controls for the RT-PCR*

reactions include a non-template control (C1) where water was added instead of the RNA and no-reverse-transcriptase control (C2) where water was added instead of enzyme.

Based upon the IOD of g3pdh at 30 cycles and MT-1E at 40 cycles, MT-1X at 30 cycles and MT-2A at 35 cycles, the relative abundance (IOD of the specific isoform/IOD of g3pdh) of each MT mRNA was 0.72 for 1E, 0.57 for 1X, and 0.45 for MT-2A (Fig. 15 A, B, C) Immuno-blotting with an MT antibody that recognizes both the MT-1 and MT-2 isoforms, but not the other isoforms of MT, demonstrated that the MCF-10A cells expressed 3.3 ± 0.6 ng (n = 6) of MT-1/2 protein/ μ g total cell protein (Fig. 15 D).

Figure 15. Relative expression of MT-1E, -1X and -2A mRNA, and Protein in two samples (sample A and Sample B) of human breast tissue and MCF-10A cells compared with the g3pdh house keeping gene. (A) *Relative expression of MT-1E mRNA.* (B) *Relative expression of MT-1X mRNA.* (C) *Relative expression of MT-2A mRNA.* (D) *MT-1/2 protein levels in two samples of human breast tissue and MCF-10A cells.*

Basal Expression of MT-1 and MT-2 mRNA and Protein in Human Breast Tissue and MT-1 and MT-2 mRNA in Microdissected Breast Duct Epithelial Cells: Total RNA and protein were prepared from human breast tissue obtained from two cases of elective reduction surgeries. The tissue was dissected free from attached fat prior to the isolation of total RNA and protein based on the different textures and colors of the tissue types. Fatty tissue was identified as gelatinous and yellow while the ductal tissue was identified as firm and tan. Using a total RNA input of 500 ng and 40 cycles of PCR it was demonstrated that there was no expression of mRNA representing the MT-1A, MT-1B, MT-1F, MT-1G, and MT-1H genes in both samples of the human breast tissue (data not shown). Using an identical total RNA input, it was demonstrated that mRNAs for MT-1E, MT-1X, MT-2A and g3pdh genes were detectable in both samples of human breast tissue at 40, 30, 35, and 30 PCR cycles, respectively (Fig 16). To allow a comparison to the MCF-10A cell line, the expression of MT-1E, MT-1X and MT-2A was expressed relative to g3pdh at identical cycle numbers to that described above for MCF-10A cells. On this basis, it was demonstrated that MT-1X mRNA was expressed at a level approximately twice that found in MCF-10A cells; MT-2A mRNA at a level approximately equal to that found in MCF-10A cells; and, MT-1E at a level approximately one-half than that found in MCF-10A cells (Fig. 15 A, B, C). The level of MT-1/2 protein in each of the human breast samples was also determined and found to be 1.1 ± 0.2 and 1.2 ± 0.3 of MT-1/2

protein/ μ g total cell protein (Fig. 15 D).

Figure 16. Accumulation of MT-1E, -1X, -2A and glyceraldehyde 3-phosphate dehydrogenase (g3pdh) mRNA in two samples of human breast tissue (A and B) and MCF-10A (M) cells. Using 500 ng of total RNA input, MT-1E, -1X, -2A and g3pdh mRNA were detected at 40, 30, 35 and 30 PCR cycles of PCR, respectively. Negative controls represent the RT-PCR reactions include a non-template control (C1) where water was added instead of the RNA and no-reverse-transcriptase control (C2) where water was added instead of enzyme.

The expression of MT-1X and MT-2A mRNA was also determined on two samples of normal breast duct epithelial cells enriched from surrounding stromal and vascular cells using the technique of laser capture microdissection. This analysis was performed to gain further evidence that the MT-isoform mRNAs were present in the duct epithelial cells. For this analysis, total RNA was prepared from ducts isolated from full-thickness (5 micron) paraffin-embedded breast tissue sections following laser capture microdissection (Fig 17 A, B).

(A)

(B)

Figure 17. Microdissection of ductal epithelial cells from normal breast tissue. (A) Five micron thick section of paraffin-embedded normal breast tissue was stained lightly with hematoxylin and eosin before laser capture. (B) Tissue section shown is after laser capture microdissection.

