Zinc inhibits magnesium-mediated human breast cancer MDA-MB-231 cell migration on fibronectin

by

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Abstract

Breast cancer is the most prevalent type of cancer in Canadian women and ranks second in mortality. The cause of breast cancer deaths is the tumor growth in secondary locations. Zinc has been suggested to alter the affinity by which cells attach to the extracellular matrix. The strength of cell adhesion is paramount to the ability of cancerous cells to migrate. The hypothesis for my thesis research was that zinc promotes the metastasic potential of human breast cancer cells. The overall objective of my thesis research was to characterize the effects of zinc on the growth and metastatic potential of human breast cancer cells. Breast carcinoma MDA-MB-231 cells were cultured in DMEM plus 10% Chelex-100 treated FBS supplemented with 0 (zinc-deficient medium), 5 (zinc-adequate medium), or 25 (zinc-supplemented medium) μmol/L of Zn^{2+} as $ZnSO_4$. After culture for 96 h, the cells were harvested for determining total cellular zinc concentration, abundance of the labile intracellular pool of zinc, and cell growth. The metastatic potential of the cells was assessed by a combination of migration rate assessed using the wound-healing assay and migration distance using the single cell migration assay. The cells were also assessed for their adhesion on fibronectin, for the involvement of specific integrin subunits using blocking antibody, and integrin activation using FACS. Zinc treatments had no effect on total cellular zinc concentration, cell growth, and viability and essentially had no effect on abundance of the labile intracellular pool of zinc. Zinc as low as 5 μmol/L inhibited MDA-MB-231 cell migration on fibronectin, reduced magnesium-mediated promotion of adhesion and thus was likely involved in inhibiting magnesium-mediated integrin activation. With the use of blocking antibodies, it was determined that α5/β1 integrin was responsible for the adhesion of the cells to

fibronectin and it was likely that zinc inhibited adhesion by blocking the activation of this specific form of integrin. Together these results suggested that zinc was an inhibitor of MDA-MB-231 migration on fibronectin, and thus likely an inhibitor of metastases, contrary to the hypothesis.

Preface

This thesis was prepared according to the University of British Columbia Faculty of Graduate Studies requirements.

For Chapter 2, I devised a study design and objectives together with Dr. Xu. I was responsible for carrying out all experiments. The flow cytometry assay for total and activated β1 integrin was carried out in the Gold and Roskelley Lab at the University of British Columbia with the assistance of Dr. Sarah McLeod. Dr. Sarah McLeod (Gold Group) also assisted me with the technique for the track "Bead Assay" with rhodamine phalloidin stain. I was responsible for all the data analysis and I wrote the thesis together with Dr. Xu. A version of Chapter 2 is in preparation for submission for publication. The anticipated authors are Sylvia Lymburner, Sarah McLeod, Calvin Roskelley, and Zhaoming Xu.

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List of Abbreviations

ATCC	American type culture collection
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
DMEM	Dulbecco's modified eagle medium
ECM	Extra-cellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial to mesenchymal transition
ES	Embryonic stem
ETS	E-twenty-six
FACS	Flow cytometry assay
FBS	Fetal bovine serum
FN	Fibronectin
Fra-1	Fos-related antigen
FRET	Fluorescence energy transfer
GTPases	Guanine triphosphatases
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF	Insulin-like growth factor
LIPZ	Labile intracellular pool of zinc
mAb	Monoclonal antibody
MDGI	Mammary-derived growth inhibitor
MIDAS	Metal ion dependent binding site
MNU	N-methyl-N-nitrosourea
MTT	3-[4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide
PAK	p21 activated kinase
PBS	Phosphate buffered saline
RFU	Relative fluorescence units
ROCK	Rho A kinase
SEM	Standard error mean

TGF β1	Transforming growth factor β1
TNM	Tumor, node and metastasis

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Introduction

Breast cancer is the most prevalent type of cancer in Canadian women and the third most common cancer overall in Canada (Canadian Cancer Society, 2011). It is predicted that in 2011 alone, 23,400 new cases of breast cancer will be diagnosed in Canada (Canadian Cancer Society, 2011). Breast cancer, representing 28% of cancer cases in women, ranks second in mortality (Canadian Cancer Society, 2011). Sadly, breast cancer claims thousands of lives and is responsible for 14.4% of all cancer deaths in women (Canadian Cancer Society, 2011). The cause of these deaths is not the primary tumor, but rather tumor growth in secondary locations (Weigelt *et al*., 2005). Advances in cancer management including better mammographic screening, which helps to detect tumors in earlier stages, have reduced the number of deaths caused by breast cancer metastases. Unfortunately, of the 11% of Canadian women diagnosed with breast cancer, 40% will still die from breast cancer metastases (Weigelt *et al*., 2005). For this reason, strategies to reduce metastases in breast cancer patients are of great interest. However, traditional therapies aimed at limiting primary tumor growth are still the predominant mode of treatment and method for reducing the chance of metastasis.

Zinc is essential for growth, and cancer is characterized by uncontrolled rapid growth. Not surprisingly, zinc's importance in growth has made it a topic of research in breast cancer. In fact, studies have previously shown that zinc concentrations are higher in cancerous tissues compared to normal breast tissue (Margalioth *et al*., 1983; Cavallo *et al*., 1991; Gupta *et al*., 1991; Ng *et al*., 1993; Jin *et al*. 1999; Geraki *et al*., 2002). Lee *et al*. (2004) have shown that low dietary zinc intake results in suppression of chemically

induced rat mammary tumorigenesis. More recently, ZIP10, a zinc importer, has been implicated in breast cancer metastases to lymph nodes and further analysis showed that the ZIP10 expression and the presence of zinc were required for breast cancer metastases to occur (Kagara *et al*., 2007).

Past research, therefore, suggests the involvement of zinc in breast cancer progression. However, the role of zinc in breast cancer progression, especially metastasis, has been minimally researched. This thesis is intended to assess the effects of zinc on the metastastic potential of human breast cancer cells and to explore the mechanism by which zinc has these effects.

Chapter 1 Background, Hypothesis and Objectives

1.1 Literature Review

This literature review provides an overview of the role of zinc in cell growth and its potential importance in breast cancer development and progression. More specifically this review will explore metastasis, the role of integrins (the primary mediators involved in cell adhesion) in metastasis and how divalent cations are potentially important in cell motility through their regulation of integrin activation. The background review of integrins extends beyond breast cancer in order to provide a clearer understanding of their role in breast cancer metastasis. Finally, this review provides evidence in support of a role of zinc in cancer metastasis and cell migration.

1.1.1 Breast Cancer, Risk Factors and Development

The etiology of breast cancer, although not completely understood, is known to be multifactorial. Increased risk has been associated with family history of the disease and age as well as environmental factors such as consumption of alcohol, high body mass index, prolonged exposure to estrogen, most often due to early menarche and late menopause, high dietary fat, etc. (Singletary, 2003). Only small differences, however, are shown when the effects of these environmental factors are tested individually. This small but statistically significant difference in the individual environmental risks fails to explain the five-fold variation in worldwide risk that has been observed when the lowest and highest risk groups are compared (Singletary, 2003). Support for an environmental component in the development of breast cancer is seen by evidence that women of Asian

descent who have migrated to North America have a greater risk of developing breast cancer than women who remain in their countries of origin. In fact, the more generations a family of Asian descent has lived in North America, the more their risk of developing breast cancer approaches that of white North American women (Ziegler *et al.,* 1993).

Breast cancer occurs when a number of mutations occur in the regulatory genes, which maintain the appropriate balance between programmed cell death (apoptosis) and cell proliferation in breast tissues (Kenemans *et al.,* 2004). These can be inherited germline mutations, as in familial breast cancer. More frequently random mutations accumulate in breast tissues giving rise to sporadic breast cancer. A number of genes have been identified as central to the initiation and progression of breast cancer. Among the most common are BRCA1 and BRCA2 in familial breast cancer and HER2/neu, cyclin D, and p53 in sporadic breast cancer (Kenemans *et al.,* 2004; Dickson *et al.,* 2005; Roses, 2005). Currently, complex analysis into the genetic profile of breast cancer cells is underway in search for genes which can act as predictors for how an individual's breast cancer will behave. Findings of this analysis could provide better predictions of long-term cancer growth rates and chance of metastasis (Weigelt *et al.,* 2005). This knowledge will guide doctors in choosing the treatment path, which will be most effective in each cancer case. At present, guidelines followed to predict cancer outcome such as the tumor, node and metastasis (TNM) scale and screenings for the genes mentioned above are only successful at predicting outcomes in the best 15% and worst 15% of cases (Weigelt *et al.,* 2005).

1.1.2 Zinc and Zinc Nutrition

Zinc is part of group IIb on the periodic table along with the toxic metals cadmium and mercury (Plum et al., 2010). Zinc is an essential trace miner for humans and animals. It is a component of more than 300 different enzymes. Zinc is required for the synthesis of DNA and RNA (Jin *et al.,* 1999; Macdonald, 2000), the activity of DNA and RNA polymerase (Prasad and Oberleas, 1974) and thymidine kinase (Prasad and Oberleas, 1974; Chesters *et al.,* 1990; Prasad *et al.,* 1996; Macdonald, 2000), and is therefore essential for cell growth, division and function. Zinc also plays an important role in protein, lipid and carbohydrate metabolism (Formigari *et al*., 2007). Zinc deficiency in animals and humans alike is marked by stunted growth, and dysfunction of rapidly proliferating cells, such as those required in wound healing and the immune response (Lee *et al*., 2004).

The total body content of zinc is approximately 1.5-2.5 g (Plum *et al*., 2010). It is ubiquitous in the body but zinc is at its highest concentration in the liver, kidney, muscle, skin and bones (Institute of Medicine, 2001). The plasma contains a small circulating pool of zinc with a concentration of around 15 μmol/L at adequate zinc status (Simon-Hettich, 2001). Zinc deficiency has been defined as having a plasma zinc concentration of below 11 μmol/L.

The Dietary Reference Intake for men and women (14 - > 70 yr) is 11 and 8 mg/day, respectively (Institute of Medicine, 2001). The best sources of zinc include red meat, organ meat and oysters. Non-meat sources of zinc include fortified whole grains,

and leafy and root vegetables (Groff JL and Gropper SS, 2000). Unfortunately the presence of zinc binders, such as phytates, in vegetables makes zinc bioavailability low in these sources (Health Canada, 1979).

Zinc deficiency is most often found in populations that consume a predominantly vegetarian diet and whose zinc intake depend on foods with poor zinc bioavailability, such as non-fortified cereals (Health Canada, 1979). The Tolerable Upper Intake Level of zinc is set at 40 mg/d (Institute of Medicine, 2001), however reports of zinc toxicity are rare (Plum *et al*., 2010).

1.1.3 Zinc and Cell Proliferation

Zinc and DNA Synthesis Zinc is essential for cell proliferation, as exemplified by the stunted growth observed in zinc deficient humans and animals. However, despite the well-established fact that zinc is essential for growth, its exact role in cell proliferation has not been elucidated.

Zinc is required for the synthesis of DNA and RNA (Jin *et al*., 1999; Macdonald, 2000) as it is necessary for the activity of DNA and RNA polymerase (Prasad and Oberleas, 1974) and thymidine kinase (Prasad and Oberleas, 1974; Chesters *et al*., 1990; Prasad *et al*., 1996; Macdonald, 2000). The effects of zinc deficiency on cell proliferation are shown in a study by Prasad *et al*. (1996), where HUT 78 lymphoblasts grown in zinc deficient medium have a growth rate half that of cells grown at physiologically normal zinc concentrations. This suppressed cell proliferation can be

explained in part by the reduction in thymidine kinase mRNA levels and its enzymatic activity. The reduction in thymidine kinase activity results in a reduced rate of DNA synthesis, delaying cell doubling. In support of these observations, a significantly greater percentage of HUT 78 cells are arrested in the G_1 and S phase of the cell cycle, the stage where DNA doubling occurs (Prasad *et al.,* 1996). This zinc deficiency induced growth retardation can be reversed by zinc supplementation, but not by addition of other essential trace elements including iron, copper and manganese (Prasad *et al*., 1996). These results show a zinc specific effect on growth in HUT 78 lymphoblasts.

Zinc, IGF and Growth There is also some evidence that zinc deficiency affects growth hormone and insulin-like growth factor (IGF), both of which are required for somatic growth (Macdonald, 2000). IGF-1 and IGF-2 are involved in promoting growth and inhibiting apoptosis via the IGF-receptor. Two *in vivo* studies, one in humans (Cossack, 1991) and one in Wistar rats (Cavallo *et al.,* 1991), have shown that low levels of cellular zinc inhibit IGF-1 and its binding proteins, reducing its binding to the IGF-receptor and inhibiting cell growth. However, a lack of further research in this area leaves the relationship between dietary zinc deficiency and IGF-1 function largely unclear.

1.1.4 Zinc and Apoptosis

In addition to its crucial role in cell proliferation, zinc also acts as a modulator in apoptosis. p53, which is involved in the progression of 40% of sporadic breast cancers (Dickson *et al.,* 2005), is a tumor suppressor gene. p53 plays a role in the regulation of

DNA repair and cell proliferation at the G1 and G2 checkpoints in the cell cycle (Fanzo *et al*., 2002; Clegg *et al*., 2005; Dickson *et al*., 2005; Roses, 2005). When p53 expression is altered, either over or under expressed, damaged DNA is able to proceed through the cell cycle unchallenged, allowing cells with a damaged genetic code to proliferate. p53 exerts its effects through upregulating Bax, an apoptotic inducer, and down regulating Bcl-2, an apoptotic inhibitor. It also increases expression of p21, which causes cell cycle arrest (Fanzo *et al*., 2002).

p53 is a zinc finger transcription factor (Kagara *et al*., 2007). Zinc is located in its DNA binding region and, therefore, is needed for its stability and DNA binding. Cells grown in a zinc deficient medium (0.8 μmol/L Zn as $ZnSO_4$) have significantly higher levels of p53 mRNA than those grown in a zinc adequate medium (3.0 μmol/L Zn as $ZnSO_4$) (Fanzo *et al*., 2002). A similar finding has also been reported in a number of studies as reviewed by Clegg *et al*. (2005). The inverse relationship between zinc concentrations and p53 expression is clearly counter intuitive. One possible explanation is that during zinc deficiency p53 adopts an altered conformation, which has a reduced ability to bind DNA (Clegg *et al*., 2005). This failed regulatory process would lead to an increase in DNA damage possibly alerting the cell that more p53 is needed. This increased expression in p53, in turn, could increase the expression of Bax and suppress the expression of Bcl-2, ultimately triggering apoptosis (Fanzo *et al*., 2002; Seve *et al*., 2002; Clegg *et al*., 2005).

An influencing factor in whether a cell undergoes apoptosis is the Bcl-2: Bax

ratio. When this ratio is low, the mitochondrial membrane becomes more permeable to proteins, which trigger the process of apoptosis (Kumar *et al*., 2005). Zinc deficiency decreases the ratio of Bcl 2:Bax, favoring cell death (Fanzo *et al*., 2002). Caspases are a family of proteases involved in the regulation of apoptosis (such as caspase 8 and 9) and execution of apoptosis (such as caspase 3). A low Bcl-2:Bax ratio,activates caspase 9, which then activates caspase 3. Once caspase 3 is activated, it carries out the destruction of the cell, targeting its organelles, including the nucleus and its cell replicating machinery (Kumar *et al*., 2005). So it is possible that zinc depletion promotes apoptosis through activating caspase 9 and 3 (Seve *et al*., 2002; Clegg *et al*., 2005), while high levels of zinc reduce apoptosis through inhibiting caspase 3 activation (Perry *et al*., 1997; Clegg *et al*., 2005). The complete story of zinc and apoptosis, however, remains unclear. Zinc's indirect effect on apoptosis through the induction of a series of caspases still needs to be experimentally proven.

1.1.5 Zinc Homeostasis

Zinc metabolism is under tight homeostatic control. Tissue zinc concentration is subject to homeostatic control involving, a family of zinc exporters (ZnTs) and a family of zinc importers (ZIPs) (Liuzzi and Cousins, 2004; Cousins *et al*., 2006). The expression of zinc transporters in many cells is influenced by extracellular and intracellular zinc concentrations (Gaither and Eide, 2001). In a lactating rat model, feeding rats a marginally zinc deficient diet led to a decrease in protein levels of ZnT1, located on the plasma membrane in mammary glands, while ZnT1 mRNA levels remain unchanged. Conversely, both mRNA and protein levels of ZnT2 and ZnT4, both located

on intracellular vesicle membranes, are increased in the mammary glands (Kelleher and Lonnerdal, 2002). As ZnT1 is involved in zinc export from cells to the extracellular space, decreasing its levels should reduce zinc transport out of the cell thus increasing cytosolic zinc concentration. ZnT2 and ZnT4, on the other hand, are involved in export of zinc from the cytosol into intracellular vesicles. Up-regulation in the expression of these zinc transporters should, therefore, increase the amount of zinc stored in the vesicles and its concentration in breast milk. Here the expression of zinc transporters is altered to maintain an adequate zinc concentration in the milk despite a reduction in total zinc available due to feeding the zinc deficient diet.

Collectively, these studies make it clear that cellular zinc concentration is under homeostasic control through alterations in the expression of zinc transporters which change in response to cellular zinc status. This fine tuned regulation likely differs among cell types and is a probable explanation for the discrepancies in zinc sensitivity among cell lines, malignant and non-malignant alike.

1.1.6 Zinc and Breast Cancer

Zinc has been linked to breast cancer development and growth. In human breast cancer patients, a reduction in serum/plasma zinc concentration from approximately 15 μmol/L, considered a normal level, to 11 μmol/L appears to be associated with breast cancer progression (Gupta *et al.,* 1991; Ng *et al.,* 1993; Borella *et al.,* 1997; Kuo *et al.,* 2002), especially in advanced stages of breast cancer (Ng *et al.,* 1993; Borella *et al.,* 1997). However, other research has shown no correlation of serum/plasma zinc

concentrations with the presence of breast cancer (Garofalo *et al*., 1980; Piccinini *et al*., 1996; Koksoy *et al*., 1997), or with its stage of progression (Garofalo *et al*., 1980; Yucel *et al*., 1994; Seven *et al*., 1998), nor with other malignancies (Garofalo *et al*., 1980; Koksoy *et al*., 1997). More recently, Tinoco-Veras *et al*. (2011) have reported that plasma zinc concentration is unaffected by the presence of breast cancer; however, zinc levels in erythrocytes are lower in pre-menopausal women with breast cancer than in women without breast cancer.

In mice, plasma zinc concentration was significantly reduced with a tumor burden of 1% of body weight and further reduced to 54% of normal levels as tumors reached 16% of total body weight (Philcox *et al*., 1993). It is likely that the growth needs of the tumor induce the drop in plasma zinc concentration. The zinc needs of a tumor mass that is 16% of total body weight, are predicted to be equivalent to the needs of the muscle mass of the entire mouse, approximately 35% of total body weight (Philcox *et al*., 1993). This exemplifies an increased need for zinc of malignant cells. Together these results suggest that low serum zinc concentration may be a characteristic feature of cancer, perhaps because of the high zinc demands of proliferating cancer cells.

In contrast to reduced serum/plasma zinc concentrations, zinc concentration in cancerous breast tissue is elevated compared to normal breast tissue (Margalioth *et al*., 1983; Cavallo *et al*., 1991; Gupta *et al*., 1991; Ng *et al*., 1993; Jin *et al*., 1999; Geraki *et al*., 2002). Zinc concentration has a 2.4 to 1 ratio in matched pair samples, comparing a cancerous breast tissue sample and its corresponding non-cancerous breast tissue from

the same breast cancer patient, while a ratio of 4.4 to 1 is shown for zinc concentrations in cancerous breast tissue compared with healthy breast tissue obtained from breast reduction surgeries (Geraki *et al*., 2002). Increasing zinc accumulation in cancerous breast tissue appears to be correlated with cancer severity, quantified by the tumor, node and metastasis (TNM) scale (Cavallo *et al*., 1991). The TNM scale looks at tumor size, involvement of lymph nodes and whether or not the cancer has metastasized to give the tumor a stage, which represents its severity and threat to the health of the patient.

In N-methyl-N-nitrosourea (MNU) -induced mammary tumors, an increased zinc concentration compared with normal tumor-free mammary glands is correlated with an increase in ZnT-1 mRNA levels (Lee *et al*., 2003). Similar results are shown with the ZIP importer family. When TPEN, an intracellular zinc chelator, is added to the medium, intracellular zinc in THP-1 cells, a human acute monocytic leukemia cell line, is reduced while ZIP2 mRNA levels are increased (Cao *et al*., 2001).

The link between zinc deficiency and impaired growth has led to experiments, which assess the effects of zinc deficiency on tumor growth. We have demonstrated that rats fed a moderately low zinc diet showed a reduction in MNU-induced mammary tumor incidence and total tumor number (Lee *et al*., 2004). Tumor incidence in rats fed a zinc-deficient diet was reduced by 45% compared with rats fed the adequate zinc diet. The total tumor number in zinc deficient rats was reduced by 77% compared with the adequate zinc groups.

Studies to date suggest an increased need for zinc in human breast cancer tumors and induced mammary tumors in an animal model. The susceptibility of human breast cancer cells to zinc deficiency or zinc supplementation *in vitro,* however, is unknown.

1.1.7 Breast Cancer Metastasis

Although breast cancer is recognized as a life threatening disease, it is only once the breast cancer cells have metastasized to the rest of the body that this threat can be realized. Metastasis is the spread of cancer cells from the place of the tumor origin, the breast for example, to a distant site in the body. Although any size tumor can metastasize, the likelihood is believed to increase with increasing tumor size (Weigelt *et al.,* 2005; Weinberg, 2007).

The first step in breast cancer progression is the formation of a carcinoma in situ. This is the designation given to a group of malignant cells that is still within the confines of the epithelial tissue from which it originated. For example, mammary ductal carcinoma in situ refers to a cancerous tumor which is growing inside the breast duct, in its epithelial lining. To achieve metastasis these cells must gain access to the blood circulation or lymphatic system by penetrating through the basement membrane. However, before this can occur tumor cells must undergo a change in phenotype to increase their motility, a transformation referred to as the epithelial to mesenchymal transition.

Epithelial cells give rise to 80% of life threatening cancers (Weinberg, 2007).

However, the biology of normal epithelial cells does not allow them the freedom of movement required for migration across the adjacent basement membrane and through the surrounding stroma. Normal epithelial cells are arranged in a sheet formation, tightly connected to one another via tight and adherens junctions and strongly expressing the cell-to-cell adhesion protein E-cadherin. These cells are tethered in a static fashion by focal adhesions making movement impossible.

Mesenchymal cells, on the other hand, have few of the same constraints compared to epithelial cells, giving them the necessary mobility to play an important role in embryogenesis (Roses, 2005; Tannock, 2005; Weinberg, 2007). Unlike the static cell adhesion of epithelial cells, mesenchymal cells depend on the dynamic adhesion of the cell's forward extensions to migrate. It is important to keep in mind that the amount and type of adhesion is crucial for migration (Li *et al.,* 2005). High levels of adhesion inhibit motility. Epithelial cells, for instance, are so tightly connected to their neighboring cells and the extra-cellular matrix (ECM) that they lack the ability to migrate. However, a threshold level of adhesion is necessary for cells to attach and pull themselves along the ECM, a process crucial for successful migration (Akiyama *et al.,* 1995). Therefore, as mentioned above, the ability of cancer cells to migrate is thought to be the result of a tumor cell undergoing an epithelial to mesenchymal transition. Epithelial cell markers such as tight and adherens junctions, and E-cadherin in this selected cell are lost and replaced by the increased presence of N-cadherin, vimentin, actin, and mesenchymal ECM proteins, fibronectin and collagen, all characteristics of the mesenchymal phenotype (Weaver 1997; Tannock *et al.,* 2005; Christiansen and Rajasekaran, 2006;

Weinberg *et al*., 2007).

A number of signaling pathways as well as cues from the tumor microenvironment are thought to initiate the first step in the migration process by inducing this epithelial to mesenchymal transition. The end result of this process is a shift in cell phenotype from square and stationary to elongated and motile. This shift is often difficult to identify, as only a small percentage of the cells in a growing tumor will undergo this transition. There are a number of models developed to find out the origin of these unique migratory cancer cells (Weigelt *et al*., 2005). The two models that best fit with current research understanding of metastasis are the traditional model and the stem cell model.

The traditional model argues that during tumor progression a small subset of the cell population will undergo mutations, which increase the metastatic ability of these cells. This model implies that all tumor cells start out as equal and the longer the tumor is allowed to grow and the larger it gets, the greater the likelihood that further genetic mutations will occur, allowing for metastasis.

