

**Zinc depletion induced apoptosis through Ca²⁺-dependent mitochondrial
apoptotic pathway in human breast cancer MDA-MB-231 cells**

by

YI – HSUAN LIN

B.Sc., The University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2012

© Yi - Hsuan Lin, 2012

Abstract

Breast cancer is the most frequently diagnosed cancer among Canadian women. Despite the use of advanced therapeutics, breast cancer remains the second leading cause of cancer death among Canadian women. Therefore, the development of novel and effective therapeutics to treat breast cancer remains an important goal. Defective or inhibited apoptosis is a major causative factor in the development and progression of cancer. Zinc is considered an apoptotic regulator. Further, previous work has also shown that there is an association between zinc depletion-induced apoptosis and an elevated intracellular Ca^{2+} level in human breast cancer MDA-MB-231 cells. Ca^{2+} is a known mediator of the mitochondrial apoptotic pathway. The overall objective of my thesis research was to investigate the role of intracellular Ca^{2+} and its involvement of the mitochondria in zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells. MDA-MB-231 cells were cultured in DMEM containing FBS (10%) followed by depletion of intracellular zinc using N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN; 20 μM) with or without the presence of intracellular Ca^{2+} chelator, 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM; 10 or 20 μM). Apoptosis was assessed by caspase-9 and -3 activities using corresponding fluorogenic substrates and the proportion of cells with fragmented DNA using PI-staining flow cytometry assay. Intracellular Ca^{2+} was assessed using Fura-2 assay. Mitochondrial membrane potential was assessed by DiOC₆-staining flow cytometry assay. Cytochrome c release was detected by Western blot. Addition of TPEN resulted in an increase of caspase-9 and -3 activities, an increase in the proportion of cells with fragmented DNA, and a prolonged increase in intracellular Ca^{2+} level. TPEN

treatment also reduced mitochondrial membrane potential and induced cytochrome c release. Zinc replenishment (10 – 40 μ M) prevented TPEN-induced apoptosis. Intracellular Ca^{2+} chelation with BAPTA-AM suppressed TPEN-induced apoptosis, mitochondrial membrane potential loss, and cytochrome c release. Collectively these results showed that zinc depletion-induced apoptosis was mediated through the Ca^{2+} -dependent mitochondrial apoptotic pathway in human breast cancer MDA-MB-231 cells.

Preface

This thesis contains the work of a research study for a masters degree and was prepared in accordance to the University of British Columbia Faculty of Graduate Studies requirements. I was responsible for conducting all the experiments and subsequent data analysis under the supervision of Dr. Zhaoming Xu.

Table of Contents

Abstract	ii
Preface	iv
Table of Contents	v
List of Tables	vii
List of Figures	viii
List of Abbreviations	x
Acknowledgements	xii
Introduction	1
Chapter 1 Literature Review, Hypothesis, and Objective	3
1.1 Breast Cancer	3
1.1.1 Risk factors	3
1.1.2 Breast cancer types and cell line models	5
1.1.3 Treatment	7
1.2 Apoptosis	8
1.2.1 Morphological features of apoptosis	9
1.2.2 Caspases	10
1.2.3 Caspase-3 activation	11
1.2.4 Mediators of the mitochondrial apoptotic pathway	12
1.2.5 Apoptosis in cancer development and treatment	14
1.3 Zinc	17
1.3.1 Food sources and bioavailability	17
1.3.2 Physiological functions	19
1.3.3 Zinc homeostasis	20
1.3.4 Zinc as an apoptotic regulator	21
1.4 Calcium Signaling and Apoptosis	26
1.4.1 Intracellular Ca ²⁺ homeostasis	26
1.4.2 Ca ²⁺ - mediated mitochondrial apoptotic pathway	28
1.5 Summary	29
1.6 Hypothesis	30
1.7 Overall Objective and Specific Aims	30
Chapter 2 Zinc Depletion Induced Apoptosis Through Ca²⁺-Dependent Mitochondrial Apoptotic Pathway in Human Breast Cancer MDA-MB-231 Cells	40