The RT-PCR was performed as described above, except a primer pair for the β -actin housekeeping gene producing a 194 bp product was also included in the analysis. The β -actin primer pair provided a more appropriate control to the analysis since the PCR product is similar in size to the MT PCR reaction products. The g3pdh PCR product is much larger than the MT isoform mRNA reaction products and it has been reported that total RNA from formalin-fixed, paraffin-embedded tissue yields a higher proportion of fragmented RNA relative to intact RNA than fresh tissue [12]. Laser captured microdissection technique also failed to detect MT-1E mRNA from paraffin embedded slides at 40 cycle PCR while RT-PCR analysis of fresh breast mRNA at 40 cycle yield MT-1E mRNA message. The results of the analysis of total RNA isolated from the two samples of microdissected duct cells demonstrated the expression of MT-1X and MT-2A in the ductal epithelial cells (Fig 18). MT protein could not be determined since protein cannot be extracted from formalin-fixed tissue.

Figure 18. Basal expression of MT-1X and MT-2A in normal human breast. Bands representing RT-PCR products for β -actin (194 bp), MT-1X (151 bp) and MT-2A (259 bp) after 40 cycle PCR.

Expression of MT-1/2 Protein and MT-Isoform Specific mRNAs When MCF-10A Cells are Exposed to Lethal and Sublethal Levels of Cd^{+2} : The above results demonstrated that both the *in situ* normal breast epithelial cells and the MCF-10A cells expressed abundant amounts of MT mRNA, but only a very modest amount of MT-1/2 protein. Since Cd^{+2} is bound to the MT protein with high affinity and is an inducer of MT gene transcription in most mammalian cell systems, studies were designed to determine what effect Cd^{+2} exposure would have on the accumulation of MT mRNA and protein in the MCF-10A cells. This was accomplished by determining the expression of the MT-1 and MT-2 isoform-specific mRNAs and MT-1/2 protein when the MCF-10A cells were exposed to CdCl_2 over a short-term (48 h) and extended (16 day) time course. Four

concentrations of CdCl_2 were utilized: $3\ \mu\text{M}$ which produced no cell death over the 16 day time course; intermediate concentrations of $7\ \mu\text{M}$ and $15\ \mu\text{M}$, which produced approximately 50% cell death over the 16 day time course; and, $30\ \mu\text{M}$ which produced significant levels of cell death midway in the 16 day time course (Fig 19). The respective mRNA and proteins were determined at 4, 8, 12, 24 and 48 h for the short-term time course and at 1, 2, 4, 7, 10, 13 and 16 days for the extended time course. The expression of MT mRNA was normalized to a control value of 1.0 for data presentation. For each of the respective MT mRNAs analyzed there was no significant difference in the expression levels of the 48 h and 16 day time courses over the control cells (data not shown). The RT-PCR analysis of MT-isoform-specific MT-1 and MT-2 mRNAs at 500 ng total RNA inputs and 40 reaction cycles demonstrated no expression of the MT-1A, MT-1B, MT-1F, MT-1G, or MT-1H mRNA at any point in the time course (data not shown).

Figure 19. Viability of MCF-10 cells exposed to CdCl_2 . The MCF-10 cells were exposed to $0\ \mu\text{M}$ (◆), $3\ \mu\text{M}$ (□), $7\ \mu\text{M}$ (▲), $15\ \mu\text{M}$ (○) and $30\ \mu\text{M}$ (■) of CdCl_2 for 16 days of extended time course.

Cell viability was determined by automated counting of DAPI-stained nuclei and all determinations were in triplicate. Values shown are the percentages of the mean cell numbers at each time point divided by the mean cell numbers of the control cells for each triplicate determination.

Analysis of the MCF-10A cells exposed to 7, 15 or 30 μM Cd^{+2} for 48 h demonstrated that there was no significant increase in MT-1X or MT-2 mRNA and a significant increase in MT-1E mRNA at only 3 of the 21 points of acute time course (Fig 20 A, B, C). In contrast, the corresponding analysis of MT-1/2 protein expression demonstrated that following 24 h of Cd^{+2} exposure, there were significant increases in MT-1/2 protein at all three levels of Cd^{+2} exposure (Fig 20 D). The level of MT-1/2 protein showed a significant increase with increasing time of exposure between 24 and 48 h, but there was not a dose response as judged by there being no significant difference ($p > .05$) in accumulation due to the level of exposure to Cd^{+2} at either the 24, 36 or 48 h time points.