The stem cell model, on the other hand, is based on the premise that specific breast stem cells are genetically altered to become breast cancer stem cells (Weigelt *et al*., 2005). These cells are named migrating cancer stem cells and have gained the ability to disseminate via the bloodstream and form colonies at distant sites in the body (Brabletz *et al*., 2005). Similar to the theory above only a small percentage of the tumor cell

population has the appropriate characteristics to colonize abroad.

However, conceptually these two models differ, in that the stem cell model implies the presence of cells capable of metastasis from early on in the tumor's growth whereas the traditional model suggests that this is something that develops because of mutations over time. Either way, these selected cells leave the primary tumor site and travel through the blood until they adhere to the blood vessel endothelial lining of a new tissue. In the case of breast cancer the most common sites of metastases are the lungs, bone, liver and brain (Canadian Cancer Society, 2011). At this time only cancer cells with the appropriate adhesive properties for the endothelium on which they have attached will adhere strongly enough to infiltrate the new site (Molloy and Veer, 2008). Once established in the new tumor site, signals from this new environment can cause these cells to undergo a mesenchymal-epithelial transition and once again adopt the epithelial morphology (Christiansen and Rajasekaran, 2006).

1.1.8 Role of Integrins

Paramount to the ability of cancerous cells to migrate is the affinity with which they attach to the extracellular matrix. Integrins are the primary mediators of cell to ECM adhesion and are important in cell migration in embryogenesis and wound healing, but also in pathological states such as tumor progression. Integrins are composed of one α- and one β-subunit and these two subunits form a heterodimer. Currently, 18 α and eight β-subunits have been identified in mammals, which combine to form 24 distinct integrins (Hynes, 2002). Although there are many integrins, only a few are involved in

the adhesion of a particular cell type to a particular ECM protein, as each cell type expresses its own set of integrins, each of which recognizes a specific ligand.

As a transmembrane protein, the head of the integrin protein is in the extracellular space and able to interact with the ECM while the two cytoplasmic tails are located intracellularly and free to interact with cytosolic proteins and intracellular signaling molecules (Figure 1.1) (Hynes, 2002; Li *et al.,* 2005). As a bidirectional-signaling molecule, integrins are capable of transmitting signals from the outside into the cell and vice-versa. As such, the head of the integrin interacts with the ECM, leading to initiation of a signaling cascade inside the cell to control cell proliferation, survival, shape, polarity, motility, gene expression and differentiation (Hynes, 2002; Li *et al.,* 2005). Conversely, signals from within the cells can cause the separation of the α- and β cytoplasmic tails resulting in a conformational change that renders the integrin into its active state and enables binding to ECM.

One aspect of this two-way signaling is a positive feedback loop which gives cells the ability to form strong adhesions to the ECM. The binding of integrins to the ECM induces a signaling cascade, which promotes the assembly of actin filaments and stress fibers. In turn, the stress fiber formation induces the clustering of more active integrins (Giancotti *et al.,* 1999). This regulation of integrin activation is key to their important role in adhesion. The number of activated integrins will affect the type and strength of adhesion of cancer cells to the ECM. A cluster of many integrins together on the cell cytoskeleton form strong connection called a focal adhesion. This type of adhesion

attaches the cell with such avidity to the ECM that mobility is inhibited.

1.1.9 Integrins in Adhesion and Migration

The role of the β1 family of integrins in cell adhesion and migration has been studied *in vitro* through the use of β1-blocking antibodies and β1 null embryonic stem cells. The β1-blocking antibody inhibits adhesion, cell spreading and migration of fibroblast cells on a fibronectin matrix (Akiyama *et al.,* 1989). The β1-subunit complexes with the α5-subunit to form the α5/β1 integrin, which interacts with fibronectin, the primary ECM ligand of α5/β1 integrin. Blocking antibody to the α5 integrin subunit partially inhibits adhesion but has no effect on cell spreading or migration of the cell sheet (Akiyama *et al.,* 1989). In another study, β1- and α5-blocking antibodies inhibited adhesion onto fibronectin in fibrocarcinoma, bladder carcinoma, colon carcinoma and transformed embryonic lung cell lines (Yamada *et al.,* 1990). Migration is inhibited in all these four cell lines by the β1-blocking antibody, and by the α5-blocking antibody in all but the bladder carcinoma cell line (Yamada *et al.,* 1990). However, migration is reduced to a greater extent by the β1- than by the α5-blocking antibody (Yamada *et al.,* 1990). As the α5- and β1-subunits complex with one another, these results suggest that the α5/β1 integrin complex is the primary integrin mediating adhesion of the four cell lines used in these studies onto fibronectin. However, it also appears that the β1-subunit complexes with other α-subunits, which bind to other ECM proteins, as cells blocked by the α5-blocking antibody are able to participate in some cell spreading and migration. This likely occurs over time as a result of the cells producing their own alternate matrix, such as collagen, on which they can spread and migrate

(Akiyama, 1989; Yamada *et al.,* 1990).

In support of these earlier observations, β1 null embryonic stem (ES) cells fail to adhere to fibronectin. In addition, these cells took on a spherical shape with microvilli extensions, which are uncharacteristic of the wild type ES cells (Fassler *et al.,* 1995). Together these observations indicate that the β1 integrin subunit is the key mediator of cell adhesion to fibronectin across many different cell lines.

1.1.10 Integrins and Cancer

The importance of the β1 integrin in cell adhesion and migration *in vitro* has led to investigations of how this integrin may influence tumor cell phenotype, growth and metastasis. A non-malignant (normal) and malignant breast cell line derived from the same breast epithelial tissue provides an excellent model to study the association between integrin expression and the normal versus metastatic cell phenotype. Using a 3D culture system, the normal epithelial breast cells display organized cell-to-cell adhesions and lateral expression of E-cadherin, and deposit an organized collagen IV basement membrane (Weaver *et al.,* 1997). The derived tumorogenic cell line, on the other hand, forms large colonies that lack organization and the ability to form a basement membrane. The localization of E-cadherin in these cells to the cytoplasm, and away from the cell surface, reduces the cell's ability to form adherens junctions and therefore leads to a lack of cell-to-cell adhesion (Weaver *et al.,* 1997). The ratio of β1- to β4-subunit expression is increased almost three folds in the tumorigenic cells versus normal cells. The addition of β1-blocking antibody is able to reverse this morphological difference and revert the

tumorigenic cell line's phenotype to one closely resembling the normal epithelial cell. In normal epithelial cells, β1-blocking antibody treatment induces apoptosis (Weaver *et al.,* 1997).

Integrin blocking-mediated inhibition of tumorigenesis is also observed in an *in vivo* model. Pre-treating the malignant progeny of breast cancer HMT-3522 cells (T4-2 cells) with β1-blocking antibody before injecting them into nude mice lowers the number of tumors formed (44%; 7 out of 16 mice) compared to injection of tumor cells bearing active β1 (94%; 15 out of 16 mice) (Weaver *et al.,* 1997). Interestingly, mammary tumors bearing disrupted β1 gene exhibit significantly reduced capacity to metastasize to the lung in mice (Huck *et al*., 2010). In addition, administering β1-blocking antibody directly into mice biweekly is able to reduce tumorigenicity of injected breast cancer cells without affecting normal epithelial cells (Park *et al*., 2006). In support of these observations, a recent study showed that the expression of both α5/β1-integrin and fibronectin, the ligand of α5/β1-integrin, is upregulated in the T4-2 cells compared with the wild-type HMT-3522 mammary epithelial cells (Nam *et al*., 2010). Importantly, disrupting the interaction of α5/β1-integrin with fibronectin promotes apoptotic death in these T4-2 cells and sensitizes these cells to radiation-induced apoptosis. These studies raise the possibility of targeting the β1-subunit as a new therapy to treat breast cancer (Park *et al*., 2006; Nam *et al*., 2010).

Many integrins containing the β1-subunit (e.g. α5/β1 also called the fibronectin receptor) have also been implicated in tumor cell growth and metastasis. Transforming

growth factor β1 (TGF β1) is known to promote tumor growth and is often upregulated in aggressive, metastatic cancers. In addition, the ETS (E-twenty-six) transcription factor is associated with malignancy in Glioma cells. Both TGF β1 and ETS increase cell adhesion onto and migration on a fibronectin matrix mediated by upregulation of the α5-subunit mRNA and cell surface protein levels of the α5/β1 integrin in liver and kidney cells respectively (Cai *et al.,* 2000; Kita *et al.,* 2001). While these two studies support a pro-metastatic role of α5/β1 in cancer, other studies, which look at its expression *in vivo,* provide conflicting evidence (Zutter *et al.,* 1993; Yao *et al.,* 1997; Tani *et al.,* 2003). In cancerous liver cells, α5/β1 protein is decreased at locations where the primary tumor cells invade the connective tissue. In addition α5/β1 integrin has been shown to decrease with increasing liver cancer tumor size and metastatic potential (Yao *et al.,* 1997). Furthermore, in nude mice injected with Chinese hamster ovary (CHO) cells expressing various levels of α5/β1 integrin, the size of the primary tumor is inversely related to the level of α5/β1 integrin expression (Tani *et al.*, 2003). Finally, analysis of a number of normal and adenocarcinoma breast tissues shows that the level of α5/β1 integrin mRNA and protein decreases with reduced differentiation of the tumor (Zutter *et al.,* 1993). The highest level of α5/β1 integrin is seen in normal epithelial cells, while the lowest level is a characteristic of poorly differentiated tumor tissue. Poorly differentiated tumors are usually associated with higher-grade tumors with greater metastatic potential. Parallel findings have also been reported for the α2/β1 integrin (Zutter *et al.,* 1993). Collectively, these studies suggest that the α5/β1 integrin acts to maintain tissue integrity and its down regulation is associated with greater metastases.

These studies, however, are only measuring α5/β1 expression at the primary tumor site. Integrin expression may also be important as the cell goes through different stages of metastasis. α5/β1 expression is elevated in hepatocytes at the new tumor sites (Cai *et al*., 2000) and its increased expression in CHO cells promotes metastases to the kidney (Tani *et al*., 2003). These observations suggest an important role of α5/β1 in the colonizing of new tumors. Adhesion of circulating tumor cells to the endothelial cells that line the blood vessel walls is an important step in their ability to gain access to new tissues and form metastases. Blocking of the α5/β1 integrin reduces adhesion of highly metastatic breast cancer cells to a model endothelium (Bliss *et al*., 1995). This observation provides strong evidence for a role of α5/β1 in promoting metastasis by increasing the adhesion of tumor cells in the blood to the endothelial lining of the blood vessels.

Taken as a whole, the body of research on α5/β1 integrin suggests that its reduced expression may be required in the primary tumor to give the cells the freedom of movement required for dispersion in the blood to occur. However, once individual cells have broken away from the tumor mass, α5/β1 integrin may be crucial to provide the adhesion required for cells to colonize in new sites. The optimum expression of α5/β1 for breast cancer metastasis would minimize restrictions on cell motility while still providing enough adhesion for the cell to pull itself along the ECM and to adhere to the endothelial lining of the blood vessels.

1.1.11 The Polarized Actin Cytoskeleton and the Role of Integrins

A distinguishing feature of motile cells is their polarized appearance. In motile cells, the actin cytoskeleton is altered to form long protrusions. These protrusions are paramount for cell motility and invasion. Three members of the Rho family of small guanosine triphosphatases (GTPases): Rac, Rho and Cdc42, have been recognized as key regulators of cell polarization and therefore migration (Weinberg, 2007). Rac activation stimulates the formation of lamellipodia, protrusions of the actin cytoskeleton at the leading edge of migrating cells. Rho proteins are involved in the formation of stress fibers and strong focal adhesion complexes (Li *et al*., 2005; Weinberg, 2007). Finally Cdc42 regulates filopodia extensions, thin protrusions that often stick out perpendicular to the cells lamellipodia. Although both Rac and Cdc42 GTPases are involved in the formation of actin protrusions their functions are distinct (Li *et al*., 2005; Weinberg, 2007). The lamellipodia stimulated by Rac are the point of adhesion to the ECM of the cells leading edge. Cells use this point of attachment to pull themselves along the matrix during cell migration. The primary purpose of filopodia, on the other hand, is to act as sensors of the cell environment. Positioned perpendicular to the cells direction of movement, it is likely that their role is to detect barriers to cell migration or extracellular signals that would affect the cell's direction of migration (Weinberg, 2007). In migration, these cues could be as complex as directing a cell towards the location of a blood vessel. This type of direction could greatly impact a cell's ability to disseminate throughout the body.

The polarization of the actin cytoskeleton appears to be induced by growth factors

or the adhesion of integrins to the ECM, which induce complex signaling pathways able to oversee and regulate the overall process of cell spreading and migration. Extensive scientific research has gone into understanding how these processes function in normal cells as well as pathological states such as cancer. For example, in fibroblastic cells, integrin-mediated adhesion to fibronectin activates Cdc42, which in turn activates Rac (Price *et al.,* 1998). The activation of Rho family GTPase results in visible cell spreading on fibronectin within 20 min of cell seeding. No cell spreading occurs, however, when cells are allowed to attach to a non-integrin dependent adhesion substrate such as poly-L - lysine. Under these conditions no activation of the Rho pathway is induced, supporting cell spreading to be an integrin dependent effect (Price *et al.,* 1998). This effect of integrin binding is also associated with a spike in the activation of a well-known downstream mediator of Rac and Cdc42 on cell spreading and migration, the p21 activated kinase (PAK) (Price *et al.,* 1998). This dramatic increase in PAK levels occurs at 5 min after cell seeding, indicating that induction of this pathway happens very quickly after cell contact with the ECM protein, fibronectin (Price *et al.,* 1998). In fibroblasts, adhesion to fibronectin increases levels of important lipid modulators in cell migration. The levels are further increased in the presence of growth factors, but only when cells are attached (McNamee *et al.*, 1993).

Fos-related antigen 1 (Fra-1), a transcription factor, has been shown to be a modulator for the well-known oncogene, Ras (Vial *et al.,* 2003; Chiapetta *et al.,* 2007). Its presence seems to be important in the cellular transformation that leads to a cell becoming cancerous. In fact, in the breast, Fra-1 is only found in cancerous tissue

(Chiapetta *et al*., 2007). In colon carcinoma cells, Fra-1 expression is required for migration and invasiveness (Vial *et al*., 2003). Fra-1 promotes migration by inhibiting β1 integrin inhibitory signaling to Rho A (Vial *et al*., 2003). When Rho A is active, it inhibits the formation of actin stress fibers and focal adhesions by inhibiting its Rho A kinase (ROCK). The lack of these adhesion mechanisms allows excessive cell migration. Under normal conditions active β1 integrins inhibit Rho A, which allows the formation of actin stress fibers and focal adhesions to occur and maintains a normal immobile epithelial cell phenotype. When Fra-1 is expressed, β1 is inhibited, leading to the abolishment of this anti-metastatic pathway and cell migration ensues (Vial *et al*., 2003). Fra-1 is therefore an important transcription factor that implements its effects on metastasis by modulating the Rho pathway, down regulating proteins which oversee the formation of strong adhesions that reduce motility. Cells with silenced Fra-1 have increased active β- 1 integrins and adhesion to ligands resulting in an 80% reduction in migration (Vial *et al*., 2003). These studies clearly show a dynamic relationship between integrins and migration through modulation of Rac, Cdc42 and Rho. Cell adhesion promotes complex signaling via their integrins, which can be modulated by thousands of factors. In the end, this complex series of messages decide whether the cells fate is to become motile or not.

1.1.12 Role of Divalent Cations in Integrin-Mediated Adhesion

Divalent cations have been established for many years as essential for ligand binding by integrins and their presence is therefore required for cell adhesion (Draisfield *et al*., 1992; Luque *et al*., 1996; Yin *et al*., 1997). Multiple studies have shown that both

the α- and β-subunits of integrins contain divalent cation binding sites within their structures (Luque *et al*., 1996, Yin *et al*., 1997; Xiong *et al*., 2002). Manganese and magnesium are the divalent cations most effective in inducing cell adhesion, with calcium promoting adhesion in BV-173 leukemic cell line (Yin *et al*., 1997). In most literature, however, manganese and magnesium are classified as divalent cations that activate integrin function while calcium inhibits integrin function (Mould *et al*., 1995; Luque *et al*., 1996).

Manganese is shown to be the most potent activator of integrins inducing 50% of maximal binding at a concentration of 6 μmol/L compared with magnesium ranging between 45 μmol/L and 1.4 mmol/L, depending on the cell line tested (Yin *et al*., 1997). The strength of adhesion is also impacted by the divalent cation present. For example, cells bound in the presence of magnesium withstood 14 times the centrifugal force of those bound in the presence of calcium (Yin *et al*., 1997). In addition to its promotion of adhesion, manganese has also been shown to upregulate cell migration on keratinocytes in wound healing, an effect that is mediated by integrins (Tenaud *et al*., 2000).

Using the mAb 24 antibodies, which recognize only integrins in their active (binding) conformation, Drainsfield *et al*. (1992) are among the first to implicate divalent cations in the conformational change that is required for ligand binding to occur. While manganese and magnesium both stimulate the binding of mAb 24, calcium inhibits it. Probably the role of calcium is to keep integrins inactive. The addition of 1 mmol/L of calcium inhibits the inductive effects of magnesium and manganese alike; however

previously bound calcium only interferes with the effects of magnesium (Drainsfield *et al*., 1992). A study looking specifically at the effects of divalent cations on the binding of the α5/β1 integrin to fibronectin shows that manganese and magnesium both promote ligand binding, although manganese has an affinity 40 times that of magnesium for its binding site. Calcium on the other hand inhibits ligand binding (Mould *et al*., 1995). When multiple cations are added simultaneously, calcium acted as a non-competitive inhibitor of manganese on adhesion and either increased or decreased the effects of magnesium, depending on the calcium concentration used (Mould *et al*., 1995). These observations led to the creation of a model showing divalent cation dependent cell adhesion that consists of three distinct binding sites on integrins for divalent cations (Mould *et al*., 1995).

More recently, the presence of three distinct divalent cation binding sites involved in integrin binding have been confirmed with the use of an X-ray crystal structure for the α5/β3 integrin (Xiong *et al*., 2003). Two of these sites interact closely at the binding surface of the integrin with its ligand and are occupied by manganese or magnesium (Xiong *et al*., 2002; Mould *et al*., 2003). One of these two sites, named the metal ion dependent biding site (MIDAS), is required for the active conformation to be achieved, as mutations to this binding site inhibit the induction of the integrin's active conformation by manganese or magnesium (Mould *et al*., 2002). The third binding site appears only to bind calcium and mediates calcium's inhibitory effects on integrin binding (Mould *et al*., 2003). However, this site also appears to stabilize the bound integrin head to its ligand (Mould *et al*., 2003). Mutations in this site lead to an integrin, which binds and then

quickly releases its ligand (Mould *et al*., 2003). While the structural details of integrins and the role of divalent cations is becoming clearer, further studies are needed to clarify the interactions between the different divalent cations and how they work *in vivo* to activate and functionally influence the behavior of integrins.

1.1.13 Zinc and Its Role in Metastasis

Zinc, another divalent cation, is rarely mentioned in studies examining the effects of cations on integrin activation. As such, little is known about the role of zinc in cancer metastasis, especially in relation to breast cancer metastasis. Therefore, the review presented in this section is extended to colon cancer metastasis.

Zinc is well known for its role in both immune function and wound healing. These functions of zinc can be partially explained by its activation of integrins. β2 integrins are important in mediating the adhesion of monocytic cells onto fibrinogen, a process that occurs at sites of injury and inflammation. The presence of zinc can induce an upregulation in activation of this integrin suggesting a mechanism by which it positively impacts immunity (Chavakis *et al*., 1999). Similar to adhesion, cell migration necessary for wound healing is also modulated by integrin expression and activation. In human keratinocytes, zinc has been shown to induce scratch closure by its apparent affect on the integrin α3/β1 (Tenaud *et al*., 2000). Another study showed an increase in α3/β1 expression in the presence of zinc (Tenaud *et al*., 1999).

Zinc has also been shown to have a modulating effect on the actions of pro-

binding cations such as manganese. Thamilselvan *et al*. (2003) showed that 10 mmol/L zinc has a similar inhibitory effect as calcium on the adhesion of colon cancer cells promoted by 1 mmol/L of manganese. Kagara *et al*. (2007) suggested that the zinc importer ZIP10 is a key modulator of invasion in breast cancer cells. Higher gene expression of ZIP10 in cancerous breast tissue is associated with lymph node metastases. Knockdown of the ZIP10 importer leads to a decrease in zinc uptake and a reduced rate of migration in these cells. Attempts to reverse this effect on migration, such as raising intracellular zinc, has no effect, suggesting that the zinc actually transported by the ZIP10 may play a key role in cell migration (Kagara *et al*., 2007).

In contrast, high expression of ZIP6, another zinc importer, corresponded with good prognostic markers such as a low grade, I or II, tumors (Kasper *et al*., 2005). The level of ZIP6 protein corresponded with better prognosis in breast cancer patients and was associated with a longer relapse free and overall survival in breast cancer patients with invasive ductal carcinoma. Interestingly, ZIP6 attenuation in cultured ductal breast cancer T47D cells resulted in a decreased cellular zinc concentration and apoptosis, an increased tumor colony formation, and a decreased E-cadherin expression, indicating that ZIP6 attenuation promotes epithelial-to-mesenchymal transition (Lopez and Kelleher, 2010). These apparently contradictory effects between ZIP10 and ZIP6 on breast cancer metastasis demonstrate the role of zinc in metastasis is complex and remains unclear.

In colon cancer cells zinc supplementation at 100 μmol/L reduces migration and invasiveness. These reductions in motility are associated with changes to the cell

cytoskeleton such as reduced F-actin, shortened microtubules and a rounded cell appearance (Rudulf *et al.,* 2008). Changes to the actin cytoskeleton are also induced with zinc (10 μmol/L) in canine kidney cells (Mills *et al.,* 1992). In this study, cell rounding is apparent as well as a loss of cell stress fibers and an increase in F-actin. Although cell death is not increased, cells have a higher tendency to detach from the matrix, possibly due to the reduction in stress fibers. Changes in a cell's aptitude for migration and its cytoskeleton rearrangement can also be assessed by measuring proteins often implicated in the epithelial to mesenchymal transition such as E-cadherin and Vimentin. Although Vimentin expression decrease would be expected in less metastatic cells, E-cadherin, normally a marker of a normal stationary epithelial cell, was also decreased (Rudolf *et al.,* 2008). The conflicting results on the action of zinc on metastasis suggests that zinc has many roles, some perhaps conflicting, in the complex metastatic process.

1.1.14 Summary

Zinc's essentiality for normal growth has been known for many years. The exact mechanism, especially how it influences cancer cell growth, is still not fully understood. The apparent need for zinc in tumor growth, however, supports a role for this mineral in cancer progression. In studies using human breast tissue, a greater accumulation of zinc in cancerous compared to normal breast tissue is observed (Margalioth *et al.,* 1983; Cavallo *et al.,* 1991; Gupta *et al.,* 1991; Ng *et al.,* 1993; Jin et al. 1999; Geraki *et al.,* 2002). In addition, reduced dietary zinc intake in an animal model for human breast cancer does indeed reduce tumor growth and incidence (Lee *et al.,* 2004). It is also known that quickly proliferating tissues are particularly sensitive to zinc deficiency

(Prasad *et al*., 1996). As cancer is defined by its fast and unregulated growth it is likely that cancer cells are highly sensitive to altered zinc availability.

A possible role of zinc in cancer metastasis is a much newer topic. Kagara *et al*. (2007) have shown that zinc uptake by the ZIP10 transporter is required for metastasis and that zinc depletion halted metastasis. In colon carcinoma cells, zinc supplementation (100 μmol/L) opposed migration apparently through its affects on the cell's cytokeleton (Rudulf *et al*., 2008). Furthermore, in normal cells, zinc has been shown to promote migration (Tenaud *et al*., 2000). Overall, past research suggests that zinc may play an important role in the growth of cancer cells. More current studies imply that zinc may also be involved in cancer progression, by affecting cancer cell metastasis. Clearly studies are needed to clarify how zinc acts in both cancer cell growth and migration.