2.1 Introduction	40
2.2 Material and Methods	42
2.2.1 Cell culture system	42
2.2.2 Induction of zinc depletion	43
2.2.3 Zinc replenishment.....	43
2.2.4 Cytosolic Ca ²⁺ chelation	43
2.2.5 Caspase-9 and -3 activities	44
2.2.6 DNA fragmentation	45
2.2.7 Intracellular Ca ²⁺ content	46
2.2.8 Mitochondrial membrane potential	47
2.2.9 Mitochondrial cytochrome c release	48
2.2.10 Statistical analysis	51
2.3 Results	51
2.3.1 Zinc prevented TPEN-induced apoptosis	51
2.3.2 TPEN increased intracellular Ca ²⁺ content	52
2.3.3 Ca ²⁺ chelation with BAPTA-AM reduced intracellular Ca ²⁺ content and suppressed TPEN-induced caspase-9 and -3 activity and DNA fragmentation	53
2.3.4 Chelation of intracellular Ca ²⁺ with BAPTA-AM protected cells from TPEN-induced mitochondrial membrane potential loss	53
2.3.5 Chelation of intracellular Ca ²⁺ with BAPTA-AM suppressed TPEN-induced mitochondrial cytochrome c release	54
2.4 Discussion	55
2.4.1 Zinc depletion with TPEN induced apoptosis	55
2.4.2 Zinc depletion induced apoptosis through the Ca ²⁺ -mediated mitochondrial apoptotic pathway	58
2.4.3 Summary	60
Chapter 3 General Discussion, Limitations, and Future Directions	74
3.1 General Discussion	74
3.2 Limitations	76
3.3 Future Directions	78
References	81
Appendices	93

List of Tables

Table 1.1: Examples of breast cancer cell lines	31
Table 1.2: Zinc content of selected foods	32

List of Figures

Figure 1.1: Morphological features of apoptosis	33
Figure 1.2: The receptor-apoptotic pathway	34
Figure 1.3: The mitochondrial-apoptotic pathway	35
Figure 1.4: Mechanisms causing mitochondrial outer membrane permeabilization	36
Figure 1.5: Intracellular Ca ²⁺ homeostasis	37
Figure 1.6: Ca ²⁺ -mediated mitochondrial apoptotic pathway	38
Figure 1.7: Hypothetical model of zinc depletion-induced apoptosis in MDA-MB-231 cells	39
Figure 2.1: TPEN increased caspase-9 and -3 activity in MDA-MB-231 cells	62
Figure 2.2: Zinc replenishment suppressed TPEN-induced increase in caspase-3 activity in MDA-MB-231 cells	63
Figure 2.3: TPEN induced DNA fragmentation in MDA-MB-231 cells	64
Figure 2.4: Zinc replenishment suppressed TPEN-induced DNA fragmentation in MDA-MB-231 cells	65
Figure 2.5: TPEN induced intracellular Ca ²⁺ elevation in MDA-MB-231 cells	66
Figure 2.6: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced intracellular Ca ²⁺ elevation in MDA-MB-231 cells	67
Figure 2.7: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced increase in caspase-9 and -3 activity in MDA-MB-231 cells	68
Figure 2.8: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation	69
Figure 2.9: TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells	70
Figure 2.10: Intracellular Ca ²⁺ chelation with BAPTA-AM prevented TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells	71
Figure 2.11: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced release of mitochondrial cytochrome c into the cytosol	72
Figure 2.12: Zinc depletion induced apoptosis through the Ca ²⁺ -mediated mitochondrial apoptotic pathway in MDA-MB-231 cells.....	73
Figure 3.1: Involvement of Ca ²⁺ in mediating zinc depletion-induced apoptosis in MDA-MB-231 cells	80
Figure A.1: TPEN induced DNA fragmentation in MDA-MB-231 cells (flow cytometry data)	94
Figure A.2: Zinc replenishment suppressed TPEN-induced DNA fragmentation in MDA-MB-231 cells (flow cytometry data)	95

Figure A.3: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation (48 h; flow cytometry data)	96
Figure A.4: Intracellular Ca ²⁺ chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation (72 h; flow cytometry data)	97
Figure A.5: TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells (flow cytometry data)	98
Figure A.6: Intracellular Ca ²⁺ chelation with BAPTA-AM prevented TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells (flow cytometry data)	99