Figure 20. Relative expression of MT-1E, -1X, -2A mRNA, and MT 1/2 protein levels in MCF-10A cells after short-term exposure to CdCl₂. MCF-10 cells were exposed to 7 μ M (grey bars), 15 μ M (white bars), and 30 μ M (black bars) CdCl₂ for 48 hrs. The expression of MT-1E, MT-1X and MT-2A mRNA is shown relative to that of the g3pdh housekeeping gene and then normalized to a control value of 1.0. The determinations were performed in triplicate.

Exposure of the MCF-10A cells to 3 μ M Cd⁺² for 16 days demonstrated no increase in the expression of mRNAs for the MT-1E, MT-1X and MT-2A genes (Fig 21 A, B, C). An increase in exposure level to 7 μ M Cd⁺² for 16 days produced no increase in MT-1X mRNA and a modest, but significant increase in MT-2A mRNA on days 13 and 16 and of MT-1E mRNA on day 4 and 10 of the time course. A further increase in exposure level to 15 μ M Cd⁺² resulted in a significant increase in MT-1X on day 2; MT-2A on days 10, 13 and 16; and, MT-1E on days 4 and 13. These increases were approximately 2-fold over control. The highest level of Cd⁺² exposure (30 μ M) which was 100% lethal to the cells by day 7 of the time course produced no significant increase in MT-2A mRNA and a significant increase in MT-1E and 1X on day 2. There was no evidence of a dose response between mRNA expression of the MT-2A, MT-1X or MT-1E genes and Cd⁺² level. In contrast, the level of MT-1/2 protein was significantly increased at all levels of Cd⁺² exposure by day 4 of the time course (Fig 21D). The MT-1/2 protein level at the lowest level of Cd⁺² exposure that produced no cell lethality over the 16 day time course, increased from 4 to 37 ng/ μ g total protein by day 16 of exposure. There was an incremental increase in MT-1/2 protein at day 4, 7 and 10 with a plateau at day 10 through 16. A similar pattern of MT-1/2 protein accumulation occurred at the 7 and 15 μ M levels of Cd⁺² exposure, except the increase and plateau occurred earlier in the time course (day 2 and 7, respectively) and levels of accumulation were approximately 2-fold higher (5 to 6% of total cell protein). There was no evidence of a dose response between MT-1/2 protein expression and level of Cd⁺² exposure.

Figure 21. Relative expression of MT-1E, -1X, -2A mRNA, and MT 1/2 protein levels in MCF-10A cells after long-term exposure to 3 μ M, 7 μ M, 15 μ M and 30 μ M of CdCl₂. MCF-10 cells were exposed to 3 μ M (striped bars) 7 μ M (grey bars), 15 μ M (white bars), and 30 μ M (black bars) CdCl₂ for 48 hrs. The expression of MT-1E, MT-1X and MT-2A mRNA is shown relative to that of the g3pdh housekeeping gene and then normalized to a control value of 1.0. The determinations were performed in triplicate.

DISCUSSION

The initial goal of this study was to determine if the immortalized, but non-tumorigenic, MCF-10A human breast epithelial cell line could serve as a model to study the dysregulation of MT in human breast cancer. The need for a model of the breast epithelial cell that recapitulates the *in situ* expression pattern of the first and second isoforms of MT is based on the wealth of information showing MT-1/2 expression is altered in patients with breast cancer. In retrospective patient-based studies it has been shown that the overexpression of MT-1/2 in breast cancer is associated with a poor prognosis [1, 2, 3, 6, 13, 14]; higher histological grade of the tumor [2, 5, 14, 15]; estrogen receptor status [2, 3, 7, 8]; and lymph node involvement [3, 16]. MT measurement these studies was based on an antibody that recognizes both the MT-1 and MT-2 isoforms of the MT gene family, but not the MT-3 and MT-4 isoform family members [17]. In all these studies, normal breast epithelium did not react with MT-1/2 antibody. As such, the first investigation of the MCF-10A cells was to determine if the expression of MT-1/2 protein was similar to that found in tissue obtained from the normal human breast. The MCF-10A cells were found to express approximately 3.3 ng of MT-1/2 protein/ μ g total protein compared with 1.15 ng of MT-1/2 from normal breast tissue. These levels of

expression are in good agreement with one another, and are in even closer agreement when consideration is given to the fact that the *in situ* sample would be contaminated with stromal and vascular cell types. Thus, the basal expression of the MT-1/2 protein is one indication that the MCF-10A cell line might model MT expression in the normal breast epithelial cell.