1.2 Hypothesis and Rationale

The hypothesis for my thesis research is that zinc promotes the metastasis potential of human breast cancer cells. The rationale is that zinc has been shown to decrease wound-healing time, likely by increasing the affinity by which cells attach to the ECM. The strength of this adhesion is paramount to the ability of cancerous cells to migrate. Integrins are the primary mediators of cell to ECM adhesion and, therefore, are required for cell migration in both normal physiological processes (e.g. embryogenesis and wound healing) and pathological states (e.g. tumor progression and metastasis). Zinc can induce an upregulation in activation of β2 integrin to mediate the adhesion of monocytic cells to fibrinogen (Chavakis *et al*., 1999) and induce scratch closure by

affecting the expression of α3/β1 integrin (Tenaud *et al*., 1999) and its activity in human keratinocytes (Tenaud *et al*., 2000). Furthermore, the zinc importer ZIP10 has been suggested to be a modulator of invasion in breast cancer cells (Kagara *et al*., 2007). These studies support a pro metastatic role of zinc. In contrast, elevated expression of ZIP6 is associated with a longer relapse free and overall survival in breast cancer patients with invasive ductal carcinoma (Kasper *et al*., 2005), apparently related to a suppressed epithelial-to-mesnchymal transition (Lopez and Kelleher, 2010), an early event in cancer metastasis.

1.3 Overall Objective and Specific Aims

The overall objective of my thesis research is to characterize the effects of zinc on the growth and metastatic potential of human breast cancer cells. The specific aims were:

1) To assess the effects of zinc on the growth of human breast cancer MDA-MB-231 cells.
2) To investigate the effects of zinc on the migration of human breast cancer MDA-MB-231 cells; and
3) To investigate the effects of zinc on the adhesion of human breast cancer MDA-MB-231 cells.

In answering these objectives, my thesis hopes to add to the current knowledge of zinc's role in cell migration and metastasis.

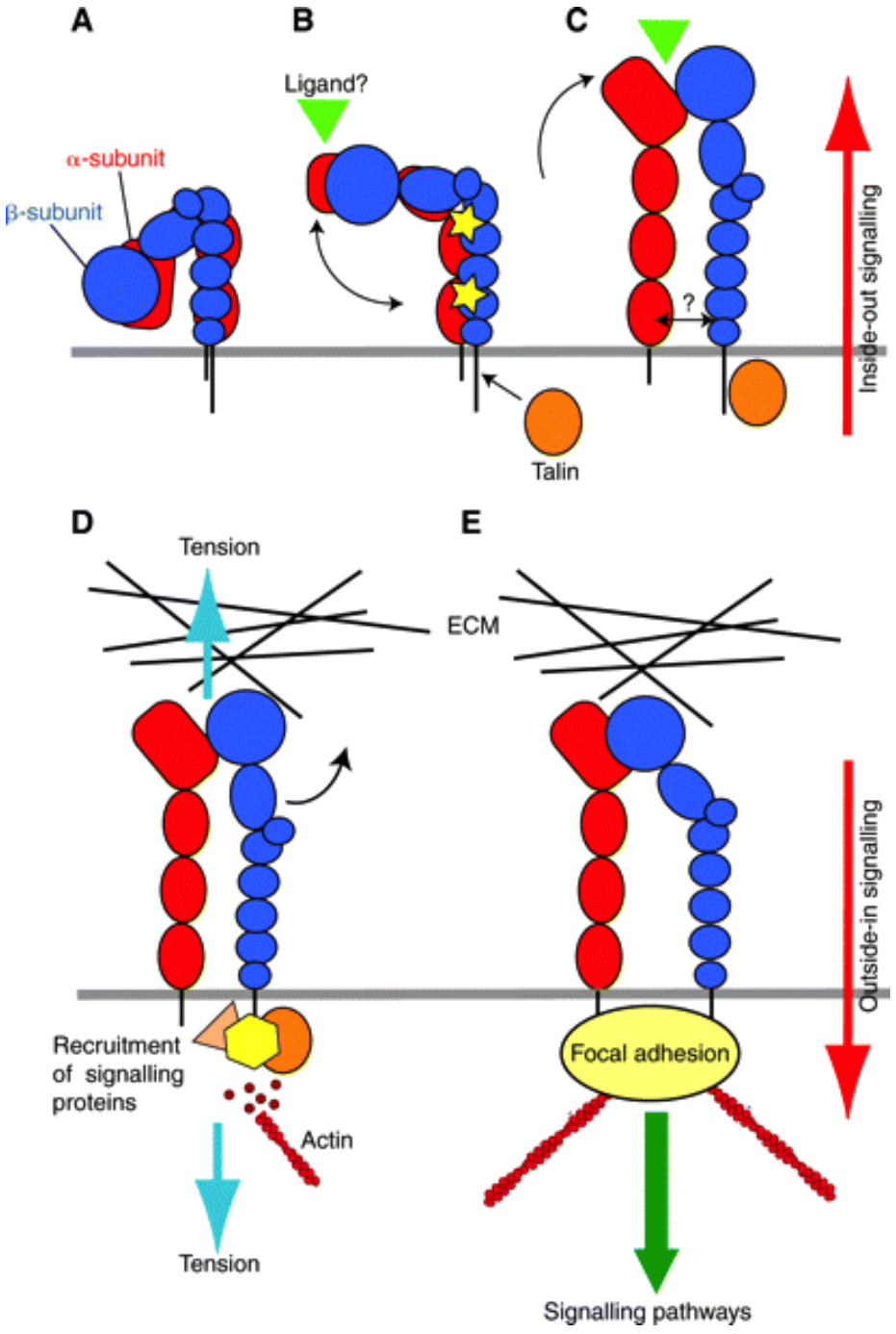


Figure 1.1 Integrin structure and function relationship (Askari *et al.*, 2009. Reproduced with permission from the Journal of Cell Science.)

Chapter 2 Zinc Inhibits Magnesium-Mediated Human Breast Cancer MDA-MB-231 Cells Migration on Fibronectin[1]

2.1 Introduction

Breast cancer is the most prevalent type of cancer in Canadian women and ranks second in cancer mortality (Canadian Cancer Society, 2011). The cause of breast cancer mortality is not the primary tumour, but rather tumour growth in secondary locations (Weigelt *et al*., 2005). For this reason, strategies to reduce metastases in breast cancer patients are an important area in the battle against breast cancer.

To achieve metastasis cancerous cells must undergo a phenotype change from epithelial cells to mesenchymal cells, which have increased motility and are capable of gaining access to the blood circulation or lymphatic system by penetrating through the basement membrane. Paramount to the ability of cancerous cells to migrate is the affinity with which they attach to the extracellular matrix (ECM).

Integrins are the primary mediators of cell to ECM adhesion and are important in cell migration during embryogenesis and wound healing, and in pathological states such as cancer metastasis. Each integrin consists of an α- and a β-subunit forming a heterodimer (Hynes, 2002). Presently, there are a total of 24 known distinct integrins. The expression of integrin is cell-type specific and each integrin recognizes a particular ECM protein. As a bidirectional-signalling molecule, the head of the integrin interacts with the ECM initiating a signalling cascade inside the cell which controls numerous

[1] A version of this chapter is in preparation for submission for publication.

cellular processes, including cell proliferation and motility (Hynes, 2002; Li *et al.,* 2005). Conversely, signals from within the cell can cause a conformational change in the integrin that renders it to its active state and enables binding to the ECM.

Many integrins of the β1 family (e.g. α5/β1 also called the fibronectin receptor) have been implicated in cancerous cell growth and metastasis. Adhesion of circulating tumour cells to the endothelial cells that line the blood vessel walls is an important step in their ability to gain access to new tissues and form distant metastases. Blocking of α5/β1 integrin reduces adhesion of a highly metastatic breast cancer cells to a model endothelium (Bliss *et al.,* 1995), providing strong evidence showing a role of α5/β1 integrin in promoting metastasis. Interestingly, mammary tumours bearing disrupted β1 gene exhibit significantly reduced capacity to metastasize to the lung in mice (Huck *et al.*, 2010).

Divalent cations such as Mn^{2+} and Mg^{2+} are essential for integrin-ligand binding and their presence is required for cell adhesion and cancer metastasis (Draisfield *et al.,* 1992; Luque et al, 1996; Yin *et al.,* 1997). In contrast, Ca^{2+} promotes adhesion in a leukemic cell line (Yin *et al.,* 1997) and inhibits the adhesion in other cell lines (Mould *et al.,* 1995; Luque *et al.,* 1996).

The role of Zn^{2+} in integrin-mediated cell adhesion in breast cancer cells and its role in the process of breast cancer metastasis is essentially unknown. However in other cell types, zinc has been shown to activate β2 integrin to mediate the adhesion of

monocytic cells to fibrinogen (Chavakis *et al.,* 1999) and promotes the expression (Tenaud *et al.,* 1999) and activation (Tenaud *et al.,* 2000) of α3/β1 in human keratinocytes. Furthermore, Kagara *et al*. (2007) reported an association between elevated expression of ZIP10, a zinc importer, in cancerous breast tissue and lymph node metastases. Knockdown of ZIP10 in MDA-MB-231 cells reduces cellular zinc uptake and migration, suggesting zinc is needed for migration of breast cancer cells. In contrast, expression of ZIP6, another zinc importer, corresponds with good prognostic markers and lower grade tumours (Kasper *et al*., 2005) and is associated with a longer relapse free and overall survival in breast cancer patients with invasive ductal carcinoma. Importantly, ZIP6 attenuation in ductal breast cancer T47D cells results in a decreased cellular zinc concentration and apoptosis, increased tumour colony formation and decreased E-cadherin expression. The results of this study suggest that zinc inhibits the epithelial-to-mesenchymal transition, a necessary step in preparing breast cancer cells for metastasis (Lopez and Kelleher, 2010). Collectively, it is apparent that zinc can influence breast cancer metastasis; but whether it acts as a promotor or inhibitor of breast cancer metastasis is presently unclear. With zinc's known importance in growth and evidence of its influence on increasing cell mobility in normal tissue types (Chavakis *et al.,* 1999; Tenaud *et al.,* 2000) it is likely that zinc enhances the metastastic potential of human breast cancer cells. The objectives of this study were: 1) to assess the effects of zinc on the growth of human breast cancer MDA-MB-231 cells; 2) to investigate the effects of zinc on the migration of human breast cancer MDA-MB-231 cells; and 3) to investigate the effects of zinc on the adhesion of human breast cancer MDA-MB-231 cells.

2.2 Materials and Methods

2.2.1 Cell Culture

Human breast carcinoma MDA-MB-231 cells, derived from a lung metastasis, were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). These cells were routinely maintained as suggested by ATCC in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, New York), 4500 mg/L glucose and 584 mg/L L-glutamine, and supplemented with sodium pyruvate (110 mg/L), sodium bicarbonate (1500 mg/L), and penicillin streptomycin (50,000 Units/L Penicillin and 50,000 μg/L streptomycin) at 37 °C, 5% CO_2. This medium is referred as the 'regular medium' throughout this thesis. The cells were grown in T-75 culture flasks or 100 mm cell culture-treated Petri dishes. The initial seeding density was 5 x 10^5 cells/T-75 flask or 100 mm Petri dish. Cells with a passage number of 39-45 were used for all experiments.

2.2.2 Zinc Depleted Medium

In the regular medium, zinc came exclusively from the FBS, which provided zinc at a concentration between 3.5 and 5 μmol/L as zinc concentration varied slightly between batches. To allow for controlling the amount of zinc in each treatment and trial conducted, FBS was treated with Chelex-100 resin (Bio-Rad, Hercules, CA), a divalent cation chelator, to remove zinc from the FBS. The chelating process was carried out as previously reported (Paski and Xu, 2001) with modifications as follows. Briefly, FBS (500 mL) was stirred continuously at 0 °C for 24 h with 50 g of Chelex-100 resin. The FBS-Chelex-100 mixture was then spun down (1,000 rpm, 15 min at 4 °C) followed by filtration to remove the Chelex-100 resin and to sterilize the FBS. Since Chelex-100 is

also capable of chelating other cations besides zinc, a mixture of salts was added back to the Chelex-100-treated FBS to restore Ca, Mg, P, Mn , Mo, Cu and Fe concentrations present in the FBS prior to chelation. The addition of 10% Chelex-100-treated FBS to DMEM yielded a final medium zinc concentration of 0.04 μmol/L. This concentration was referred to as "0 μmol/L" zinc group throughout this thesis.

2.2.3 Zinc Treatments

MDA-MB-231 cells were seeded at 5 x 10^5 cells/T75 flask or 100 mm Petri dish in the regular medium for 24 h allowing the cells to attach to the culture flasks or the culture dish. At the end of the 24 h, the culture medium was removed and replenished with the Chelex-100-treated medium supplemented with 0 (zinc-deficient medium), 5 (zinc-adequate medium), or 25 (zinc-supplemented medium) μmol/L of Zn^{2+} as $ZnSO_4$. Cells were maintained in the assigned medium for 96 h before determining total cellular zinc concentration, abundance of labile intracellular pool of zinc, and cell growth.

For the cell migration and adhesion experiments, cells were cultured in regular medium. After the cells reached confluence, the regular medium was replaced by serum-free DMEM or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer; pH 7.4) to carry out the experiment. The omission of FBS was for the purpose of eliminating growth factors that might influence migration and cell proliferation. Both serum-free DMEM and the HEPES buffer were able to maintain cell viability for the duration of the experiment. Both serum-free DMEM and HEPES buffer had the advantage of being zinc free, which allowed for more accurate manipulation of zinc concentrations with a true zinc-free control. The cells were treated with zinc ranging from 0 to 50 μmol/L for a

duration of 45 min for the adhesion assay and 12 h for the migration studies.

2.2.4 *Determination of the Overall Cell Growth* (Figure 2.1)

After growing for 96 h, the cells were harvested using Trypsin-EDTA (2 mL, 0.25%; Invitrogen, Burlington, ON). Once the cells were lifted from the surface of the flask, an equal volume of regular medium was added to neutralize the trypsin activity. The cell culture dish was then washed five times with the regular medium/Trypsin-EDTA solution mixture to ensure complete detachment of the cells from the surface. The cells were then transferred to a centrifuge tube and pelleted by centrifugation (500 x g, 5 min; 4 °C). After removing the supernatant, the pellet was re-suspended in 5 mL of regular medium by repeated pipetting up and down several times. A 50 μL aliquot of the cell suspension was then diluted 200 times in phosphate buffered saline (PBS; pH 7.4) and counted using a particle counter (Z1 Coulter Particle Counter, Beckman Coulter, Fullerton, California). To avoid counting cell debris, the cutoff was set at 8.0 μm. Each sample was counted twice and their average was used to represent the cell counts for the sample. This count was multiplied by 200 to get the total number of cells/mL followed by further multiplication by five to obtain the total number of cells/cell culture dish.

2.2.5 Total Cellular Zinc Content (Figure 2.1)

Following the 96 h zinc treatment, total cellular zinc concentration was measured as follows. Cells were harvested and pelleted as described above followed by rinsing with PBS to remove residual medium. The cell pellet was then resuspended in 500 μL PBS followed by gentle mixing. A small fraction of the cell suspension (50 μL) from

each sample was removed and diluted in 10 mL PBS for cell counting as described above. A total of 1.5 x 10^6 cells/sample were then transferred to a micro-centrifuge tube and pelleted for a second time by centrifugation (300 x g, 5 min, 4 °C). After discarding the supernatant, the pellet was then acid digested with concentrated nitric acid (100 µL) overnight. The digesta was then diluted by 900 µL of 0.1 N nitric acid, yielding a final volume of 1 mL, before obtaining absorbance using a flame atomic absorption spectrophotometer (Model 2380, Perkin Elmer, Norwalk, CT). The absorbance was converted to zinc concentration using zinc standards prepared using standard zinc solution (Fisher, Ottawa, ON). Total cellular zinc concentration was normalized to 1 x 10^6 cells.

2.2.6 Abundance of Labile Intracellular Pool of Zinc (LIPZ) (Figure 2.1)

Following the 96 h zinc treatment, cells were harvested, pelleted, rinsed, resuspended, and counted as described above. The cells (2.2 x 10^6) were then transferred to a micro-centrifuge tube and pelleted (300 x g, 5 min, 4^0C). The cells were re-suspended in 400 µL of Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY). Two aliquot of samples (180 µL each) were transferred to a 96-well plate (black) yielding 1 x 10^6 cells/well.

Abundance of the labile intracellular pool of zinc was assessed using Zinquin elthyl ester (Zinquin; Sigma-Aldrich, St. Louis, MO). The Zinquin stock solution (5 mmol/L) was prepared using dimethyl sulfoxide (DMSO) and stored at -20 °C until used. Before use, the Zinquin stock solution was further diluted with HBSS (pH 7.4) to prepare

a working solution (250 μmol/L). To carry out the assay, the Zinquin working solution (20 μL) was added to each well in the 96-well plate (black) yielding a final Zinquin concentration of 25 μmol/L and a total volume of 200 μL. Subsequently, the cells were incubated at 37 °C for 30 min in the dark using a thermal shaker (Eppendorf Thermomixer R, Brinkman Instruments, Westbury, NY). The intensity of Zinquin-dependent fluorescence was measured at an excitation and emission wavelength of 365 nm and 485 nm, respectively, using a micro-plate reader (SpectraMAX GEMINI XS, Molecular Devices, Sunnyvale, CA). The reagent control contained no cells. The fluorescence intensity obtained from this control served as the background and was subtracted from the fluorescence intensity obtained from the samples.

2.2.7 Cell Viability (Figure 2.2)

Cells were seeded at 1,500 cells/well in a 96-well plate and maintained in the regular medium for 24 h followed by growing the cells in one of the three zinc-treatment media described above for 96 h. To assess cell viability, the medium was replaced with 100 μL of phenol free DMEM containing 10% 3-[4,5]-dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) followed by incubation at 37 °C for 3.5 h or until visible purple crystals had formed around the cells. MTT is transformed from a soluble yellow compound to purple crystals by enzymatic action of live cells. Finally, isopropanol (100 μL) was added to each well and cells were incubated for another 40 min before obtaining the absorbance at 570 nm using a microplate reader (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA). Background absorbance (phenol-free DMEM containing 10% MTT) was also measured and subtracted from the absorbance of the samples.

2.2.8 Wound Healing "Scratch Assay" (Figure 2.3)

The wound-healing assay was carried out following the protocol reported by Liang *et al*. (2007) with modifications. Briefly, Petri dishes (10 mm) were coated with fibronectin (5 μg/mL; Sigma-Aldrich, St. Louis, MO) or poly-D-lysine (100 μg/mL; Sigma-Aldrich) overnight at 4 °C followed by blocking with bovine serum albumin (BSA; 2 mg/mL) for 1 h. Cells were then seeded at 1 x 10^6 cells/dish and grown in the regular medium until the cells formed a monolayer, which took approximately 4 d. At that point, the medium was removed and a scratch was made in the monolayer with a 1 mL pipette tip to void an area of cells. The scratch was evened out by washing with a serum-free DMEM. Subsequently, serum-free DMEM supplemented with 0, 2.5, 5, 10 or 25 μmol/L of Zn^{2+} was added to each fibronectin- or poly-D-lysine-coated dish. The serum-free DMEM was used to eliminate growth factors present in the FBS and, hence, to minimize cell proliferation. The dishes were viewed and photographed under a light microscope (100 X magnification; Axiovert 200M; Carl Zeiss, Germany) for examining the scratch and measuring the scratch width at 0 and 12 h after scratching. At each time point, three pictures per scratch (1 scratch per dish) were taken. The width of the scratch was measured using the measurement function of the Axiovision 4.6 software. Due to the variability in scratch width, four measurements were taken at each of the three photographed locations along the scratch at the 0 h time point and six measurements were taken per location at the 12 h time point to ensure an accurate measurement of the width of the scratch. Coordinates for each location were recorded and used to ensure that the subsequent measurements were taken at the same three locations at the 0 h and 12 h time

points in each dish. The average width of the scratch calculated at 12 h was subtracted from the average width of the scratch at 0 h to obtain the distance that cells migrated during the 12 h period. This procedure was carried out for both the fibronectin- and poly-D-lysine coated dishes. The average migration distance of each of the poly-D-lysine coated dishes was subtracted from its coresponding fibronectin-coated pair and then divided by 12 to obtain the rate of fibronectin specific migration distance (μm/h).

2.2.9 Track "Bead Assay" with Rhodamine Phalloidin Stain (Figure 2.4)

The bead assay was carried out by following the established protocol (Windlerr-Hart *et al*., 2005) with modifications. Briefly, glass cover slips were coated overnight with fibronectin (5 μg/mL) by submerging them in fibronectin solution in a 12 well plate. Subsequently, while still in the 12-well plate, the cover slips were washed with PBS (pH 7.4) and coated evenly with 50 μL 0.2% FluoSpheres beads (Invitrogen, Burlington, ON). After allowing the FluoSpheres to dry for 1 h at room temperature, extra beads were removed by washing with 1 mL PBS twice. Cells were then seeded onto the glass cover slips at a density of 5 x 10^3 cells/well. The cells were incubated in HEPES buffer (pH 7.4) supplemented with 1% FBS, 1 mmol/L Mg^{2+} and 0, 2.5, 5, 10 or 50 μmol/L Zn^{2+} for 12h. At the end of this incubation period, HEPES buffer was carefully removed and the cells were fixed in para-formaldehyde (3.7%) for 20 min. After removing para-formaldehyde, the fixed cells were gently washed with 1 mL PBS twice. The cells were then permeabilized with Triton-X-100 (0.3%, 750 μL; VWR international, Missisauga, ON) for 15 min. Subsequently, the cells were washed with 1 mL PBS twice and stained with rhodamine-phalloidin (Invitrogen, Burlington, ON) for 20 min in a dark, humidified chamber at room temperature. Finally, the cover slip was rinsed with PBS and mounted

onto a histological slide to be viewed under the florescent microscope (100 X magnification; 10 x 10) with a blue filter (cutoff at 440 nm). Cells phagocytozed the FluoSpheres in the spot where they adhered and along their migratory path, leaving a clear dark track where the cells had migrated. To quantify migration distance, the track length of 30-50 cells/cover slip were measured using the measurement function of the Axio Vision 4.6 software. The average migration distance of these cells on three different cover slips was used to determine the migration in each zinc treatment group.

The actin polymers (F-actin) stained by the rhodamine phalloidin dye was visualized under the microscope (400 X magnification; 40 x 10) with the red filter (cutoff at 565 nm). Pictures of representative cells were taken to demonstrate the affects of zinc treatments on the structure of the cell's F-actin.

2.2.10 Adhesion Assay and Antibody Blocking Assay (Figure 2.5)

The adhesion assay was carried out using the protocol reported previously by Luque *et al*. (1996), with modifications. Briefly, cells were first grown in the regular medium in 100 mm Petri dishes at a seeding density of 1 x 10^6 cells/dish. When it reached 80% confluency, the cells were harvested and pelleted as described previously. The pellet was re-suspended in serum-free medium or HEPES buffer (pH 7.4) and the density was adjusted to 2.2 x 10^6 cells/mL. To prepare for the adhesion assay, wells of a 96-well plate were coated with fibronectin (5 μg/mL) and left at 4 °C overnight. After removing excess fibronectin, the wells were blocked with 1% BSA for 1 h at room temperature and then washed with 200 μL PBS twice. Depending on the experiment either serum-free medium with Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) or HEPES buffer (50 μL)

containing Mg^{2+} (1 mmol/L), Ca^{2+} (2 mmol/L), Mg^{2+} (1 mmol/L) + Ca^{2+} (2 mmol/L), or Mn^{2+} (1 mmol/L) with Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) was added to each well. Cells (50 μL/well) were then transferred to the 96-well plates at a final cell density of 1 x 10^5 cells/well. After incubation for 45 min at 37 ºC, the 96-well plates were washed twice with 200 μL of warm HEPES buffer (pH 7.4) per wash followed by fixation with 95% ethanol for 15 min. Subsequently, ethanol was removed and the cells were stained with 0.1% crystal violet for 20 min at room temperature. At the end of this incubation period, the crystal violet solution was removed and the wells were rinsed thoroughly with double de-ionized water. Finally, acetic acid (10%) was added to the wells to solubilize crystal violet stained cells followed by determining absorbance at 590 nm using a microplate reader (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA). Absorbance from the controls, which contained no cells, was subtracted from the absorbance from the treated cells to account for background absorbance.

Integrin blocking antibodies for the integrin subunits α1 - α6 and αV (β1 Integrin Partners Kit, Chemicon International, Temecula, CA) were used to establish the involvement of integrin subunits in the adhesion of MDA-MB-231 cells to fibronectin. Cells were prepared as described above for the adhesion assay, except that prior to seeding the cells into fibronectin coated wells, the cells were incubated in one of the above antibodies (2 μg/mL) for 30 min at room temperature following the manufacturer's instruction. HEPES buffer (pH 7.4) containing Mg^{2+} (1 mmol/L) was added to each well (50 μL/well) followed by the addition of antibody-blocked cells (50 μL/well). The final cell density was 1 x 10^5 cells/well and the blocking antibody concentration was 1 μg/mL.