List of Abbreviations

Ac-DEVD-AFC	N-Acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin)
Ac-LEHD-AFC	N-Acetyl-Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin)
ANT	Adenine nucleotide
BAPTA-AM	1, 2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester
Cu/Zn SOD	Copper/zinc superoxide dismutase
DAG	Diacyl glycerol
DiOC₆	3, 3'-dihexyloxacarbocyanine iodide
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DMT1	Divalent mineral transporter 1
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ER+/-	Estrogen receptor-positive/-negative
GSH	Glutathione
HEPES	Hydroxyethyl piperazineethanesulfonic acid
InsP₃	Inositol 1, 4, 5-trisphosphate
InsP₃R	Inositol 1, 4, 5-trisphosphate receptor
LIPZ	Labile intracellular pool of zinc
MMP	Mitochondrial membrane potential
MT	Metallothionien
PBS	Phosphate buffered saline
PI	Propidium iodide
PLC	Phospholipase C
PtdInsP₂	Phosphatidylinositol 4, 5-bisphosphate
PTP	Permeability transition pore
RDA	Recommended dietary allowance
ROC	Receptor-operated channel
RyR	Ryanodine receptor
SOC	Store-operated channel
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPEN	N,N,N', N'-tetrakis(2-pyridylmethyl)ethylenediamine
TTBS	Tris-tween buffered saline
UL	Upper intake level
UP	Uniporter

VOC

ZIP

ZnSO₄

ZnT

Voltage-operated channel

ZRT, IRT-like protein

Zinc sulfate

Zinc transporter

Acknowledgements

My graduate study was an unforgettable journey. I would like to thank everyone at the University of British Columbia who has helped me throughout the process of completing my master degree. Especially, I would like to acknowledge and thank my supervisor, Dr. Zhaoming Xu, for his ongoing support and guidance throughout my research and the completion of this thesis. I would also like to thank my supervisory committee members, Dr. Christine Scaman and Dr. Yvonne Lamers, for their expertise and valuable insights into the research project and my external examiner, Dr. David Kitts, for his involvement and encouragement. I would also like to acknowledge the UBC flow facility as supporting the work, with special thanks to Andy Johnson. Many thanks to my lab mates and colleagues, Deanna Ibbitson, Wendy Hempstock, Melinda Bakker, Li He, Markus Purtzki, Tina Li, Amy Liu and Ingrid Elisia, for their support and friendship during this time. Lastly, special thanks to my family, especially my parents and grandfather for their financial support, unconditional love and encouragement.

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among Canadian women. In 2012, it is estimated that 22,700 new cases of breast cancer will be diagnosed with 5,100 deaths in Canadian women. (Canadian Cancer Society, 2012). Despite early diagnosis and aggressive therapies, the incidence and mortality rate of breast cancer among Canadian women remains high. Therefore, the need for development of novel and effective therapeutic strategies against breast cancer remains an important goal.

Zinc is required for a wide range of physiological functions. One of the most profound functions of zinc is its regulatory role in growth (Clegg *et al.*, 2005; MacDonald, 2000). Growth can be viewed as the net effect between cell proliferation and cell death (Saikumar *et al.*, 1999). Apoptosis, also known as programmed cell death, follows a genetically encoded sequence of events leading to the self-destruction of the cell without triggering inflammation (Saikumar *et al.*, 1999).

Zinc is considered a regulator of apoptosis (Truong-Tran *et al.*, 2001). Impaired apoptosis is a major causative factor in the development and progression of cancer, such as breast cancer (Saikumar *et al.*, 1999). Zinc depletion has been demonstrated to induce apoptosis in a variety of cell types (i.e. rat and human thymocytes), while zinc replenishment prevents apoptotic cell death (Clegg *et al.*, 2005; McCabe *et al.*, 1993). We have previously observed that depletion of intracellular zinc using N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), an intracellular zinc chelator, induces

apoptosis in human breast cancer MDA-MB-231 cells (Wu, 2003; Xu & Purtzki, 2010a). The precise mechanisms involved in zinc depletion-induced apoptosis remains unclear. However, an altered intracellular Ca^{2+} homeostasis has been associated with zinc depletion-induced apoptosis in MDA-MB-231 cells (Xu & Purtzki, 2010a). Therefore, a better understanding of the mechanisms whereby zinc functions as an apoptotic regulator of breast cancer involving the dysregulation of Ca^{2+} may contribute to the development of a novel and more effective therapeutic strategy against breast cancer.