The expression of the human MT-1 and MT-2 isoform proteins is theoretically supported by seven active MT-1 genes and one active MT-2 gene; designated as MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X and MT-2A [18, 19]. The expression of these genes has not been previously determined in the normal breast epithelial cell or the MCF-10A cell line. The MCF-10A cells were found to express no mRNA for the MT-1A, MT-1B, MT-1F, MT-1G and MT-1H genes, but did express mRNA for the MT-1E, MT-1X and MT-2A genes. The MT-1X, MT-2A and MT-1E were found to be very abundant transcripts and in large excess to what would be expected for the accumulation of the low amount of MT-1/2 protein (3.3 ng/μg total protein) that was found in MCF-10A cells. The major reason for concluding that MT-1/2 mRNA was in large excess over that needed to support MT-1/2 protein accumulation was the fact that transcripts for MT-1X were detected at 22 cycles, MT-2A at 25 cycles and MT-1E at 30 cycles of PCR. With a total RNA input of 500 ng these cycle levels are indicative of very abundant transcripts and the levels were much more abundant than that of mRNA from the g3pdh housekeeping gene, which was detected at 30 cycles. Furthermore, amounts of MT-1X, -1E, -2A and g3pdh mRNAs are probably underestimated due to the use of higher temperatures required for the MT RT-PCR reactions; a condition necessary to assure primer specificity among the highly homologous MT isoforms [11]. This finding generated initial concern that the MCF-10A cells might have inappropriate MT mRNA expression and be compromised as a model system. While it is difficult to predict with certainty the amount of

mRNA necessary to support the synthesis of a given amount of any protein, the concern that MCF-10A cells might have inappropriately overexpressed MT-1 and MT-2 mRNA was alleviated when MT-1 and MT-2 mRNA was analyzed in normal breast tissue. Normal breast tissue also expressed mRNA for the MT-1E, MT-1X and MT-2A genes at levels similar to that of the MCF-10A cell line. Laser capture microdissection was used to further support that this expression originated from ductal epithelial cells. Thus, it was demonstrated that the MCF-10A cell line has basal expression of the MT-1 and MT-2 mRNA isoforms similar to that of the *in situ* breast epithelial cell.

It is well-documented from studies with rodents, and in cell lines derived from rodent and human sources, that MT-1 and MT-2 are stimulated by a wide variety of factors and are primarily regulated at the level of transcription [20]. However, there is recent evidence in rodents following treatment with Cu^{+2} and Cd^{+2} which shows that MT protein levels can be regulated at the level of translation and that protein level often has no clear relationship with mRNA levels [21]. To determine if the high levels of basal MT-1 and MT-2 mRNA found in MCF-10A cells might be evidence for translational regulation of MT-1/2 protein accumulation, the MCF-10A cells were exposed to lethal and sub-lethal levels of Cd^{+2} under both acute and extended periods of exposure. Cadmium is a metal pollutant that binds MT with high affinity and is a classic inducer of MT transcription [19, 22]. For the MCF-10A cells, it was demonstrated that Cd^{+2} elicited only a marginal increase in MT-1E, MT-1X or MT-2A mRNA abundance at either lethal or sub-lethal levels of exposure under both acute and extended periods of exposure. However, Cd^{+2} exposure did result in a large increase in MT-1/2 protein under identical conditions, reaching levels of 6% of total cell protein following extended exposure to Cd^{+2} . The only other organ that has been examined in a manner similar to that of the human breast is the human bladder urothelium. The *in situ* human bladder was shown to

express no MT-1/2 protein, but to have expression of the MT-1E, MT-1X, and MT-2A mRNAs, with each mRNA level being substantially less than that of the breast [23]. A human immortalized, but non-tumorigenic cell line (UROtsa), was shown to have a similar expression of the MT-1E, MT-1X and MT-2A genes and MT-1/2 protein as that of human urothelium [24]. Treatment of the UROtsa cell line with Cd^{+2} under similar conditions to that of the MCF-10A cells resulted in only a modest increase in the MT mRNAs and an intermediate increase in MT-1/2 protein; a result suggesting that both transcriptional and post-transcriptional regulation could be a factor in the human bladder [25]. The present findings with the MCF-10A cells are strong evidence that translational regulation could be a major mechanism in determining MT-1/2 protein levels in human breast epithelial cells.