The cells were incubated at 37 °C for 45 min allowing them to adhere to the wells. The 96-well plate was then washed with HEPES buffer, fixed with ethanol, stained with crystal violet and absorbance read following the exact procedure detailed in the paragraph above for the adhesion assay. Results were presented as % control of adhesion compared to HEPES buffer containing Mg^{2+} (1 mmol/L) alone with no antibody.

2.2.11 Flow Cytometry Assay for Total and Activated β1 Integrin (Figure 2.6)

The flow cytometry assay was performed using the protocol described in Luque et al. (1996) with modifications. Cells were first grown to 80 % confluence in the regular medium in 100 mm Petri dishes and then harvested using a dissociation buffer (0.34 mmol/L EDTA, 68 mmol/L NaCl, 1.34 mmol/L KCl, 4.05 mmol/L Na_2HPO_4, 0.73 mmol/L KH_2PO_4, and 0.56 mmol/L glucose). The cells were then divided into two groups. One group was used for β1 integrin testing and the other group was used for HUTS 4. Cells in the β1 integrin group were kept on ice, and diluted and washed with cold FACS buffer. Cells in the HUTS 4 group were kept at room temperature, diluted and washed with warm FACS buffer throughout the experiment. Cells in both groups were pelleted by centrifugation (300 x g, 5 min) at 4 °C for the β1 integrin group and at room temperature for the HUTS 4 group followed by resuspension in 5 mL of FACS buffer (PBS containing 1% BSA and 0.02 mol/L glucose; pH 7.4) and counted using a hematocytometer. Cells were then diluted to 5 x 10^5 cells/mL with the FACS buffer and transferred (500 μL) to microcentrifuge tubes yielding 2.5 x 10^5 cells/tube. Cells were subsequently pelleted by centrifugation (300 x g, 5 min), washed with 500 μL FACS buffer and re-pelleted by centrifugation (300 x g, 5 min). Finally, the cells were

resuspended in 50 μL FACS buffer and the appropriate antibody added (β1 or HUTS 4 conjugated FITC).

HUTS 4 activated integrin antibody (BD Biosciences Pharmingen, San Diego, CA) was added at 2.5 μg/50 μL of sample in the presence of divalent cations (same as the adhesion assay described above) and incubated for 30 min at 37 °C. Subsequently, the antibody was diluted out by adding FACS buffer (450 μL), pelleted by centrifugation (300 x g, 5 min) and then washed with FACS buffer (500 μL) and centrifuged (300 x g; 5 min) again to remove excess primary antibody. After the final centrifugation, the cells were kept on ice for the remainder of the procedure. Anti-mouse alexa-488 (Invitrogen, Burlington, ON) was used as the secondary antibody at 0.25 μg/50 μL FACS buffer. Upon adding the secondary antibody, the cells were incubated for 30 min in the dark at 4 °C. At the end of the incubation period, the secondary antibody was diluted out with FACS buffer (500 μL) and cells were washed and centrifuged twice as described above for removing the excess primary antibody.

For quantifying the presence of total β1 integrin, only one antibody incubation step was required using Anti-integrin β1 conjugated FITC antibody (1 μg/sample in 50 μL FACS) (Immunotech, Vaudreuil-Dorion, Quebec). Cells were incubated at 4 °C for 30 min in the dark.

For analysis using flow cytometery, samples from both antibody groups were suspended in 500 μL FACS buffer and transferred to FACS tubes. The cell fluorescent

output was quantitated using a FACScan (Becton-Dickinson, Franklin Lakes, NJ). Histogram of cell fluorescent output was for 10,000 cells. To assess the background fluorescence output, samples containing no primary and secondary antibodies were also included and Mn^{2+} (1 mmol/L) was used as a positive control for integrin activation.

2.2.12 Statistical Analysis

The differences among the means of zinc concentration groups in each experiment were analyzed by ANOVA followed by Tukey's Honesty Test (JMP, Release 5.1; $p < 0.05$).

2.3 Results

2.3.1 Zinc Status and Growth

Total Cellular Zinc Status Cellular zinc status was assessed by total cellular zinc content and the abundance of LIPZ. Total cellular zinc content was 126 ng Zn/10^6 cells and was not affected by zinc treatment (Figure 2.7). The abundance of LIPZ was similar between the cells cultured in the zinc-deficient medium and that in the zinc-adequate medium (Figure 2.8). In contrast, the abundance of LIPZ was increased by about 20% in the zinc-supplemented medium compared to that in the zinc-adequate medium ($p < 0.05$).

Cell Number and Cell Viability Effects of zinc on overall cell growth were assessed by total cell number and cell viability. When the cells were cultured in the zinc-adequate medium, total cell numbers reached 3.9 ± 0.2 x 10^6 cells/dish (Figure 2.9). Neither lowering the medium zinc concentration to deficient levels nor raising the

medium zinc concentration above adequate zinc levels had an effect on total cell number. Similarly, zinc also had no effect on cell viability (Figure 2.10).

2.3.2 Metastatic Potential

The metastatic potential of the MDA-MB-231 cells was assessed by a combination of migration distance assessed using the single cell migration assay and migration rate assessed using the wound-healing assay.

Zinc Inhibited Single Cell Migration MDA-MB-231 cells cultured in the zinc-deficient medium migrated 74 ± 3.6 μm on fibronectin in 12 h (Figure 2.11). This migration distance was reduced by 68% (24 ± 4.8 μm) and 86% (10 ± 1.4 μm) when the zinc-deficient medium was supplemented with 10 and 50 μmol/L, respectively ($p < 0.05$). The reduction in migration distance when zinc supplementation increased from 10 to 50 μmol/L (Figure 2.11) was not statistically significant. To tease out the lowest effective level of zinc supplementation causing a reduced cell migration distance, a lower range of zinc supplementation (0 - 10 μmol/L) was tested. The migration distance remained unchanged when the zinc-deficient medium (48 ± 4.4 μm) was supplemented with 2.5 μmol/L of zinc (49 ± 2.53 μm); however, further increase in the supplementation level to 5 and 10 μmol/L resulted in a 43% (27 ± 0.02 μm) and 63% (18 ± 0.14 μm) reduction in the migration distance, respectively ($p < 0.05$). The reduction in cell migration distance when zinc supplementation was increased from 5 to 10 μmol/L (Figure 2.11) was not statistically significant.

Zinc Inhibited Fibronectin Specific Cell Migration MDA-MB-231 cells cultured in the zinc-deficient medium migrated on fibronectin at a rate of 6.03 ± 1.0 μm/h (Figure 2.12). This fibronectin specific migration rate remained unchanged when the zinc-deficient medium was supplemented with 2.5 μmol/L zinc. However, the migration rate was reduced by 72% (1.70 ± 1.3 μm/h), 75% (1.46 ± 0.93 μm/h), and 90% (0.6 ± 0.45) μm/h) when the medium was supplemented with 5, 10, and 25 μmol/L of zinc, respectively, ($p < 0.05$; Figure 2.12). The differences when zinc supplementation was increased from 5 μmol/L to 10 or 25 μmol/L (Figure 2.12) were not statistically significant.

2.3.3 Cell Adhesion

Divalent Cations Varied in Their Ability to Promote Cell Adhesion to Fibronectin The absence of divalent cations prohibited MDA-MB-231 cell adhesion to fibronectin (Figure 2.13). Presence of Mg^{2+} (1 mmol/L) alone facilitated the adhesion of MDA-MB-231 cells to fibronectin ($p < 0.05$). Replacing Mg^{2+} with Mn^{2+} (1 mmol/L) increased cell adhesion by approximately 40% ($p < 0.05$) while replacing Mg^{2+} with Ca^{2+} (2 mmol/L) reduced cell adhesion by 60% ($p < 0.05$; Figure 2.13). When Mg^{2+} was replaced with Zn^{2+} (10 μmol/L), cell adhesion was no longer supported with results similar to that in the absence of divalent cation ($p < 0.05$; Figure 2-13).

Zinc Inhibited Cell Adhesion to Fibronectin The serum free medium used in this assay contained no zinc. Adhesion of MDA-MB-231 cells to fibronectin was not affected in the presence of 2.5, 5, or 10 μmol/L of Zn^{2+} to the medium (Figure 2.14).

However, adhesion of the cells to fibronectin was decreased by 44 and 48% in the presence of 25 or 50 μmol/L of Zn^{2+}, respectively, compared to that in the absence of Zn^{2+} ($p < 0.05$).

Zinc Inhibited Mg^{2+}-Facilitated Cell Adhesion to Fibronectin In the presence of Mg^{2+} alone (1 mmol/L) MDA-MB-231 cells adhered to fibronectin (Figure 2.15). Inclusion of Zn^{2+} at 5 μmol/L had no effect on Mg^{2+}-facilitated cell adhesion to fibronectin; however this Mg^{2+}-facilitated cell adhesion was reduced by 33, 42, and 68 % in the presence of 10, 25, and 50 μmol/L of Zn^{2+}, respectively ($p < 0.05$). This zinc-mediated inhibition in Mg^{2+}-facilitated MDA-MB-231 cell adhesion to fibronectin was inversely correlated to Zn^{2+} concentration, showing a dose-dependent inhibition ($R^2 = 0.90$).

Zinc Promoted Ca^{2+}-Facilitated Cell Adhesion In the presence of Ca^{2+} alone (2 mmol/L) MDA-MB-231 cells adhered to fibronectin (Figure 2.16). Inclusion of Zn^{2+} at 5 μmol/L had no effect on Ca^{2+}-facilitated cell adhesion to fibronectin. However, increasing the concentration of Zn^{2+} to 10, 25 and 50 μmol/L promoted Ca^{2+}-facilitated cell adhesion to fibronectin by 50, 80 and 77%, respectively ($p < 0.05$). Ca^{2+}-facilitated cell adhesion to fibronectin was not significantly affected when the concentration of Zn^{2+} was increased from 10 to 50 μmol/L ($p < 0.05$). This Zn^{2+}-mediated promotion in Ca^{2+}-facilitated cell adhesion was correlated to Zn^{2+} concentration, showing a dose-dependent promotion ($R^2 = 0.69$).

Zinc Inhibited Mg^{2+}/Ca^{2+}-Facilitated Cell Adhesion. The presence of Mg^{2+} (1 mmol/L)/Ca^{2+} (2 mmol/L) facilitated the adhesion of MDA-MB-231 cells to fibronectin (Figure 2.17). Inclusion of Zn^{2+} at 5 and 10 μmol/L had no effect on Mg^{2+}/Ca^{2+}-facilitated cell adhesion. However, further increase in Zn^{2+} concentration to 25 and 50 μmol/L inhibited Mg^{2+}/Ca^{2+}-facilitated cell adhesion by 10 and 18%, respectively ($p < 0.05$). This Zn^{2+}-mediated inhibition in Mg^{2+}/Ca^{2+}-facilitated cell adhesion was inversely correlated to Zn^{2+} concentration, showing a dose-dependent inhibition ($R^2 = 0.91$).

Zinc Had No Effect on Mn^{2+}-Facilitated Cell Adhesion The presence of Mn^{2+} (1 mmol/L) facilitated the adhesion of MDA-MB-231 cells to fibronectin (Figure 2.18). Inclusion of Zn^{2+} from 5 to 50 μmol/L had no effect on the Mn^{2+}-facilitated cell adhesion to fibronectin ($p < 0.05$).

The effects of Zn^{2+} on Mg^{2+}-, Ca^{2+}-, Mg^{2+}/Ca^{2+}- and Mn^{2+}-induced adhesion of MDA-MB-231 cells to fibronectin are summarized in Table 2.1. Zn^{2+} at 25 or 50 μmol/L inversely inhibited Mg^{2+}- and Mg^{2+}/Ca^{2+}-induced adhesion ($p < 0.05$). In contrast, Zn^{2+} at 10, 25 or 50 μmol/L promoted Ca^{2+}-induced cell adhesion ($p < 0.05$).

Integrin Subunits α5 and β1 Were Required for Cell Adhesion to Fibronectin

Presence of anti-integrin α5- or anti-β1-subunit antibody inhibited Mg^{2+}-facilitated adhesion of MDA-MB-231 cells to fibronectin by 95 and 99%, respectively ($p < 0.05$; Figure 2.19). In contrast, presence of anti-integrin α3-subunit antibody increased Mg^{2+}-

facilitated cell adhesion by 43% ($p < 0.05$). Presence of anti-integrin α1-, α2-, α4-, α6- or αV-subunit antibody had no effect on Mg^{2+}-facilitated cell adhesion ($p < 0.05$).

2.3.4 Presence and Activation of Integrin β1-Subunit

Zn^{2+} Had No Effect on the Presence of Total Cell Surface Integrin β1-Subunit

The proportion of cells stained with the anti-integrin β1 subunit antibody and the fluorescence intensity were the same in cells incubated in the presence of 0 μmol/L of Zn^{2+} compared to that in the presence of 10, 25 or 50 μmol/L of Zn^{2+} (Figure 2.20A-C).

Divalent Cations Had Variable Effects on the Activation of Integrin β1- Subunit

Compared to the control with no mineral, the presence of Mn^{2+} (1 mmol/L) increased fluorescence intensity during HUTS 4 binding, suggesting an increase in activated integrin β1-subunit (Figure 2.21A). Conversely, the presence of Ca^{2+} (2 mmol/L) decreased integrin activation (Figure 2.21B). The presence of Mg^{2+} (1 mmol/L) had no effect on the activation of integrin β1-subunit as shown by the lack of a shift in fluorescence intensity (Figure 2.21C). When adding EGTA (2 mmol/L), a chelator for Ca^{2+}, together with Mg^{2+} (1 mmol/L), there was a small increase in the activation of integrin β1 subunit as shown by a small shift in fluorescence intensity (Figure 2.21D).

Zn^{2+} Had No Effect on the Activation of Integrin β1-Subunit The intensity of fluorescene during HUTS 4 binding remained unchanged in the presence of 5, 10, 25, or 50 μmol/L of Zn^{2+} compared to that in the absence of Zn^{2+} (Figure 2.22A-D). In contrast,

presence of Mn^{2+} (1 mmol/L) increased the fluorescence intensity during HUTS 4 binding, suggesting an increased activation of integrin β1-subunit (Figure 2.22E).

2.3.5 F-Actin Staining with Rhodamine Phalloidin

Zn $^{2+}$ Induced Cell Rounding in Cells Attached to a Fibronectin After adhering and migrating on fibronectin for 12 h in the presence of 0 or 2.5 μmol/L of Zn^{2+}, the MDA-MB-231 cell's actin cytoskeletons appeared to be elongated with obvious lamellipodia and fillopodia protrusions (Figure 1.23A and B). When cells were allowed to adhere and migrate for 12 h in the presence of 5 μmol/L of Zn^{2+}, cells appeared as a florescent ball with a lack of actin protrusions (Figure 1.23C). When the Zn^{2+} concentration was further increased to 10 μmol/L, loss of cell actin appeared to occur as the florescence intensity was greatly diminished and the cells outline becomes difficult to determine (Figure 2.23D). A similar effect was observed when the Zn^{2+} concentration was further increased to 50 μmol/L (Data not shown).

2.4. Discussion

2.4.1 Zinc Status and Growth

Cellular Zinc Status Was Unaffected by Zinc Treatments Tested *LI*PZ abundance was increased by 20% in cells cultured in the zinc-supplemented medium compared to that in the zinc-adequate medium, while total cellular zinc content remained unchanged. LIPZ represents the pool of intracellular zinc that is either loosely bound to macromolecules such as proteins or in its free ionic form (Zn^{2+}). LIPZ is a small fraction of total cellular zinc as its abundance is in picomole range. For example, LIPZ

abundance is 20, 31, 14, and 35 pmol/10^6 cells in human chronic lymphocytic leukaemia cells, rat splenocytes, rat thymocytes (Zalewski *et al*., 1993), and human breast cancer MDA-MB-231 cells (Wu, 2003), respectively. Using ^{65}Zn, it is estimated that the exchangeable compartment of zinc in erythrocytes constitutes approximately 3% of the total cellular zinc (Van Wouwe *et al*., 1990). Therefore, it is unlikely that a 20% increase in the abundance of LIPZ would impact on the total cellular zinc content.

It is interesting to note that total cellular zinc content was unaffected by culturing the cells in either zinc-deficient or zinc-supplemented medium. This lack of response to zinc treatment could be due to an altered zinc homeostasis. Zinc is important to many cellular processes, such as gene expression and regulation, cell proliferation and cell death. Inadequately low cellular zinc status could impair normal cellular functions while excess cellular zinc is toxic. Hence maintaining a relatively stable total cellular zinc content is paramount to normal cellular zinc function. Zinc homeostasis is achieved by a coordinated process involving both zinc importers and zinc exporters (Cousins et al., 2006; Liuzzi and Cousins 2004). When neuronal PC12 cells are exposed to a fatally high zinc environment, the expression of ZnT1, a zinc exporter, is upregulated resulting in an increased zinc efflux and a reduced cell death (Palmiter et al., 1996). In mice, feeding a zinc-deficient diet results in an elevated expression of ZIP10 while zinc repletion causes a reduced expression of ZIP10 (Lichten *et al*., 2011). Thus, it is possible that the lack of response of total cellular zinc content to zinc deficiency or zinc supplementation in MDA-MB-231 cells was due to altered zinc homeostasis achieved through altered expression of zinc importers (i.e. ZIP 6 (LIV 1), ZIP7 and ZIP10) and/or zinc exporters

(i.e. ZnT1, ZnT2 and ZnT4) (Liuzzi and Couins 2004; Kagara *et al*., 2007; Taylor *et al*., 2008).

Growth of MDA-MB-231 Cells Was Unaffected by Zinc Treatment Tested In this study, growth of MDA-MB-231 cells cultured in the zinc-deficient medium was similar to that in zinc-adequate medium. Similarly, growth of MOLT-3 malignant lymphoblast cells is also unaffected by zinc deprivation (Prasad *et al*., 1996). However, zinc deficiency induced by depletion of medium zinc, similar to our study, has previously been shown to slow the growth of HUT 78 malignant lymphoblast cells (Prasad *et al*., 1996). This apparent inconsistency in the effect of zinc deficiency on growth might indicate that the susceptibility of cells to zinc-deficiency-induced growth suppression is cell type specific.

Zinc is essential for growth and cancerous cells are marked by rapid growth. Lee *et al*. (2003) showed that the expression of ZnT1, a zinc exporter, is reduced by 55% in mammary tumours compared to normal mammary tissues while the expression of metallothionein, a putative cellular zinc storage protein, is increased by 3.5 times. These observations suggest that zinc homeostasis in the mammary tumour is altered allowing an accumulation of zinc to ensure sufficient zinc supply to sustain tumour growth. Interestingly, total cellular zinc content in MDA-MB-231 cells cultured in zinc-deficient medium was similar to that in zinc-adequate medium. Therefore, it is possible that zinc homeostasis was altered allowing the cells to accumulate a sufficient amount of zinc to sustain its needs for growth. However, studies are needed to find whether zinc

homeostasis is indeed altered in these cells under the same culture condition.

2.4.2 Zinc Inhibited Integrin-Specific Migration on a Fibronectin

Single cell migration assesses the individual cell invasiveness by measuring the length of the track path each cell is able to clear by phagocytosing the fluorescent beads coating the matrix on which they travel. On the other hand, migration in a confluent monolayer is more representative of *in vivo* cell populations where the ability of a cell to migrate is first dependent on the cell breaking away from its neighbouring cells. In this way, measuring migration in a confluent monolayer allows the impact of cell-cell interactions to be factored in (Liang *et al*., 2007). Therefore, assessing the migration potential through assessing both single cell migration and migration in a confluent monolayer provided a system to quantify of the influence of zinc in migration on an extracellular matrix in MDA-MB-231 cells.

MDA-MB-231 cells migrated on fibronectin (Figure 2.11 and 2.12) in the presence of 0 and 2.5 μmol/L of zinc; however, in the presence of adequate level of zinc (5 μmol/L) or higher (5 - 50 μmol/L of zinc), the migration was significantly reduced. In contrast, at the same zinc concentrations, there was a lack of inhibitory effect of zinc on migration when the assays were carried on a poly-D-lysine matrix (Figure A1), suggesting that the inhibitory effect of zinc on the migration of MDA-MB-231 cells was specific for cells adhering to a fibronectin matrix. MDA-MB-231 cells are known to secrete fibronectin (Saad *et al*., 2002) and therefore express adhesion molecules such as integrins is necessary for cells to adhere and subsequently migrate along this matrix. As poly-D-lysine matrix has been used as a substrate for non-integrin-dependent adhesion

(Price *et al*., 1998), the specificity of the inhibitory effect of zinc on the migration on fibronectin indicated that the reduction in motility of the MDA-MB-231 cells in the presence of zinc was integrin dependent.

2.4.3 Zinc Inhibited Integrin-Specific Adhesion to Fibronectin through Interacting with Other Divalent Cations.

The ability of cells to migrate is dependent on an intermediate level of integrin presence and activation. With too high levels of activation, cells become fixed to their adhesion substrate. In contrast, with too little activation, cell fillopodia are unable to make the connections they need with the matrix to drive migration (Akiyama *et al*., 1995). For this reason, it becomes important to determine whether the inhibitory effect of zinc on MDA-MB-231 cell migration was due to an inhibition or promotion of integrin activation.

The presence of divalent cations is necessary for integrin activation and are therefore required for cell binding to the ECM and migration (Draisfield *et al*., 1992; Luque *et al*., 1996; Yin *et al*., 1997; Mould *et al*., 2002). Manganese has been shown to possess the strongest promoting effect on integrin binding followed by magnesium (Drainsfield *et al*., 1992; Mould *et al*., 1995; Yin *et al*., 1997; Thamilsevan *et al*., 2003), while calcium in certain conditions, supports low level adhesion, but often acts to inhibit adhesion (Mould *et al*., 1995; Yin *et al*., 1997). In this study, manganese induced the strongest adhesion of MDA-MB-231 cells to fibronectin followed by magnesium while calcium promoted a much weaker adhesion of MDA-MB-231 cells to fibronectin

compared to manganese and magnesium (Figure 2.13). These observations were consistent with previous reports. Interestingly, in the presence of zinc alone, there was no measurable adhesion of MDA-MB-231 cells to fibronectin (Figure 2.13). It then is apparent that zinc did not promote the adhesion of MDA-MB-231 cells to fibronectin, which in turn suggests that zinc's influence over cell migration is not mediated via integrin activation.

Evidence obtained thus far showed that zinc was capable of inhibiting the migration of MDA-MB-231 cells on fibronectin; but it was not capable of promoting intergrin activation and subsequent adhesion. This suggests that zinc's affect on adhesion may be through its interference of adhesion promoting cations known to activate integrins. Indeed, when the assay was performed in a serum-free medium, zinc exerted a dose-dependent inhibition of the adhesion of MDA-MB-231 cells to fibronectin (Figure 2.14). In serum-free medium, magnesium (0.8 mmol/L) and calcium (1.8 mmol/L) are the only two divalent cations known to modulate adhesion; therefore the inhibitory effect of zinc on adhesion was likely through its interference with magnesium and/or calcium. Interestingly, zinc exerted a dose-dependent inhibition of adhesion in the presence of magnesium alone (Figure 2.15). Conversely, zinc promoted adhesion in the presence of calcium alone (Figure 2.16). When both magnesium and calcium were present, zinc also exerted a dose-dependent, but much weaker inhibition on the adhesion (Figure 2.17). These observations collectively demonstrated that zinc was capable of interfering with magnesium-mediated integrin activation and subsequent adhesion. The inhibitory effect of zinc on adhesion in serum-free medium was the net effect of the interaction between

zinc and magnesium and zinc and calcium. This interaction further suggests that zinc had some modulating effect on the integrin protein's ability to actively bind to its ligand (i.e. fibronectin), which manifests itself differently depending on the other divalent cations present.

Mould *et al*. (1995), on the interactions among Mn^{2+}, Mg^{2+} and Ca^{2+}, proposed a three divalent cation-binding sites model for integrins: the metal ion-dependant adhesion site, the adjacent metal ion-dependant adhesion site, and the ligand-associated metal-binding site. This model has been supported more recently by an X-ray crytal structure of integrin (Xiong *et al*., 2002). Occupancy of the metal ion-dependant adhesion site by Mn^{2+} or Mg^{2+} is required for integrin ligand binding. The adjacent metal ion-dependant adhesion site is not required for ligand binding (Mould *et al*., 2003). Rather, it stabilizes the active integrin state and occupancy by Ca^{2+} acts to allosterically inhibit the binding of Mn^{2+} to the metal ion-dependant adhesion site (Mould *et al*., 2003). The exact role of the ligand-associated metal-binding site in adhesion has not yet been elucidated. One theory is that this site may be a high affinity Ca^{2+} binding site as it has been previously shown that low concentrations of Ca^{2+} are able to increase the affinity of the metal ion-dependant adhesion site for Mg^{2+}, resulting in an increased ligand binding (Mould *et al*., 1995). Based on this three divalent cation-binding site model, it is possible that zinc was able to bind to the ligand-associated metal-binding site to alter the affinity of adjacent binding sites for Ca^{2+} and Mg^{2+}.

2.4.4 Integrin α5/β1 May Be the Target of Zinc

Integrins are heterodimers consisting one α- and one β-subunit. Currently,

eighteen α– and eight β-subunits identified have been identified in mammals, which combine to form 24 distinct integrins (Hynes, 2002). MDA-MB-231 cells express a number of integrin subunits such as α2, α3, α5, α6, αV, β1 and β7 (Doerr and Jones, 1996; Saad *et al*., 2000). Some of these integrin subunits are able to bind fibronectin while the others are not. When MDA-MB-231 cells were treated with a range of subunit-specific blocking antibodies, the blocking antibody against α5- and β1-subunits resulted in an inhibition of adhesion of these cells to fibronectin of 95 and 99%, respectively (Figure 2-19). In contrast, treating the cells with other subunit-specific blocking antibodies had no effect on the adhesion with the exception of blocking antibody against α3-subunit. The α5/β1 integrin is known to be central in cell binding to fibronectin and is often called the fibronectin receptor (Akiyama *et al*., 1995). Therefore, the near complete inhibition of adhesion caused by the integrin α5- and β1-subunit blocking antibodies suggested that the inhibitory effect of zinc on MDA-MB-231 cell adhesion to and migration on fibronectin might be through interference with the activation of the integrin α5/β1.

Surprisingly, treating MDA-MB-231 cells with integrin α3-subunit blocking antibody increased cell adhesion by 40% (Figure 2.19). Furthermore, it was also observed that these cells were clustered into groups of cells. Similarly, Bliss *et al*. (1995) also noticed an increase in adhesion of human breast cancer MCF-7 and HTB-126 cells by this blocking antibody. Although this was an interesting observation, the cause and implication of this increased adhesion associated with the use of blocking integrin β3-subunit was not apparent. However, this increased adhesion unlikely played a role in

zinc-mediated inhibition of adhesion as zinc decreased rather than increased adhesion of these cells to fibronectin.

Integrin Activation by Magnesium is Not Detected Using HUTS 4 We used the β1 activating antibody HUTS 4 to detect whether zinc's ability to inhibit adhesion and migration could be explained through a reduction of magnesium induced β1 integrin activation. The HUTS 4 antibody binds to an epitope on the β1 integrin exposed only in the integrin's active "extended" form. Although our results show a strong promotion of cell binding in the presence of magnesium, we were unable to detect an increase in HUTS 4, therefore we were unable to prove that zinc's inhibition of magnesium induced binding was dependent on activated β1 integrin. This result appears to question the hypothesis that zinc inhibits adhesion by reducing magnesium's activation of the β1 integrin. However, the relationship between integrin activation, its associated conformational change and how this relates to its binding affinity may not be as straightforward as once believed (Mould and Humphries, 2004).

A recent study using fluorescence energy transfer (FRET) technique was able to estimate the influence of divalent cations on the degree of extension of the β1 integrin (Chigaev *et al*., 2003). This study showed that each cation promotes a different range of extension and that none of the conformations required for adhesion are an integrin in full extension. These results imply that magnesium could induce a partial extension of the β1 integrin, satisfactory to induce ligand binding by the β1 integrin, but unable to fully expose the activation dependent epitopes to which HUTS 4 binds.

2.4.5 Zinc Dramatically Alters Cell Morphology and Reduces F-actin

Apparent in our study is the intimate connection between adhesion and migration, and it is well documented that an optimum level of adhesion is crucial to the ability of cells to migrate (Akiyama *et al*., 1995; Palecek *et al*., 1997). Interestingly, while adhesion in the presence of 1mM magnesium decreases linearly with increasing zinc, in both the wound healing "scratch" assay and the bead track assay, the most dramatic drop in migration occurs between 2.5 and 5μmol/L zinc, 72 and 53% respectively. The clear drop in migration rate at 5 μmol/L zinc makes it apparent that the optimum adhesion and therefore integrin activation for migration is no longer occurring. The decrease in migration with increasing zinc was visible in the dramatically altered distribution of the cells' cytoskeleton (Figure 2.23). Staining of cell actin with rhodamine phalloidin allowed for details of the cell actin to be visualized. Polarized cells with long lamellipodia, important for carrying out motility by pulling the cells along the fibronectin matrix were highly visible in the 0 and 2.5 μmol/L zinc groups, but completely absent from the 5 μM zinc groups and above. Furthermore, at concentrations of 10 to 50 μmol/L zinc the total amount of F-actin appeared to decrease, as the cell's florescence visibly faded in intensity (Figure 2.23). These retracted cells no longer fit the phenotype of the metastatic MDA-MB-231 cells. Integrin activation is essential for the formation and attachment of these lamellipodia to the ECM (Grande-Garcia *et al*., 2005). Therefore the change in cell morphology with increasing zinc concentration further fits with an inhibitory role of zinc on integrin activation. Without the presence of lamellipodia

extensions, the MDA–MB-231 cells are unable to pull themselves along the matrix and therefore migration is impossible.

2.4.6 Summary

In summary, zinc at as low as 5 μmol/L significantly inhibited migration of MDA-MB-231 breast cancer cells on fibronectin. Zinc also reduced magnesium-mediated promotion of adhesion and thus was likely involved in inhibiting magnesium-mediated integrin activation. With the use of blocking antibodies, it was determined that the α5/β1 integrin was responsible for the adhesion of the MDA-MB-231 cells to fibronectin and it was likely that zinc inhibited adhesion by blocking the activation of this specific form of integrin. Zinc at 5 μmol/L also induced cell rounding in the normally elongated MDA-MB-231 cells. Together these results suggested that zinc was an inhibitor of MDA-MB-231 migration on fibronectin. Further studies are required to elucidate the precise mechanism by which zinc modulates adhesion and migration. Studies on whether the effects of zinc in cell migration were specific to this cell line and fibronectin or whether its effects could be seen in a wide variety of cell lines on a number of different extracellular matrices would help to understand the significance of these findings for cell adhesion and migration as a whole.

Table 2.1 Summary of the effects of zinc on the adhesion of MDA-MB-231 cells in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+}.

Divalent Cation	**Correlation of Increasing Zn^{2+} Concentration on Adhesion**	**R^2**	**Effective Zn^{2+} concentrations (μmol/L Zn^{2+})**
Mg^{2+}	Negative	0.90	10, 25 and 50
Ca^{2+}	Positive	0.69	10, 25 and 50
Mg^{2+} + Ca^{2+}	Negative	0.91	25 and 50
Mn^{2+}	N/A	N/A	N/A

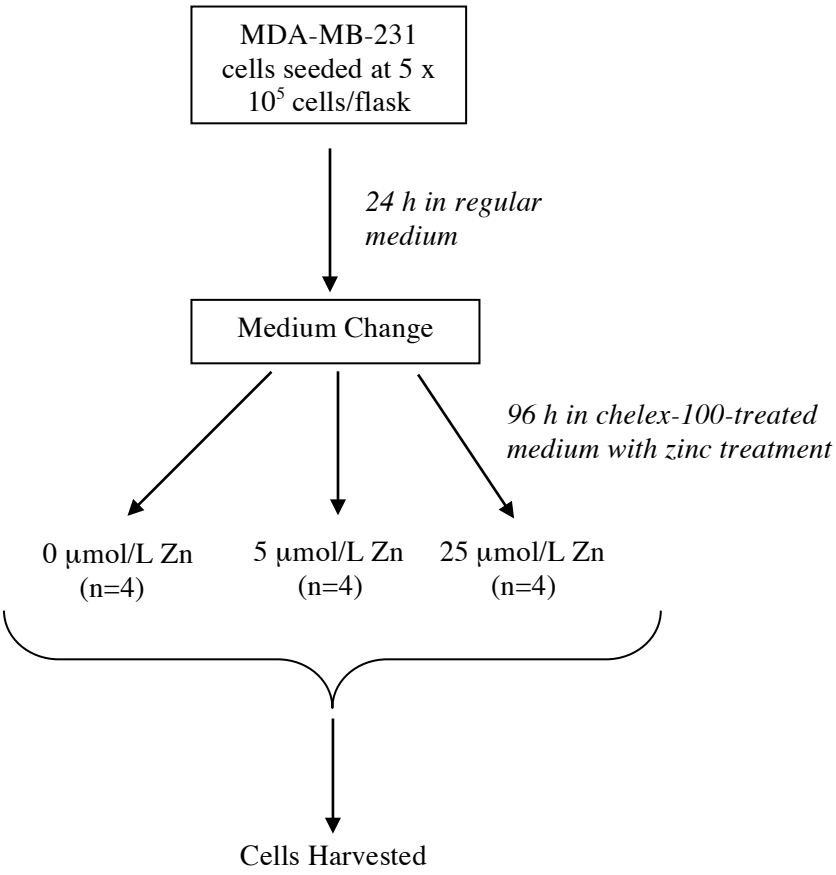


Figure 2.1 Schematic representation of the methods for the study parameters of total cellular zinc, labile zinc and cell number.

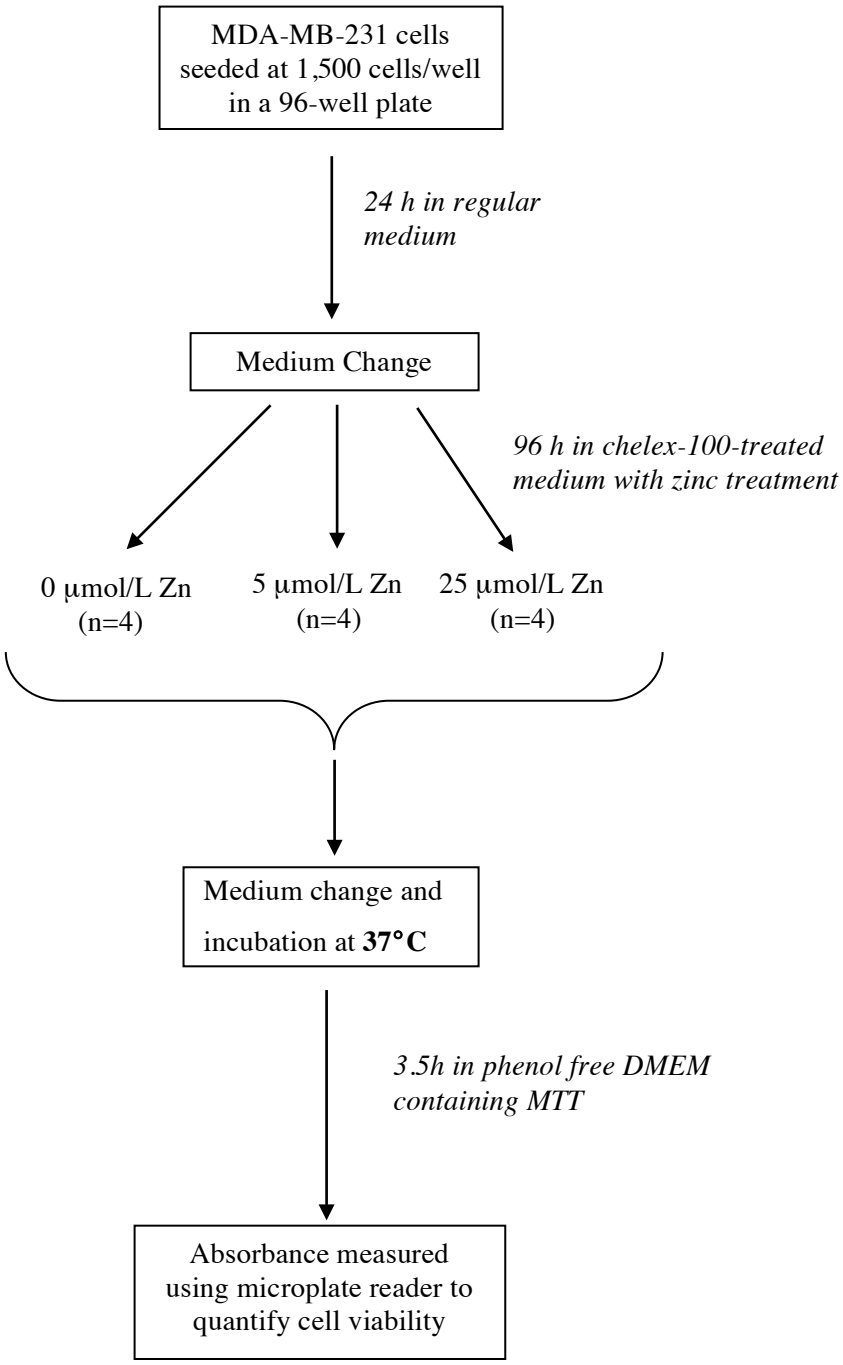


Figure 2.2 Schematic representation of the methods for cell viability.

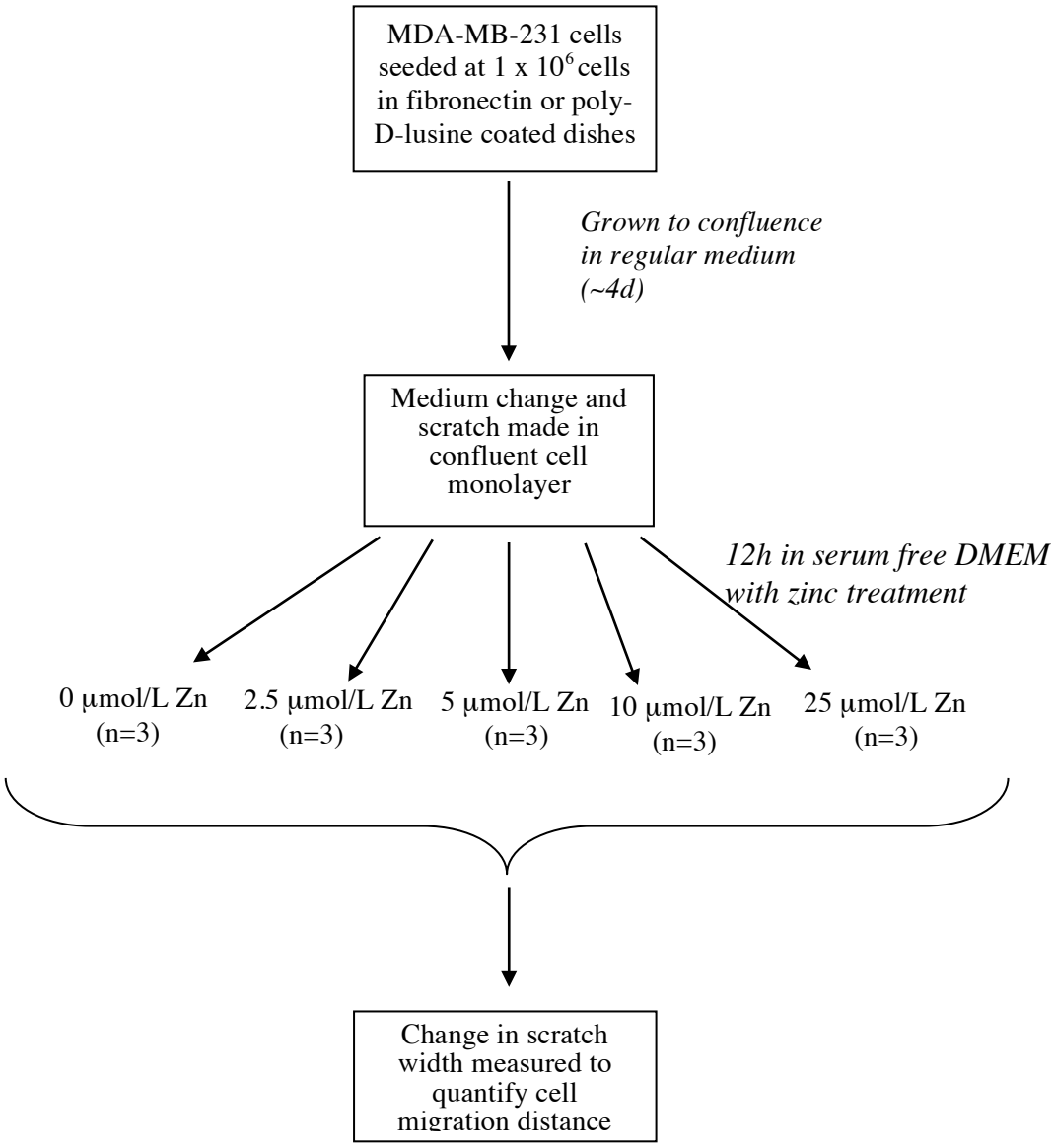
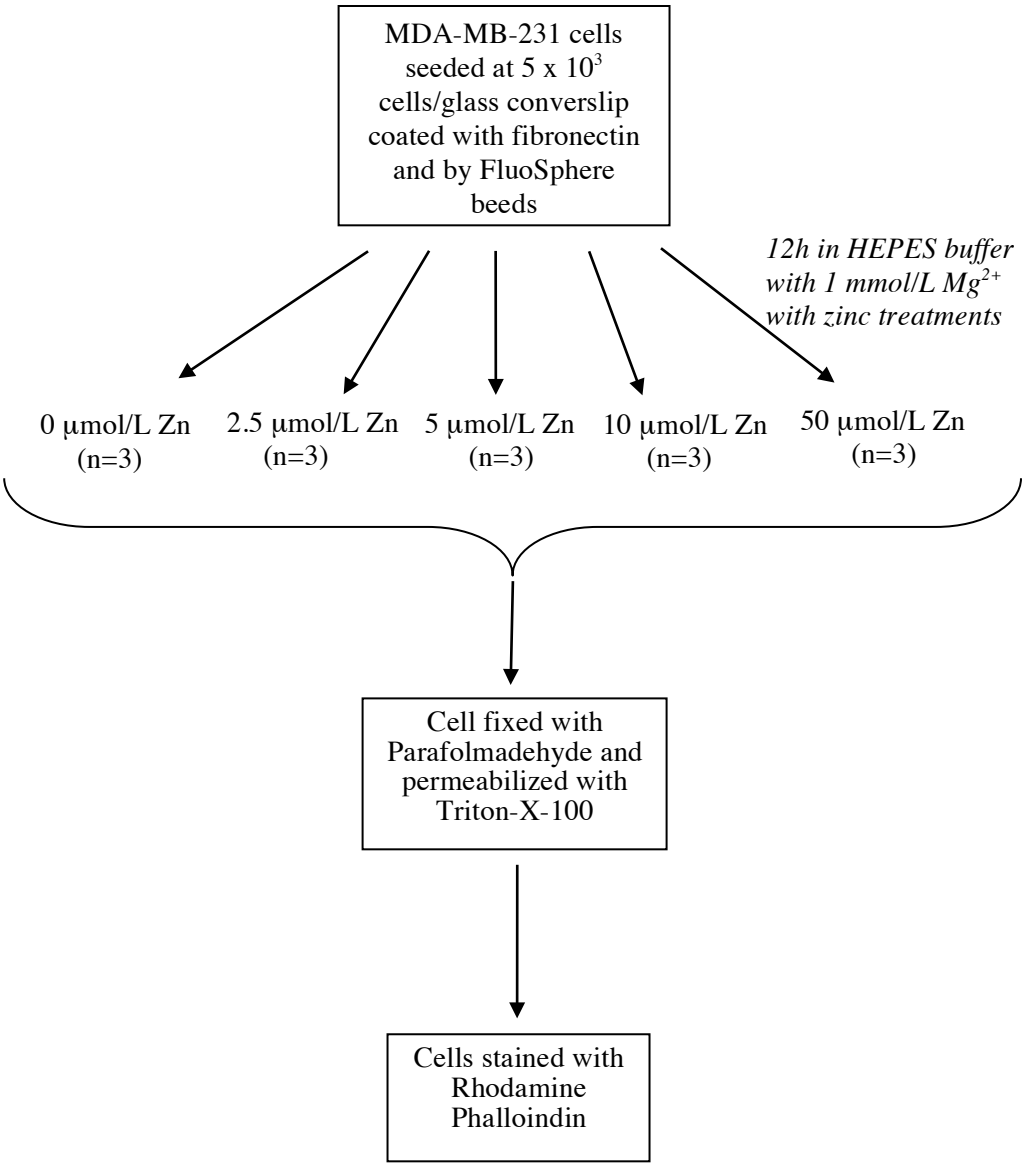


Figure 2.3 Schematic representation of the methods for the cell migration "scratch assay."



Parameters Measured

1) Cell migrated track length – florescent microscope with blue filter (cutoff at 440 nm)
2) Cell F-actin –florescent microscope with red filter (cutoff at 565 nm)

Figure 2.4 Schematic representation of the methods for the single cell migration "bead assay."

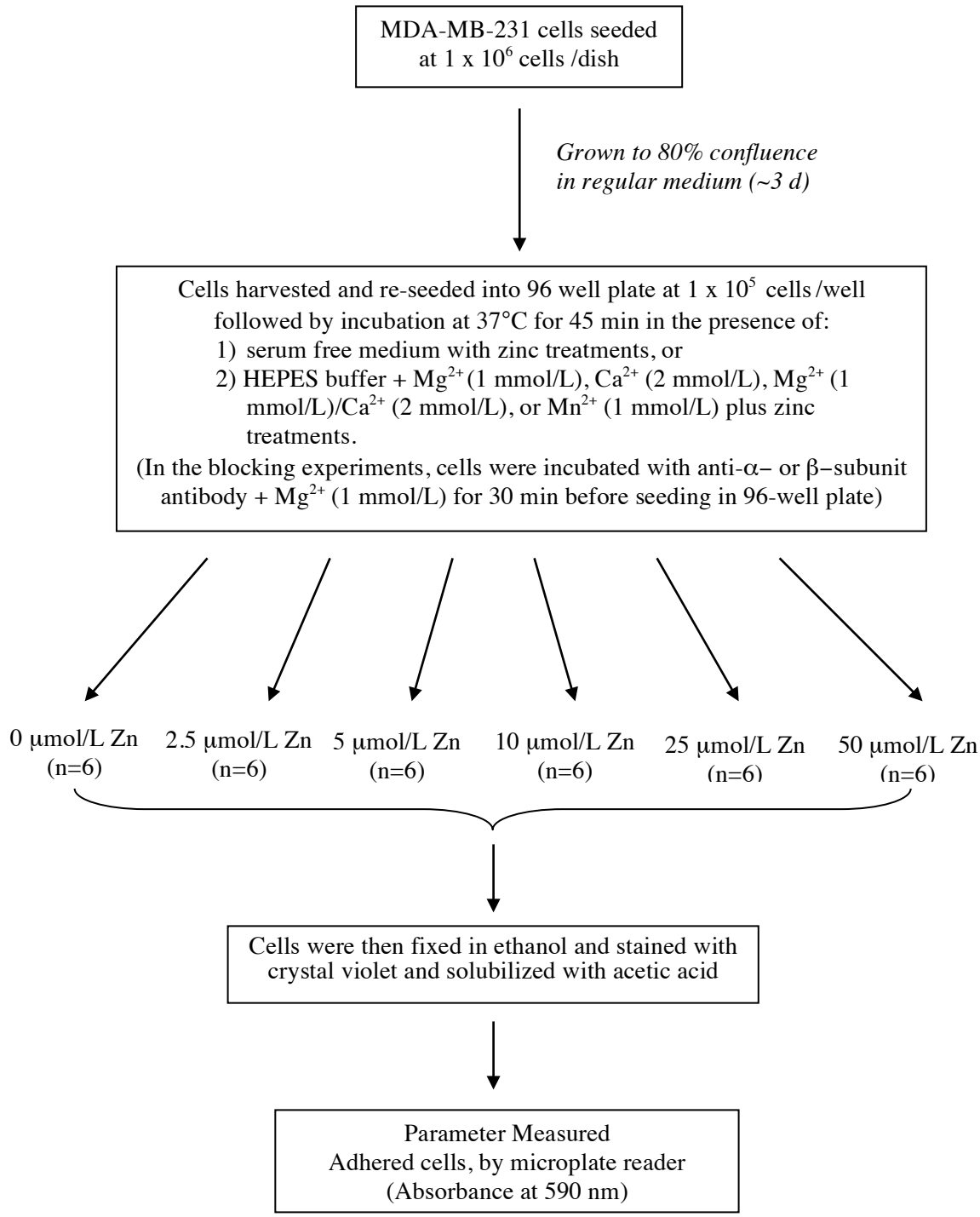


Figure 2.5 Schematic representation of the methods for the adhesion studies.