The finding that Cd^{+2} might increase MT-1/2 protein accumulation in MCF-10A cells through a post-transcriptional mechanism is interesting in light of the study from Finland which demonstrated an association between human breast concentrations of Cd^{+2} and breast cancer [26]. One consequence of a Cd^{+2} -mediated post-transcriptional accumulation of MT-1/2 protein that would be consistent with development of a cancer cell phenotype would be increased resistance of the cell to programmed cell death. Studies of cells from MT-null mice have provided direct evidence that MT expression prevents apoptosis [27], and studies in the MCF-7 breast cancer cell line have shown that the antisense down-regulation of MT-1/2 expression in the malignant breast epithelial cell increases sensitivity to apoptosis [28]. Generating further interest in Cd^{+2} exposure and breast cancer is the recent study that demonstrates estrogen receptor positive, p53 positive, breast cancer cell lines accumulate higher levels of MT-1/2 protein when exposed to Cd^{+2} than estrogen receptor negative, p53 negative, breast cancer cell lines [29]. There is also evidence that MT-1E gene expression is

altered at the level of transcription in human breast cancer. In a study of 4 breast cancer cell lines, MT-1E mRNA was highly expressed in 2 estrogen receptor negative cell lines (MCF-7 and T-47D), but absent in the 2 estrogen receptor positive cell lines (Hs578T and MDA-MB-231) [9]. Increased expression of MT-1E mRNA correlated with increased MT-1/2 protein expression [9]. This cell culture-defined relationship was confirmed in a study of fresh, surgically removed breast cancer tissue in which it was shown that MT-1E mRNA was highly expressed in estrogen receptor-negative human invasive ductal breast cancers compared to estrogen receptor-positive tumors [30]. The MCF-10A cell line should be helpful in determining the changes that occur in the regulation and expression of the MT-1 and MT-2 isoforms between the normal and malignant breast epithelial cell.

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APPENDIX A

Dulbecco's Modified Eagle Medium (DMEM)

Low glucose DMEM (Gibco-BRL, Rockville, MD., Cat No. 31600-75) with L- glutamine and sodium pyruvate (100 mg/ml) but without sodium bicarbonate, was stored in powdered form at 4⁰ C. To make 5 liters of DMEM, 49.9 gr of powder DMEM was dissolved in 4,950 ml of sterile water in a 6 liter flask by constantly stirring. Fifty ml of Penicillin/Streptomycin (Gibco-BRL, Cat No. 15140-122), 5 ml of Fungizone™ (Gibco-BRL, Cat No. 15295-017) and 18.5 gr of sodium bicarbonate (Gibco-BRL, Cat No. 11810-033) were added to the 4,950 ml solution. The solution was stirred for 45 minutes at room temperature. Then the solutions was filtered using 0,22 µm Acrocap™ filters (Gelman Laboratory, Ann Arbor, MI, USA. Cat. No. PN4480). Filtered medium was stored in 500 ml aliquots at 4⁰ C for up to three weeks.

Ham's F-12 Nutrient Mixture

Ham's F-12 Nutrient Mixture (Gibco-BRL, Cat No. 21700-109) with L-glutamine, but without sodium bicarbonate, was stored in powdered form at 4⁰ C. To make 5 liters of F-12 Medium, 53.1 gr of powder Ham's F-12 was dissolved in 4,945 ml of sterile water in a 6 liter flask by constantly stirring. Fifty ml of Penicillin/Streptomycin (Gibco-BRL, Cat No. 15140-122), 5 ml of Fungizone™ (Gibco-BRL, Cat No. 15295-017) and 5.88 gr of sodium bicarbonate (Gibco-BRL, Cat No. 11810-033) were added to the 4,945 ml solution. The solution was stirred for 45 minutes at room temperature. Then the solution was filtered usin 0.22 µm Acrocap™ (Gelman Laboratory, Ann Arbor, MI, USA. Cat. No. PN4480) filter. Filtered medium was stored in 500 ml aliquots at 4⁰ C for up to three weeks.