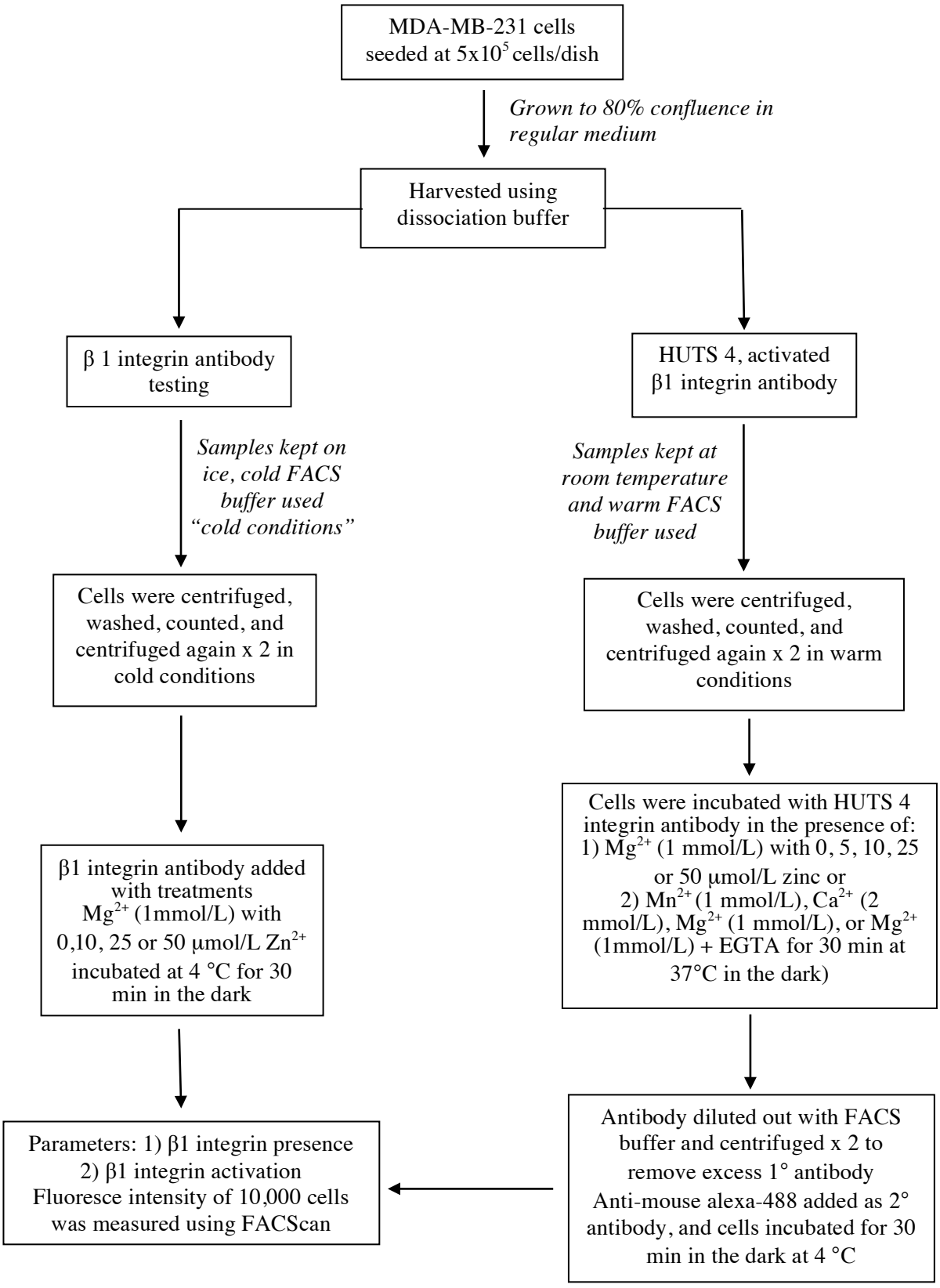


Figure 2.6 Schematic representation of the methods for the presence and activation of the β1 integrin

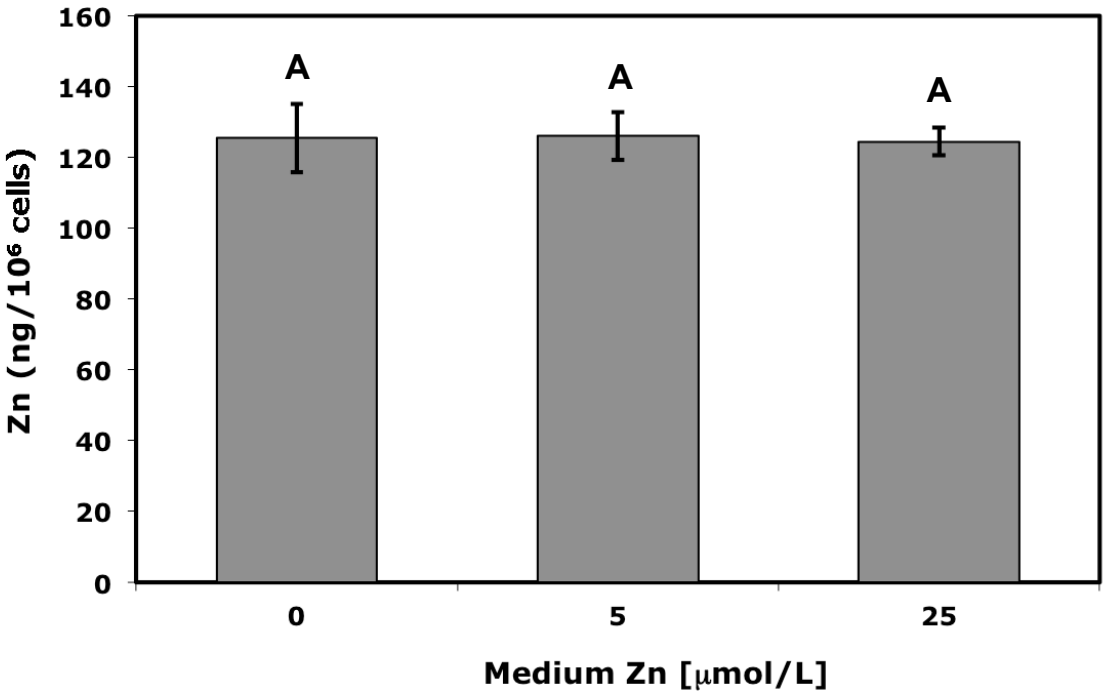


Figure 2.7 Total cellular zinc content remained unchanged in response to zinc treatments in MDA-MB-231 breast cancer cells. Cells were cultured in medium supplemented with 0, 5 or 25 μmol/L of Zn^{2+} for 96 h. Values represent means ± SEM (n = 4; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

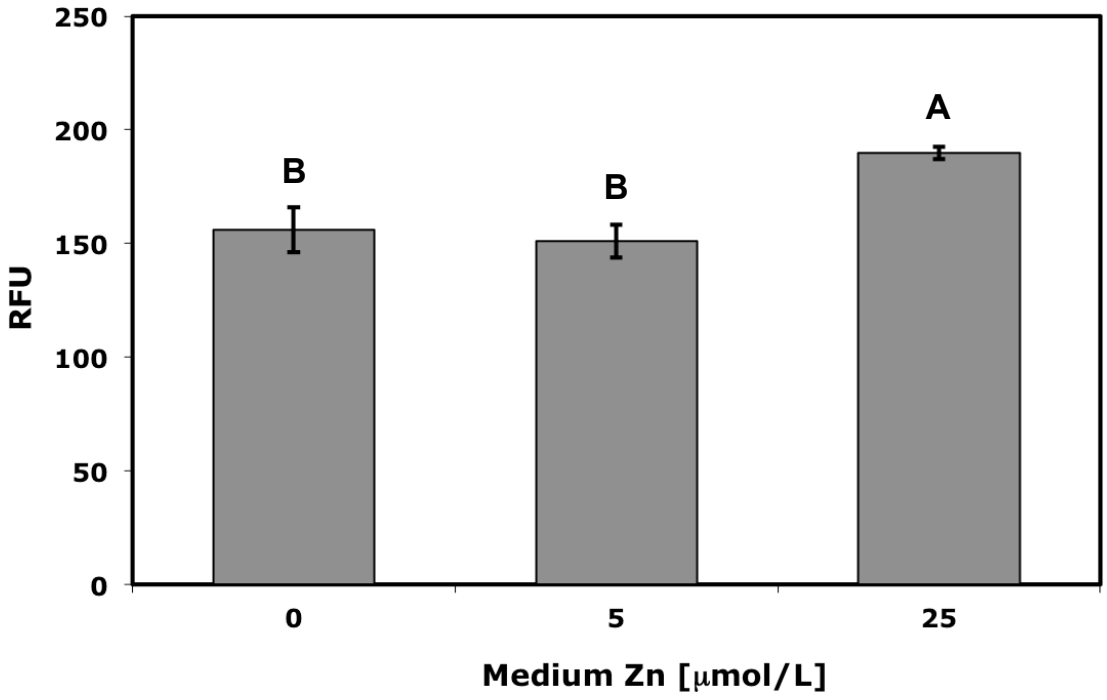


Figure 2.8 Abundance of the labile intracellular pool of zinc was elevated in response to zinc supplementation in MDA-MB-231 breast cancer cells. Cells were cultured in medium containing 0, 5 or 25 μmol/L of Zn^{2+} for 96 h. Values represent means ± SEM (n = 4; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

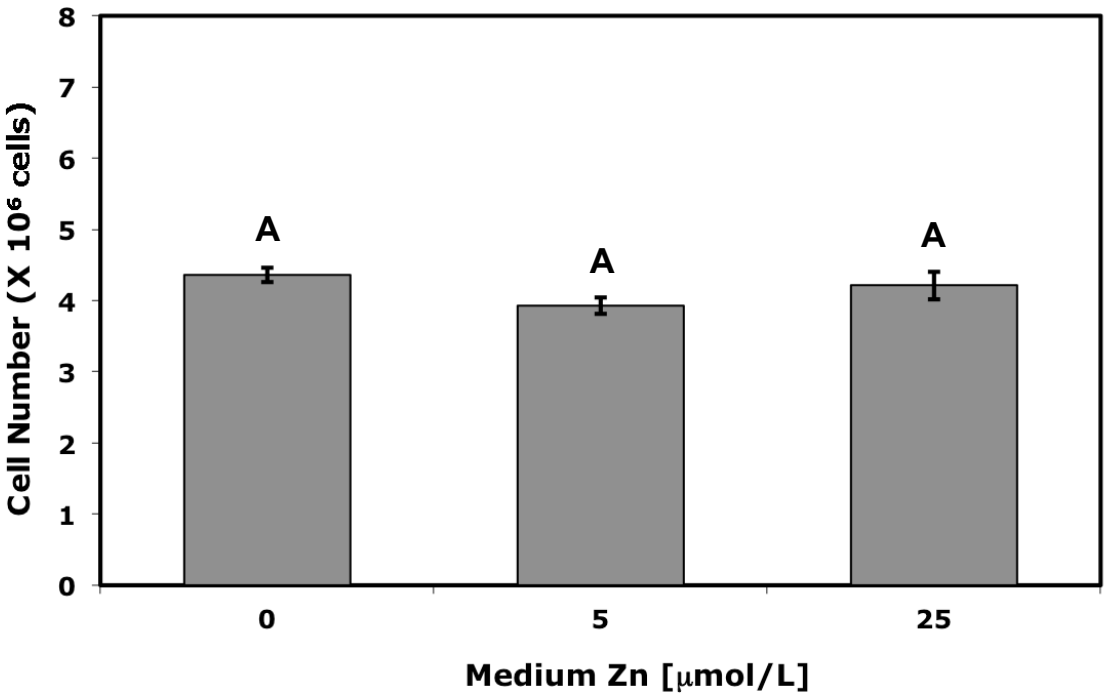


Figure 2.9 Growth of MDA-MB-231 breast cancer cells remained unaffected by zinc treatments. Cells were cultured in medium supplemented with 0, 5 or 25 μmol/L of Zn^{2+} for 96 h. Values represent means ± SEM (n = 4; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

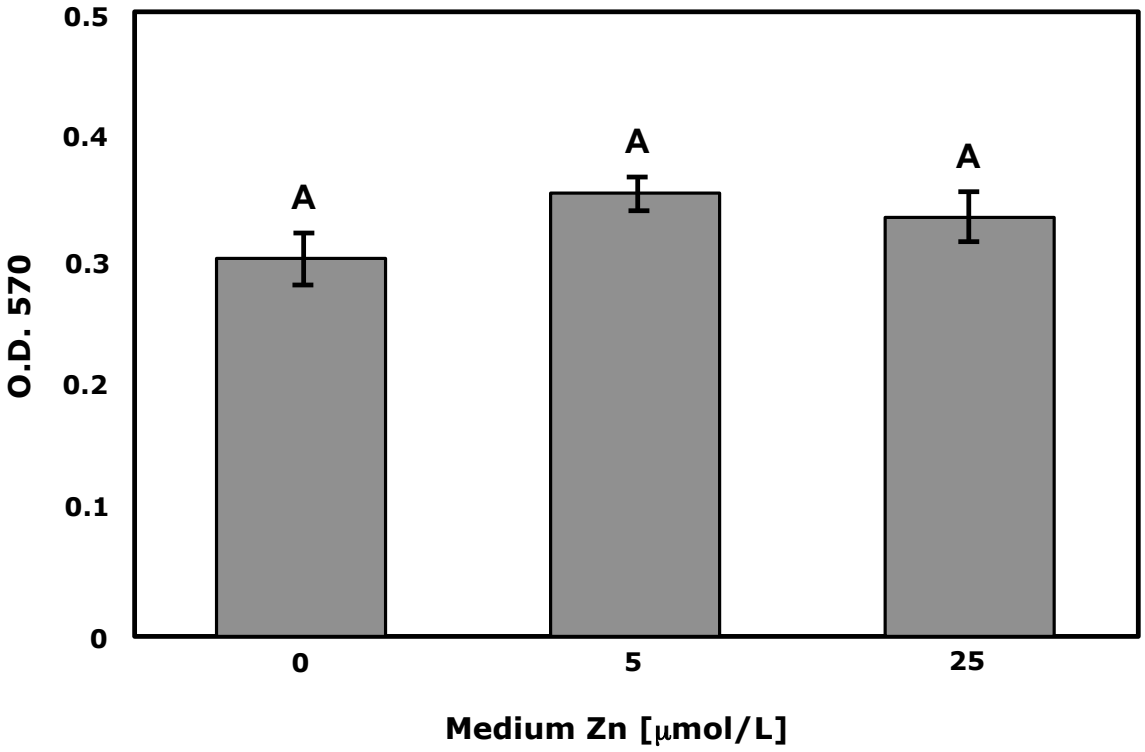


Figure 2.10 Cell viability remained unaffected by zinc treatments in MDA-MB-231 breast cancer cells. Cells were cultured in medium supplemented with 0, 5 or 25 μmol/L of Zn^{2+} for 96 h. Values represent means ± SEM (n = 4; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

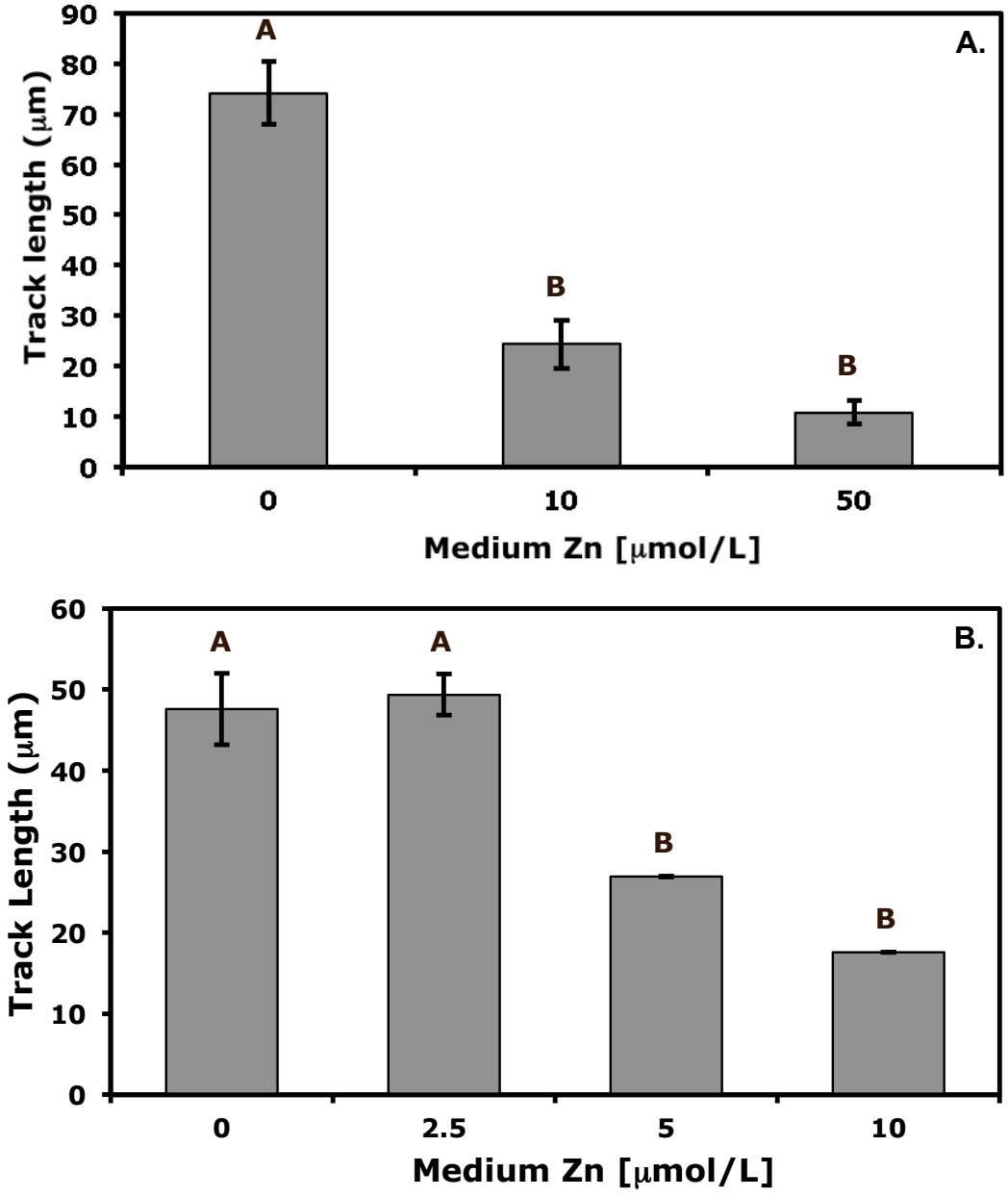


Figure 2.11 Adequate zinc and zinc supplementation reduced the distance of single cell migration of MDA-MB-231 breast cancer cells on fibronectin. Tracks left by migrating cells in the FluoroSphere coating were measured 12 h after seeding. Migration was conducted in HEPES buffer containing 1 mmol/L of Mg^{2+} and Zn^{2+} at higher concentrations (0, 10 or 50 μmol/L; upper) or lower concentrations (0, 2.5, 5 and 10 μmol/L; lower). Migration per replicate was the average of 30-50 cell tracks per dish. Values represent means ± SEM (n = 3; the experiment was repeated once independently.) Means with different letters are significantly different ($p < 0.05$).

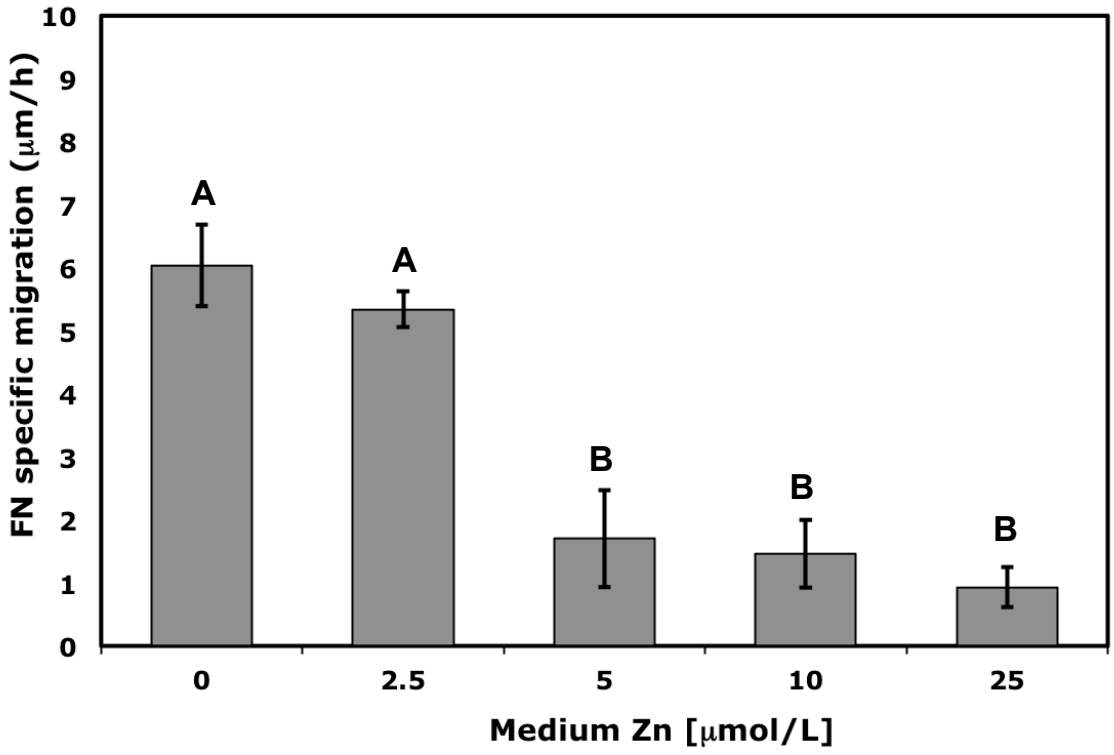


Figure 2.12 Adequate zinc and zinc supplementation reduced the migration rate of MDA-MB-231 breast cancer cells on fibronectin. Migration of MDA-MB-231 cells in a confluent monolayer were measured for a 12 h period in the presence of 0, 2.5, 5, 10 or 25 μmol/L of Zn^{2+} in serum free medium. Migration rate (μm/h) was calculated by subtracting the distance traveled on poly-D-lysine from that traveled on fibronectin (FN) to obtain fibronectin specific migration. Values represent means ± SEM (n = 3; the experiment was repeated once independently.) Means with different letters are significantly different ($p < 0.05$).

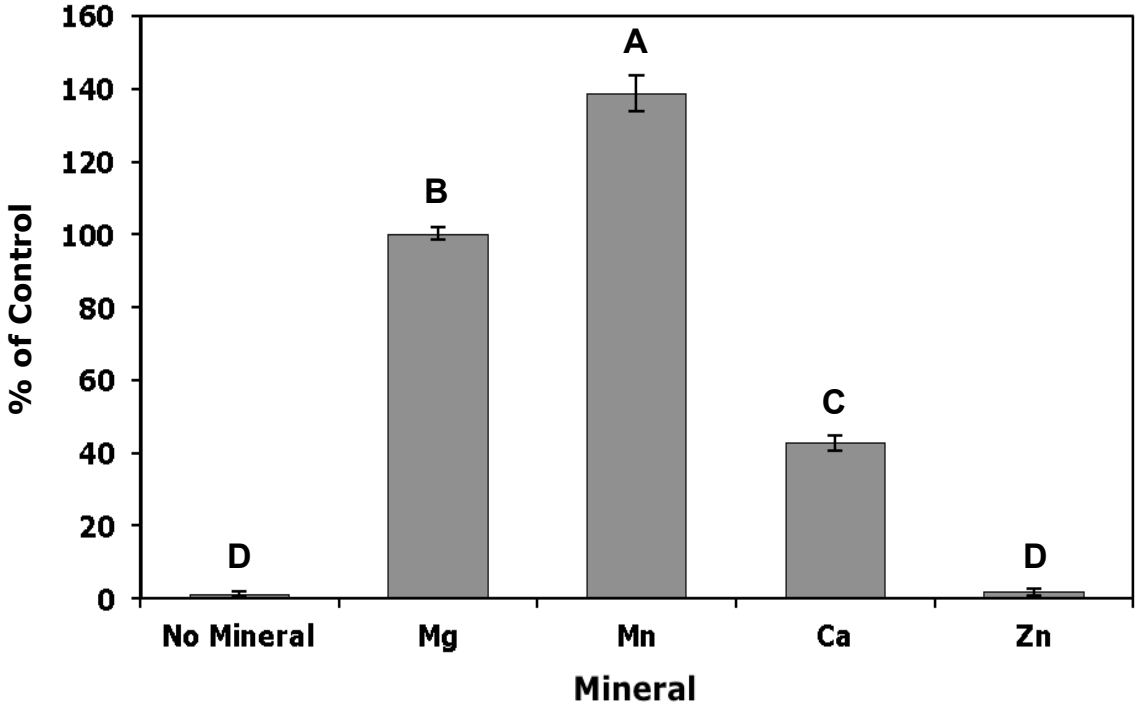


Figure 2.13 Mg^{2+}, Mn^{2+} and Ca^{2+} promoted the adhesion of MDA MB 231 breast cancer cells to fibronectin while Zn^{2+} had no effect on the adhesion. MDA-MB-231 cells were allowed to adhere to fibronectin under different divalent cation conditions at 37^0C in HEPES buffer. Adhesion in the absence of divalent cations served as the negative control and in the presence of Mg^{2+} (1 mmol/L) served as the positive control. Adhesion in the presence of Mn^{2+} (1 mmol/L), Ca^{2+} (2 mmol/L) or Zn^{2+} (10 μmol/L) was normalized on the basis of the adhesion in the presence of Mg^{2+}. Values represent means ± SEM (n = 6; the experiment was repeated once independently.). Means with different letters are significantly different ($p < 0.05$).

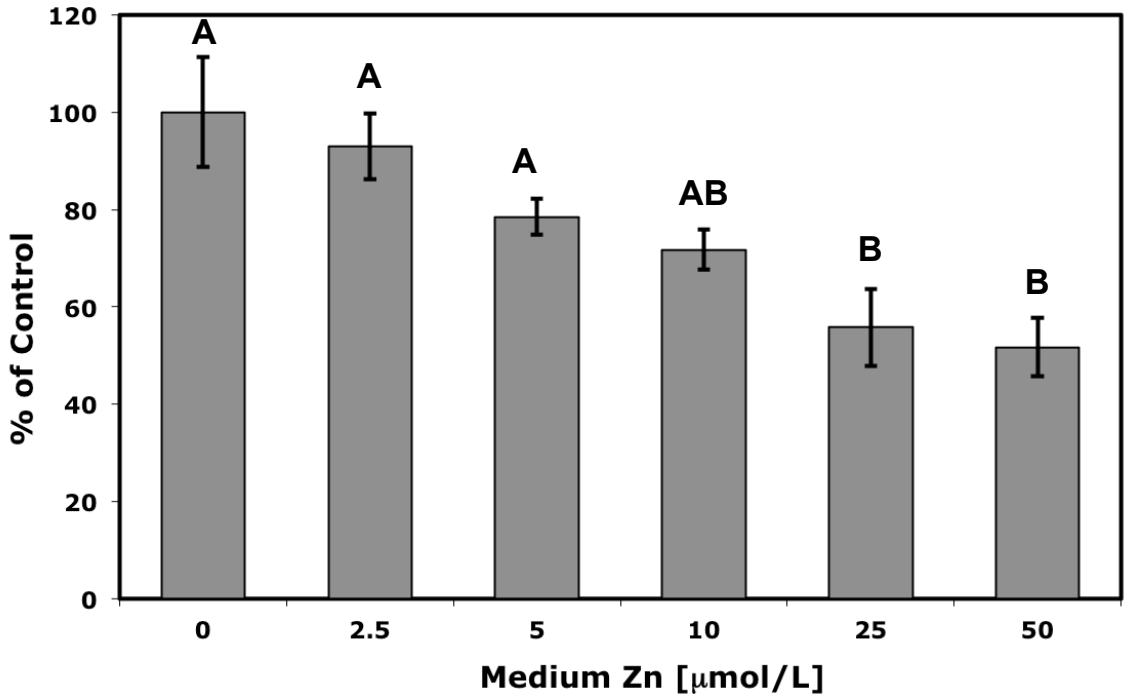


Figure 2.14 Zinc induced a dose-dependent inhibition of the adhesion of MDA-MB-231 breast cancer cells on fibronectin. MDA-MB-231 cells were allowed to adhere to fibronectin in the serum free medium containing 0, 2.5, 5, 10, 25 or 50 μmol/L Zn^{2+} at 37^0C and allowed to adhere for 45 min. Adhesion in the absence of Zn^{2+} served as the control and as the basis for normalization. Values represent means ± SEM (n = 6; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

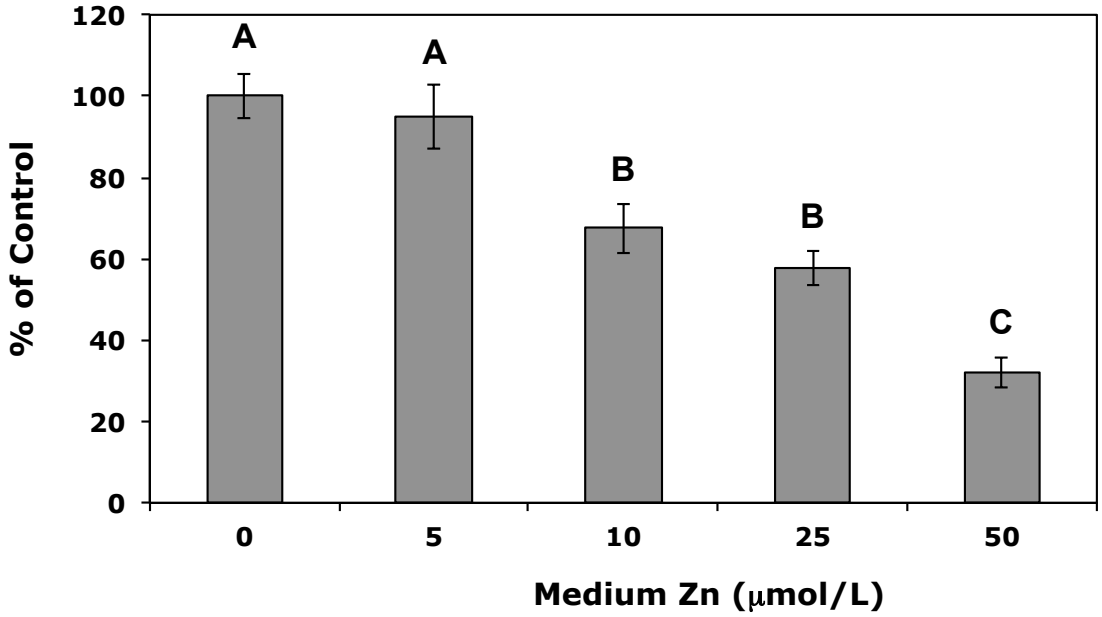


Figure 2.15 Zinc induced a dose-dependent inhibition of the adhesion of MDA-MB-231 breast cancer cells to fibronectin in the presence of Mg^{2+}. MDA-MB-231 cells were allowed to adhere to fibronectin in the presence of Mg^{2+} (1 mmol/L) and Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) at 37^0C and allowed for adhesion for 45 min. Adhesion in the presence of Mg^{2+} and 0 μmol/L of Zn^{2+} served as the control and as the basis for normalization. Values represent means ± SEM (n = 6; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

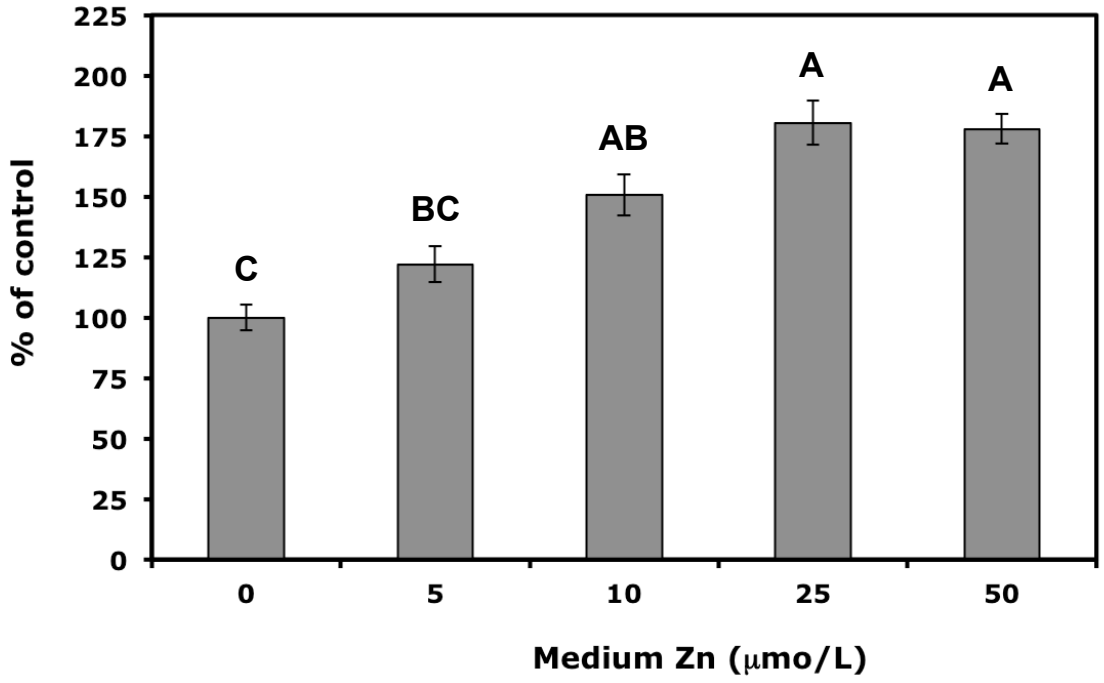


Figure 2.16 Zinc induced a dose-dependent promotion of the adhesion of MDA-MB-231 breast cancer cells to fibronectin in the presence of Ca^{2+}. MDA-MB-231 cells were allowed to adhere to fibronectin in the presence of Ca^{2+} (2 mmol/L) and Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) at 37^0C and allowed for adhesion for 45 min. Adhesion in the presence of Mg^{2+} and 0 μmol/L of Zn^{2+} served as the control and as the basis for normalization. Values represent means ± SEM (n = 6; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

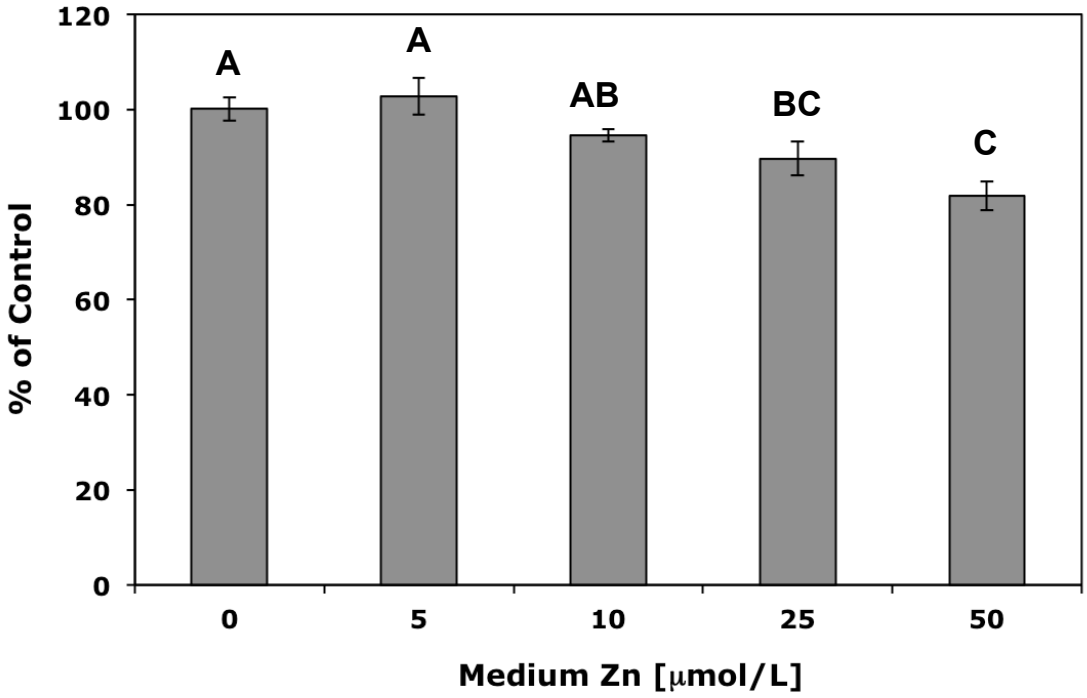


Figure 2.17 Zinc induced a does-dependent inhibition of the adhesion of MDA-MB-231 breast cancer cells to fibronectin in the presence of Mg^{2} and Ca^{2+}. MDA-MB-231 cells were allowed to adhere to fibronectin in the presence of Mg^{2+} (1 mmol/L), Ca^{2+} (2 mmol/L) and Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) at 37^{0}C and allowed for adhesion for 45 min. Adhesion in the presence of Mg^{2+}, Ca^{2+}, and 0 μmol/L of Zn^{2+} served as the control and as the basis for normalization. Values represent means ± SEM (n = 6; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

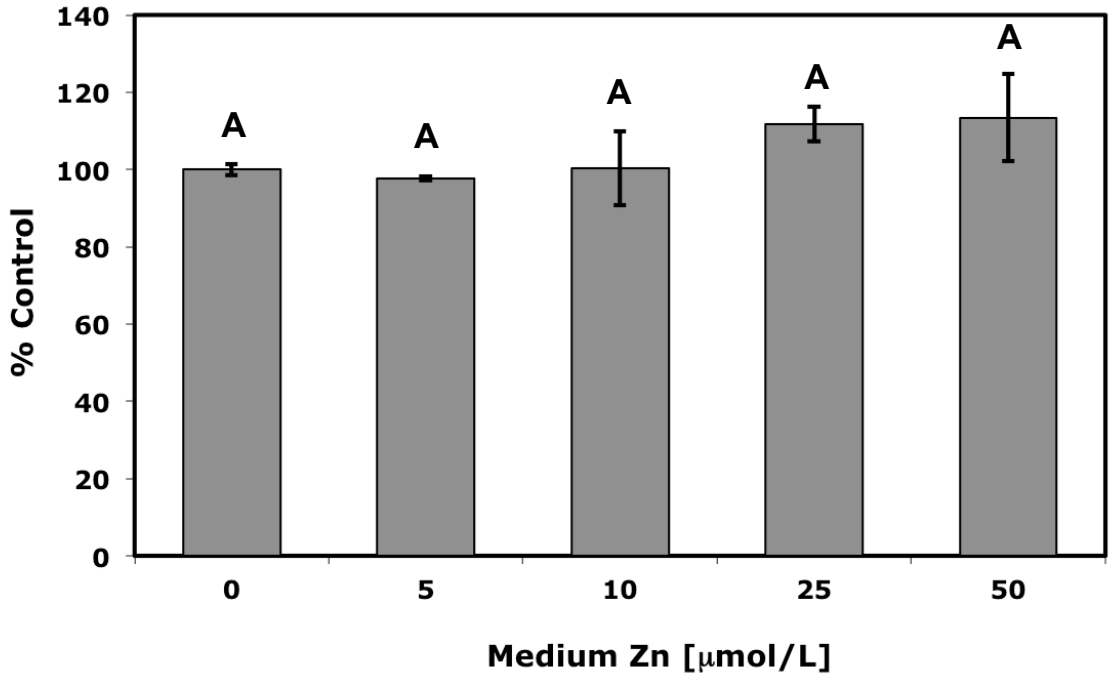


Figure 2.18 Zinc had no effect on the adhesion of MDA-MB-231 breast cancer cells to fibronectin in the presence of Mn^{2+}. MDA-MB-231 cells were allowed to adhere to fibronectin in the presence of Mn^{2+} (1 mmol/L) and Zn^{2+} (0, 5, 10, 25 or 50 μmol/L) at 37^0C and allowed for adhesion for 45 min. Adhesion in the presence of Mn^{2+} and 0 μmol/L of Zn^{2+} served as the control and as the basis for normalization. Values represent means ± SEM (n = 6; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

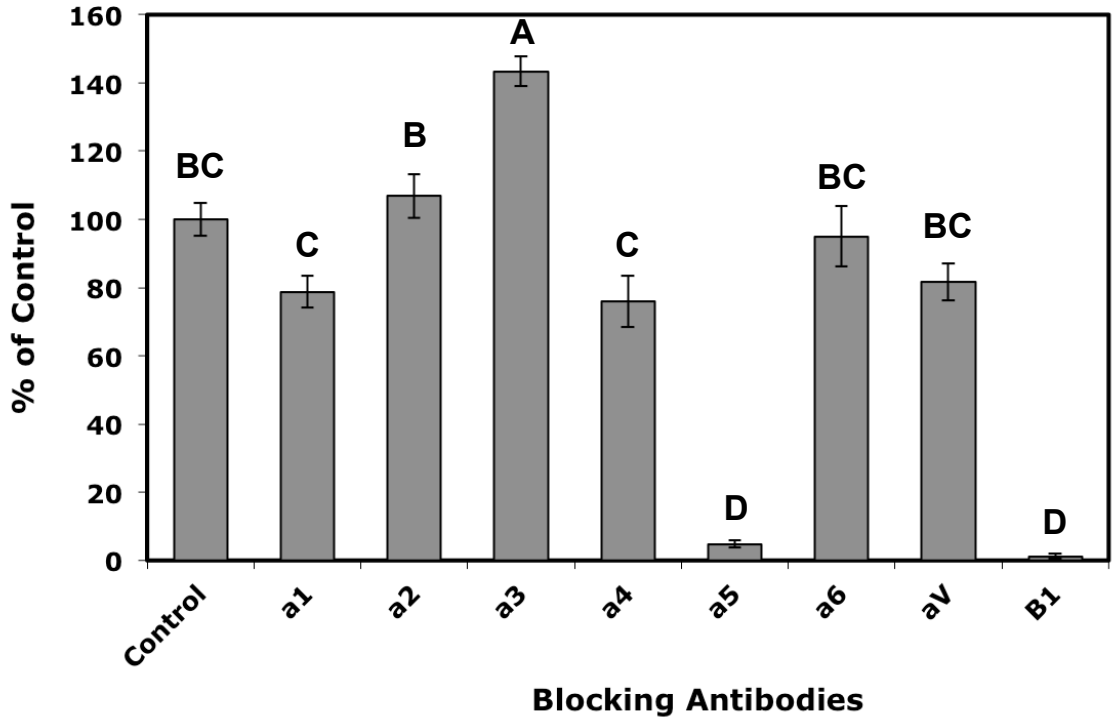


Figure 2.19 Anti-integrin α and β1-subunit blocking antibodies inhibited the adhesion of MDA-MB-231 breast cancer cell adhesion to fibronectin. Cells were incubated with an antibody against one of the eight integrin subunits for 30 min prior to the adhesion assay on fibronectin. Adhesion was carried out in HEPES buffer containing Mg^{2+} (1 mmol/L). The control contained Mg^{2+} with no antibody and served as the basis for normalization. Values represent means ± SEM (n = 3; the experiment was repeated once independently). Means with different letters are significantly different ($p < 0.05$).

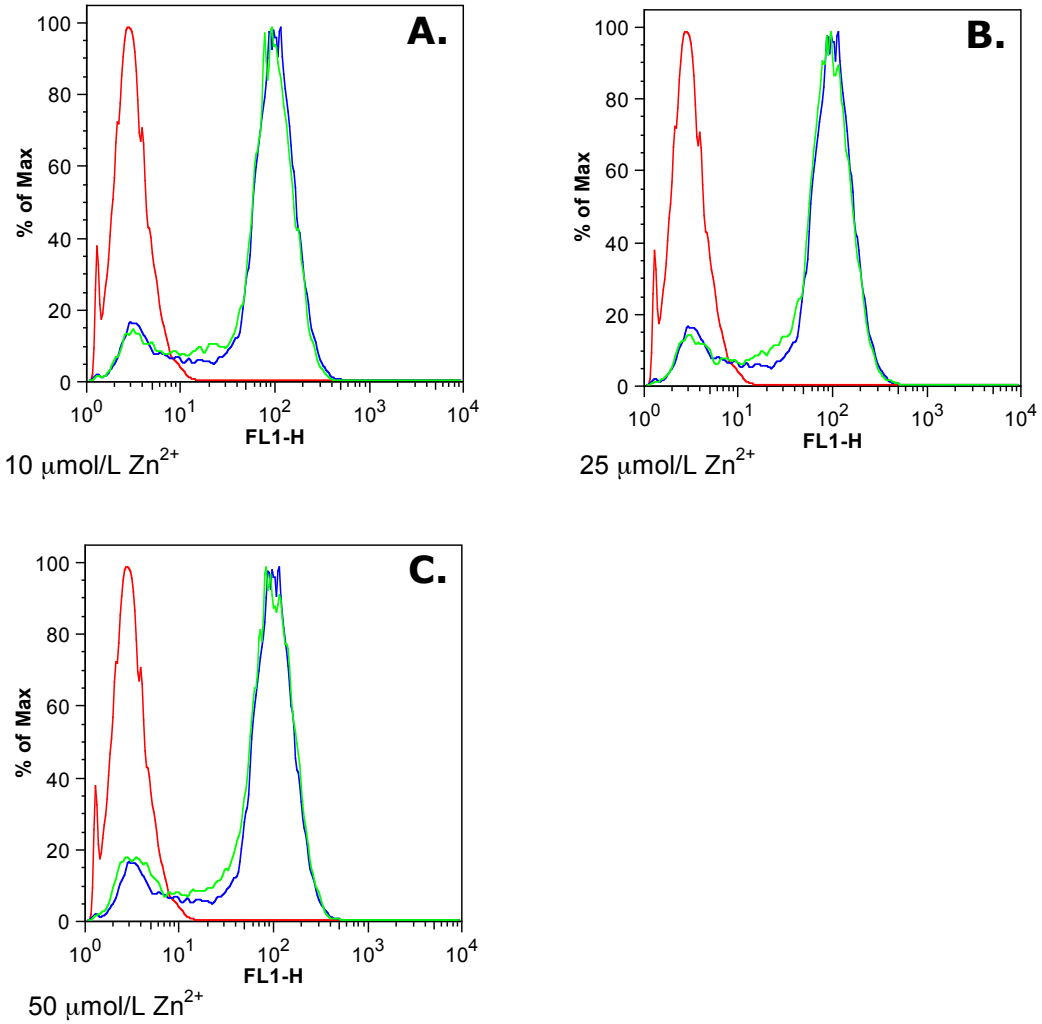


Figure 2.20 Zinc supplementation had no effect on the presence of total surface integrin β1-subunit in the presence of Mg^{2+}. Cells were incubated with the anti-integrin β1 conjugated FITC antibody in the presence of 0, 10, 25 or 50 μmol/L of Zn^{2+}. Presence of the β1-integrin subunit was assessed using a flow cytometric assay. The red line represents the control (no antibody), the blue line represents Zn^{2+} control (0 μmol/L). The green line represents the effect of Zn^{2+} at 10 (A), 25 (B), or 50 (C) μmol/L. The histogram represents the frequency (% of cells; y-axis) against fluorescence intensity (x-axis) of 10,000 cells. Histogram peaks represent the fluorescence output expressed by the largest percentage of cells.