Phosphate Buffer Saline (1 x PBS)

To make 5 liters of 1 x PBS, 42.5 gr of NaCl (Sigma, Cat No. S-9888) was combined with 0.6 gr of Na₂HPO₄ (Fisher Scientific, Fair Lawn, NJ, USA. Cat No.S-374) and 0.12 gr NaH₂PO₄ (Fisher Scientific, Cat No.S-369) in a total volume of 5 liters of sterile water. PBS was aliquoted into 500 ml bottles and autoclaved. PBS was stored up to a month at 4⁰ C.

Breast Cancer Cell Line Medium

MCF-7, T-47D, Hs578T and MDA-MB-231 cell lines were maintained in DMEM supplemented with 5% Fetal Calf Serum (Hyclone, Logan, UT, Cat No. SH30070.03) and 5mg/ml Glucose (Dextrose, Fisher, Fair Lawn, NJ Cat No. D16-500). Glucose was reconstituted in sterile water.

Immortalized Normal Breast Cell Line, MCF-10 Medium

MCF-10 cell line was maintained in 1:1 mixture of Ham's F-12 medium and DMEM supplemented with 5% (v/v) fetal calf serum (Hyclone, Logan, UT, Cat No. SH30070.03), 10 µg/ml insulin (Sigma Inc, St. Louis, MO, Cat No. 58H0375), 0.5 µg/ml hydrocortisone (Sigma Inc, St. Louis, MO, Cat No. 101K8925), 20 ng/ml epidermal growth factor (Invitrogen Life Technologies, Cat No 13247-051), 0.1 µg/ml cholera toxin(Sigma Inc, St. Louis, MO, Cat No. MFCD00212923). All the reagents were reconstituted in sterile water.

APPENDIX B

GENE	VENDOR	CONC.	SEQUENCE UPPER PRIMER	CONC.	SEQUENCE LOWER PRIMER
GA3PDH (983 BP)	CLONETE CATNO.54 06	0.1UM	5'TGAAGGTCGGTGT GAACGGATTTGGC3'	0.1UM	5'CATGTAGGCCATG AGGTCCACCAC3'
MT-1A (219 bp)	Gibco-BRL	0.1uM	5'CTCGAAATGGACC CCAAC3'	0.1uM	5'ATATCTTCGAGCAG GGCTGTC3'
MT-1B (287 bp)	Gibco-BRL	0.1uM	5'GCTTGTCTTGGCTC CACA3'	0.1uM	5'AGACAAACCGGTC AGGTAGTT3'
MT-1E (284 bp)	Gibco-BRL	0.1uM	5'GCTTGTTCTGTCTCA CTGGTG3'	0.1uM	5'CAGGTTGTGCAGG TTGTTCTA3'
MT-1F (232 bp)	Gibco-BRL	0.1uM	5'AGTCTCTCCTCGGC TTTGC3'	0.1uM	5'ACATCTCTGGGAG AAAGGTTGGTC3'
MT-1G (309 bp)	Gibco-BRL	0.1uM	5'CTTCTCGCTTGGGA ACTCTA3'	0.1uM	5'AGGGGTCAAGATT GTAGCAA3'
MT-1H (315 bp)	Gibco-BRL	0.1uM	5'CCTCTTCTCTTCTC GCTTGG3'	0.1uM	5'GCAAATGAGTCGG AGTTGTAG3'
MT-1X (151 bp)	Gibco-BRL	0.4uM	5'TCTCCTTGCCTCGA AATGGAC3'	0.4uM	5'GGGCACACTTGGC ACAGC3'
MT-2A (259 bp)	Gibco-BRL	0.1uM	5'CCGACTCTAGCCGC CTCTT3'	0.1uM	5'GTGGAAGTCGCGT TCTTTACA3'
MT-3 (325 bp)	Gibco-BRL	0.1uM	5'CCGTTACCGCCTC CAG3'	0.1uM	5'CACCAGCCACACTT CACCACA3'
MT-4 (213 bp)	Gibco-BRL	0.1uM	5'CATGGACCCAGG GAATGTGT3'	0.1uM	5'GGGGTGGGCACGA TGGA3'

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pairs were obtained from Clontech (Palo Alto, CA, USA). The selection of metallothionein primer pairs for RT-PCR was described by Mididoddi *et al.* (1996) Toxicol Lett. 85(1):17-27).

<u>PCR Product</u>	<u>Denaturation</u>	<u>Optimal Cycles</u>	<u>Optimal Cycles</u>
Clontec GA3PDH	95 ⁰ for 2 min	95 ⁰ C for 30 sec 68 ⁰ C for 30 sec	25 and 30 cycles
Metallothionein 1A, 1B, 1F, 1G 1H, MT-3, MT-4	95 ⁰ C for 2 min	95 ⁰ C for 30 sec 68 ⁰ C for 30 sec	30, 35, 40 cycles
Metallothionein 1E, 1X, 2A	95 ⁰ C for 2 min	95 ⁰ C for 30 sec 68 ⁰ C for 30 sec	22, 25, 30 cycles

APPENDIX C

ANTIBODIES FOR THE DETECTION OF METALLOTHIONEIN PROTEINS

MT 1/2, E9 Antibody (1:1000 dilution), Primary Antibody

4,5 ml of 10% BSA, 225 µl of 4% NaN₃, 450 µl of E9 MT 1/2 antibody (Dako Corp., Carpinteria, CA, Cat No. Mo639), 1 x PBS to 45 ml.

Anti-Mouse IgG Antibody (1:500 dilution), Secondary Antibody

1.8 ml of 10 % BSA, 225 µl of 4% NaN₃, 90 µl of antibody (1µg/ml) (Promega, Cat No. S372B), 1 x PBS to 45 ml.

MT-3 Polyclonal Antibody (1:100 dilution), Primary Antibody

9 ml of 10% BSA, 225 µl of 4% NaN₃, 112.5 µl of 2 µg/µl metallothionein 3 anti-rabbit antibody, 1 x PBS to 45 ml. The synthesis of the metallothionein 3 anti-rabbit antibody has been described previously (Garrett, *et al.* (1999) Toxicol Lett. 105(3):207-214). New Zealand White rabbits were immunized with a peptide (GGEAAEAEAEKC) corresponding to MT-3 amino acids 53-64 that contain the MT-3 unique amino acid insert. The dodeca-peptide was conjugated through the C-terminal sulfhydryl group to keyhole limpet hemocyanine using maleimidobenzoyl-N-hydroxysuccinimide ester. The MT-3 antibody was affinity purified using the dodecapeptide linked to a SulfoLink gel (Pierce, Rockford, IL) through the C-terminal cysteine residue. The MT-3 antibody has been shown to selectively bind to both tissue sections (immunohistochemical localization) and protein extracts (Western blot analysis) of human brain.

Anti-Rabbit Antibody (1:500 dilutions), Secondary Antibody

1.8 µl of 10 % BSA, 225 µl of 4% NaN₃, 90 ul of 1 µg/ml anti-rabbit antibody (Promega, Cat No. S3831), 1 x PBS to 45 ml.

ALKALINE PHOSPHATASE BUFFER

Alkaline phosphatase buffer for use with the Vectastin ABC-AP detection kit (Vector Laboratories, Burlingame, CA, USA) was a solution of 100 mM Tris-HCl at pH 8.2.

TRIS HARVESTING BUFFER

The cells were harvested in 100 mM Tris-HCl, 10 mM DDT at pH 8.2 for immuno-blot protein analysis.

CRYSTAL VIOLATE

Solution A: Crystal Violate	2 gr.
Ethyl Alcohol (95%)	20 ml
Solution B: Ammonium Oxalate	0.8 gr.
Distilled water	80.0 ml

The solutions A and B were mixed. Then, it was filtered through a filter paper into a staining bottle. The solution was stored 24 hours before use.

3% GLUTARALDEHYDE

Four ml of 50 % glutaraldehyde (Sigma, St Louis, MO 63178) were mixed with 96 ml of distilled water. The solution was stored at 4 °C for up to six months.