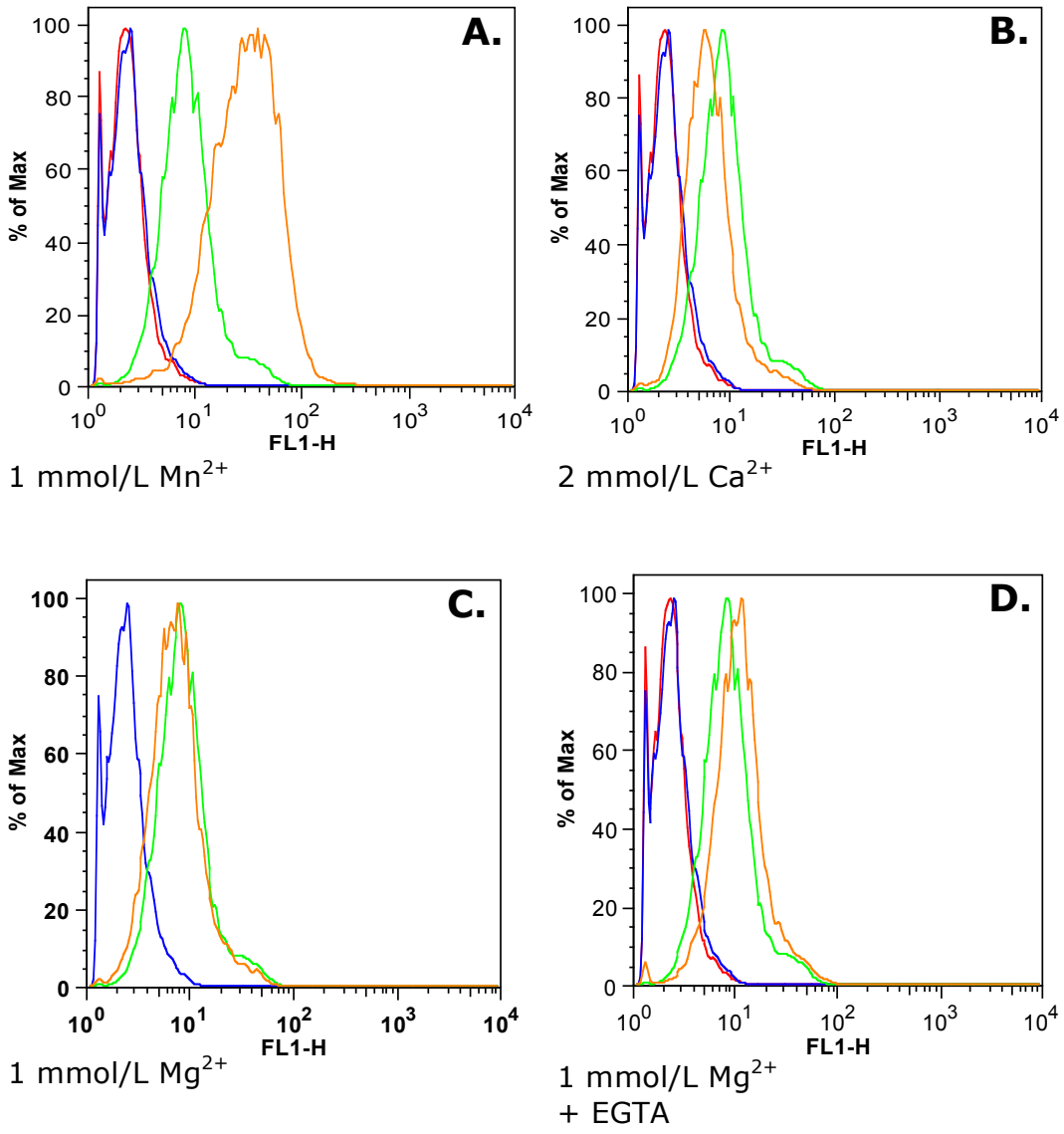


Figure 2.21 Mn^{2+} promoted integrin β1-subnit activation in MDA-MB-231 cells while Ca^{2+} reduced the activation. Cells were incubated with the anti-integrin β1 conjugated FITC antibody in the presence of Mn^{2+} (1 mmol/L), Mg^{2+}(1 mmol/L) alone, Mg^{2+}(1 mmol/L) plus EGTA (2 mmol/L), and Ca^{2+} (2 mmol/L). Activation was assessed by measuring the HUTS 4 β1 antibody-dependent fluoresce intensity using FACscan. The red and blue lines are the background florescence (no antibody) and the secondary antibody-dependent fluorescence, respectively. The green line is the fluorescence without the presence of mineral. The orange line is the fluorescence in the presence of Mn^{2+} (A), Ca^{2+} (B), Mg^{2+} alone (C), or Mg^{2+} plus EGTA (D). The histogram represents the frequency (% of cells; y-axis) against fluorescence intensity (x-axis) of 10,000 cells. Histogram peaks represent the fluorescence output expressed by the largest percentage of cells.

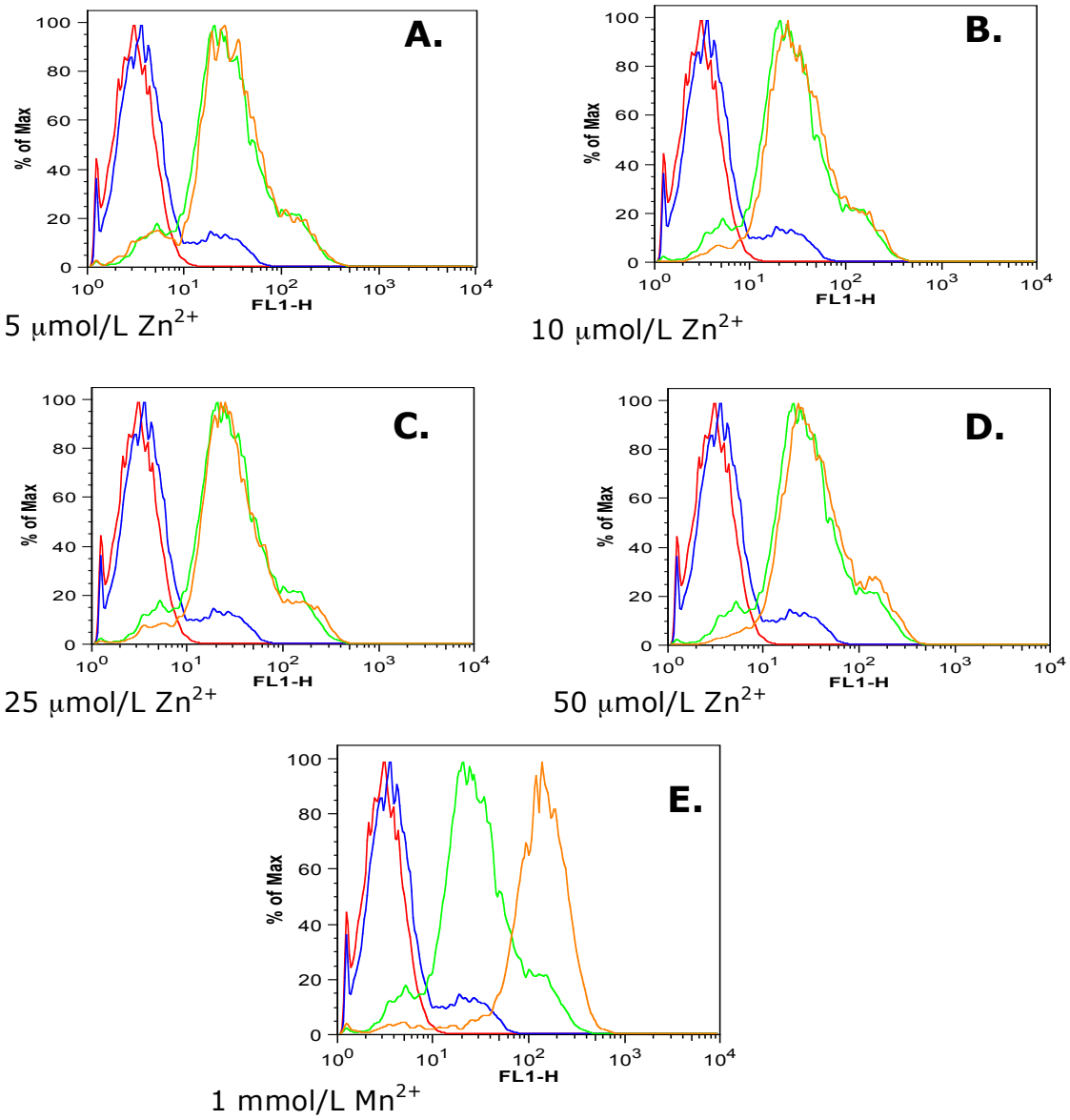


Figure 2.22 Zinc had no effect HUTS 4-induced integrin β1-subunit activation in MDA-MB-231 cells. Cells were incubated with the anti-integrin β1 conjugated FITC antibody in the presence of Mg^{2+} (1 mmol/L) and 0, 5, 10, 25 or 50 μmol/L of Zn^{2+}, or Mn^{2+} (1 mmol/L) alone. Activation was assesed by HUTS 4 β1 antibody-dependent fluoresce intensity using FACscan. The red and blue lines are the background florescence (no antibody) and the secondary antibody-dependent fluorescence, respectively. The green line is the fluorescence in the presence of Mg^{2+} and 0 μmol/L of Zn^{2+}. The orange line is the fluorescence in the presence of 5 (A), 10 (B), 25 (C) or 50 (D) μmol/L of Zn^{2}, or Mn^{2+} alone (E). The histogram represents the frequency (% of cells; y-axis) against fluorescence intensity (x-axis) of 10,000 cells. Histogram peaks represent the fluorescence output expressed by the largest percentage of cells.

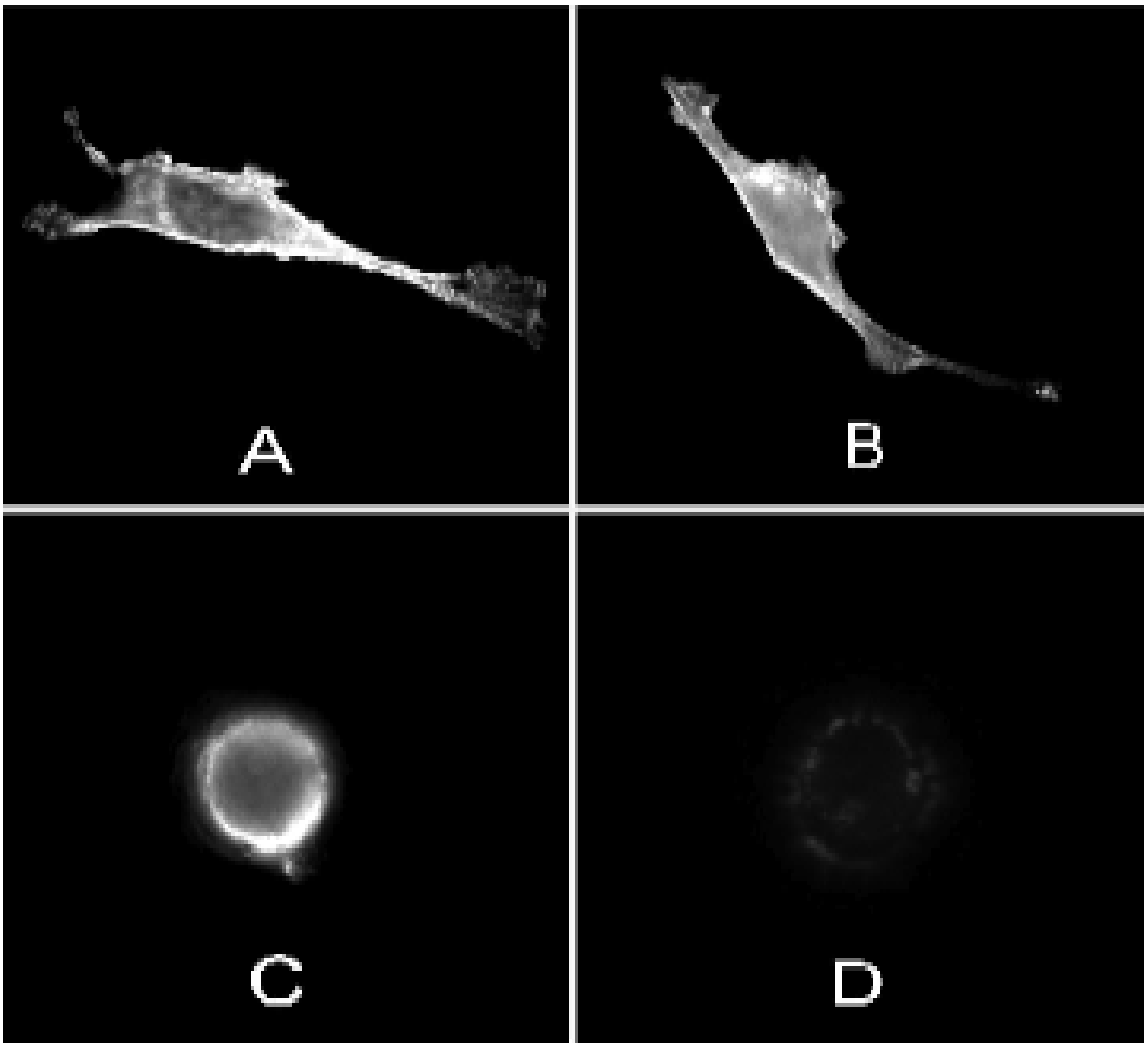


Figure 2.23 Adequate zinc and zinc supplementation caused rounding in MDA-MB-231 breast cancer cells . Cells were treated with 1mmol/L Mg^{2+} and 0 (A) 2.5 (B) 5 (C) or 10 (D) μmol/L of Zn^{2+}. MDA-MB-231 cells were grown on fibronectin-coated cover slips for 12 h before staining with rhodamine phalloidin. Photos were taken under 400 X magnification.

Chapter 3 General Discussion, Limitations and Future Directions

3.1 General Discussion

Integrin blocking antibodies have been used to treat a number of diseases in recent years, such as inhibiting platelet aggregation in acute coronary syndrome with abciximab and using natalizumab to slow the destruction of the nerve sheath in relapsing multiple sclerosis by modulating inflammatory T-cell migration (Byron *et al*., 2009). Integrins have recently become implicated in cancer progression and metastasis. Use of integrin β1-subunit blocking antibodies reduces metastasis and invasiveness in a number of breast cancer models *in vitro* (Newton *et al*., 1995; Weaver *et al*., 1997; Wang *et al*., 2002) and *in vivo* (White *et al*., 2004; Park *et al*., 2006; Huck *et al*., 2010). In contrast, mutation of suppressor genes to integrin β1-subunit lead to a more increased invasiveness of MDA-MB-231 cells (Brandt *et al*., 2009).

The role of the integrin β1-subunit in human breast cancer is still controversial. Decreasing α5/β1 integrin appeared to be associated with more poorly differentiated, more aggressive primary tumours (Zutter *et al*., 1993). In contrast, Yao *et al*. (2007) showed that breast cancers expressing the highest level of integrin β1-subunit are associated with higher expression of fibronectin and significantly reduce disease free survival and total survival at 5 and 10 years. Breast cancer patients with tumours positive for MDGI , a suppressor of integrin β1-subunit, have a better prognosis at 10 years (Nevo *et al*., 2010). These two studies provide evidence that blocking the integrin β1 subunit is directly linked to reduced mortality in breast cancer patients and strongly supports the importance of developing integrin mediated breast cancer treatment strategies.

In concordance with the above studies (Yao *et al*., 2007; Nevo *et al*., 2010), my thesis research showed that adhesion of MDA-MB-231 cells on fibronectin was inhibited by α5 and β1 blocking antibodies (95 and 99% respectively). More surprisingly, physiological levels of zinc also inhibited the adhesion and migration of these cells. Adhesion of MDA-MB-231 cells was decreased in a dose-dependent fashion with increasing concentrations of zinc, while migration dropped off dramatically with increasing concentrations of zinc. To explain the mechanism behind zinc's inhibition of adhesion and migration, we explored its effects in the presence of other divalent cations known to influence integrin adhesion. Our study showed that zinc's anti-adhesive properties varied with the adhesion promoting cation present. The variable influence of zinc at micromolar concentrations on the ability of divalent cations at millimolar concentrations to promote adhesion suggests that zinc's inhibition of adhesion and migration was through a non-competitive inhibitory effect on integrin activation. To the best of my knowledge, this study was the first to show an inhibitory role of zinc in integrin-mediated cell adhesion and migration in a breast cancer cell line.

As detailed above, antibodies against the integrin β1-subunit are beginning to look like a viable therapeutic option to reduce metastasis in breast cancer. Our results suggest a similar inhibitory action of zinc on the integrin β1-subunit. Therefore, the ability of zinc to have an inhibitory effect on breast cancer metastasis *in vivo* must be queried. In my thesis research, physiological concentrations of zinc were shown to inhibit the adhesion and migration of breast cancer cells *in vitro*. Furthermore, the zinc concentrations used in this research did not affect intracellular zinc concentration or cell

growth. These findings suggest that zinc acted extracellularly to exert its inhibitory effects. It seems possible, therefore, that increasing serum zinc concentration by oral zinc supplementation could raise serum zinc levels sufficiently to inhibit integrin activation on the cell surface, decreasing cell adhesion and migration.

Normal serum zinc in adult humans is approximately 15 μmol/L with deficiency defined as serum zinc below 10.7 μmol/L (Simon-Hettich *et al.*, 2001). Comparing serum zinc levels in healthy women to those with stage IV metastatic cancer reveals, a change of approximately 4 μmol/L, from 15 to 11 μmol/L zinc (Gupta *et al*., 1991; Kuo *et al*., 2002). Diets high in zinc have been shown to be inversely related to the risk of a number of cancers including breast cancer (Grant *et al*., 2008).

Zinc supplementation studies in humans show that serum zinc can be easily increased by oral zinc supplementation (Black *et al*., 1988; The age-related eye disease study research group, 2002). The recommended daily intake for zinc in adults is 8 and 11 mg/d for adult females and males respectively. The average daily intake of Canadian women is 10.1 mg/d.

In a cohort of women ranging from 55 to 80 years old, supplementation of 80 mg/d of zinc oxide increases serum zinc at 1 year and 5 years from 12.4 μmol/L at baseline to 14.7 μmol/L after treatment (The age-related eye disease study research group, 2002). Young healthy men show an increase in serum zinc concentration of

2 μmol/L after 2 weeks of daily zinc supplementation of 75 mg/day. Zinc supplementation for 3 months increases serum zinc to 18.1 μmol/L compared with 13.5 μmol/L in the placebo group (Black *et al*., 1988). These studies do not mention any negative side effects at the 75 or 80 mg/d doses used; however, nausea and other side effects have been reported at zinc does of 150 mg/d (Samman and Roberts, 1987). In a more recent study, supplementing patients undergoing treatment for advanced nasopharyngeal cancer with 75 mg/d for 2 months concurrently with radiation and chemotherapy showed that significant increases in serum zinc levels are associated with reduced 5 year local recurrence rates and improved 5 year survival rates. The difference in 5 year rate of metastasis was insignificant (Lin *et al*., 2009). This study did not look at the inhibitory effect of zinc on integrins, but rather tried to explain the therapeutic effect of zinc supplementation through its promotion of immune function. According to these studies cited, zinc supplementation is capable of increasing serum zinc by close to 5 μmol/L. With these studies in mind, supplementation of breast cancer patients with zinc could be a simple yet viable treatment for reducing migration of malignant cells away from their primary tumour and reducing adhesion of blood borne metastatic cells to the endothelial lining of distant tissues where they can invade new sites. In this way, the inhibitory effect of higher serum zinc on adhesion would hopefully yield a lower incidence of secondary tumours.

3.2 Limitations

Several limitations are apparent in my thesis research. The first limitation was the use of a single breast cancer cell line. Although MDA-MB-231 cells are human breast

cancer cells, it is difficult to extrapolate the findings from a single cell type. Repeating these experiments using a range of human breast cancer cells with different genetic makeup, (e.g.. MCF-7, T47D, and MDA-MB-468, etc.) would be important for establishing the role of zinc in the adhesion and migration of cancerous cells. In addition MDA-MB-231 cells originated from a lung metastatic site and therefore might be more representative of secondary cancer cells than those of the primary tumour. Also, as the studies were carried out *in vitro*, it is not clear whether a higher concentration of serum zinc would have the same inhibitory effect on metastatic cells *in vivo* or that zinc supplementation of cancer patients would be efficacious.

The second limitation is that it is unknown whether zinc can also inhibit breast cancer migration *in vivo*. If zinc does in fact inhibit the integrin β1-subunit, as is suggested in this research, reports showing the impact of reduced integrin β1-subunit expression on breast cancer survival provide support for the potential efficacy of zinc supplementation *in vivo*. Recent studies have shown that high levels of integrin β1-subunit are linked to increased mortality (Yao *et al*., 2007; Nevo *et al*., 2010); however, caution must be exercised as an earlier study found that the expression of α2/β1 and α5/β1 integrins are decreased in breast carcinoma (Zutter *et al*., 1993). Because of this apparent controversy in the relationship between integrin and breast cancer metastasis, and prognosis and survival, it becomes more important to establish the inhibitory effect of zinc on breast cancer adhesion and migration *in vivo*.

The third limitation is that although the observations obtained from my thesis

research provide strong evidence showing zinc inhibited the adhesion and migration of MDA-MB-231 cells on fibronectin, it was still not totally clear whether these effects of zinc are in fact through inhibiting the activation of the integrin β1-subunit.

In addition, activation of integrins in the presence of divalent cations suggest that the type of matrix that the cells adhere to and migrate on is crucial for the integrin to reach its maximum activation state (Mould *et al*., 2003). Cell adhesion and migration is mediated by 24 distinct integrins (Hynes, 2002) interacting with a large number of extracellular matrices. It is impossible to extrapolate the observations from my thesis research to predict how zinc supplementation would influence interactions between other forms of integrins and other matrices. Therefore, observations on the effects of zinc on the integrins involved in the mediation of MDA-MB-231 binding to fibronectin is insufficient to provide a clear understanding of the influence of zinc on cell adhesion in other cancer cells *in vitro* or *in vivo*.

3.3 Future Directions

Further studies are needed to delineate the role of zinc in breast cancer cell metastasis. Firstly, confirming that zinc is acting through mediating integrin β1-subunit activation is paramount. Studies, as designed by Mould *et al*. (2003), would allow for integrin activation to be assessed while cells are bound to fibronectin. In addition, carrying out studies using different breast cancer cell lines and adhesion substrates would confirm the breadth of zinc's inhibitory actions. Finally, investigating these findings in an *in vivo* model is crucial to determining whether the inhibitory role of zinc can be

potentially useful in developing a new strategy to treat breast cancer patients. Encouraging results were shown using zinc supplementation as an adjuvant treatment in patients treated for nasopharyngeal cancer (Lin *et al*., 2009), but no such study has been carried out in breast cancer patients. Epidemiological data show an inverse correlation between zinc and breast cancer incidence (Grant *et al*., 2008). Numerous studies are now supporting a role for using integrin β1-subunit blocking antibodies in breast cancer therapy. Our data together with these previous findings support a role for zinc in slowing breast cancer cell adhesion and metastasis. For this reason further studies investigating the role of zinc in modifying breast cancer cell metastasis are warranted.

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Appendix I

Effect of zinc on the migration rate of MDA-MB-231 breast cancer cells on a poly-D-lysine matrix

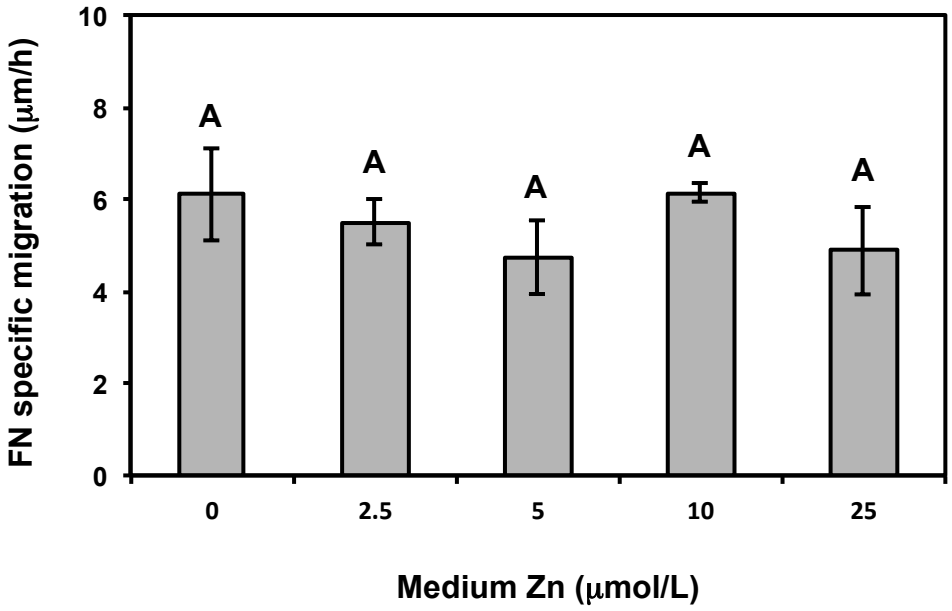

Figure A1. Effect of zinc on the migration rate of MDA-MB-231 breast cancer cells on poly-D-lysine matrix. Migration of MDA-MB-231 cells in a confluent monolayer was measured for a 12 h period in the presence of 0, 2.5, 5, 10 or 25 μmol/L of Zn^{2+} in serum free medium. Migration rate (μm/h) was calculated by dividing the distance traveled on poly-D-lysine by 12. Values represent means ± SEM (n = 3; the experiment was repeated once independently.) Means with different letters are significantly different ($p < 0.05$).