Chapter 1. Literature Review, Hypothesis, and Objective

1.1 Breast Cancer

Breast cancer is the most frequently diagnosed and the second leading cancer death among Canadian women. In 2012, there will be 22,700 new cases of breast cancer with 5,100 deaths (Canadian Cancer Society, 2012). On average, 62 Canadian women will be diagnosed with breast cancer every day and 14 of those women with breast cancer will die from it. This death rate is estimated to account for 14% of all cancer deaths among Canadian women in 2012. Moreover, it is expected that 1 in 9 Canadian women will be diagnosed with breast cancer during her life time and 1 in 29 women with breast cancer will die from it (Canadian Cancer Society, 2012).

1.1.1 Risk factors

The most documented risk factors for breast cancer are being female and an older age (Reeder & Vogel, 2008). It has been noted that women 65 or older have a 5.8 fold increased risk for breast cancer compared to women less than 65 years of age (Singletary, 2003). Other risk factors for breast cancer include early menarche, late menopause, older age at first birth, and lack of childbearing or breast feeding (Singletary, 2003). For instance, girls who start menstruation at an age younger than 12 have a 1.3 fold increased risk for breast cancer compared to girls aged 15 and older. Women having menopause over the age of 55 have a 1.2-1.5 fold increased risk for breast cancer compared to women that have menopause at younger than 45 years of age (Singletary, 2003). Menopausal hormone (i.e. estrogen and progesterone) replacement therapy given to

women to alleviate menopausal symptoms has been reported to increase risk for developing breast cancer (Nelson *et al.*, 2002). Women who use hormone replacement therapy for at least 5 years have a 1.3 fold increased risk for breast cancer compared to those that have never used it (Singletary, 2003). Moreover, obesity appears to increase the risk for developing breast cancer. Postmenopausal women (age 55 or older) with a body mass index percentile of 80th have a 1.2 fold increased risk for breast cancer compared to women with a body mass index percentile of 20th (Tretli, 1989). Obesity may be due to lifestyle factors such as a high fat diet, lack of physical activity, and alcohol consumption, which have all been identified as risk factors for breast cancer (Singletary, 2003). For instance, alcohol drinkers with one, two, and three drinks per day, have a 1.1, 1.2 and 1.4 fold increased risk for breast cancer, respectively, compared to non-drinkers (Singletary, 2003; Tretli, 1989). Other lifestyle factors such as smoking have also been reported to increase the risk for developing breast cancer (Johnson *et al.*, 2011). Long-term smokers have a 35-50 % higher risk of developing breast cancer compared to non-smokers (Johnson *et al.*, 2011). Inherited mutations, such as mutations in the *BRCA-1* (on chromosome 17q21) and *BRCA-2* (on chromosome 13q12-13), have been identified as important risk factors for developing breast cancer (Kenemans *et al.*, 2004). Women with *BRCA-1* and *BRCA-2* mutations have a 40 – 85 % chance of developing breast cancer in their lifetime (Dumitrescu & Cotarla, 2005). *BRCA-1* and *BRCA-2* mutations account for 5 – 10% of all breast cancer cases (Aguas *et al.*, 2005). Gene mutations related to breast cancer are more prevalent in certain ethnic groups, including Caucasians (Aguas *et al.*, 2005; Kenemans *et al.*, 2004).

1.1.2 Breast cancer types and cell line models

Each breast is mainly made up of lobules, ducts and fatty tissues (Sariego, 2010). Lobules are milk-producing glands. Milk produced in the lobules flows to the nipple through thin tubes known as ducts. Fatty tissues serve as the cushion and fill the areas between lobules and ducts. Each breast is also surrounded by lymph vessels and lymph nodes, which play a major role in the immune system (Sariego, 2010). Breast cancers originate from either the cells of lobules in lobular carcinoma, or the cells of ducts in ductal carcinoma (Simpson *et al.*, 2005). Ductal carcinoma is the most common type of breast cancer. When the development of breast cancer remains localized in the lobules or ducts, it is considered to be a non-invasive breast cancer. However, when breast cancer in the lobules or ducts spreads to the nearby lymph nodes or metastasizes to other parts of the body (e.g. lung), it becomes an invasive breast cancer (Simpson *et al.*, 2005). Breast cancer that forms new tumors at other parts of the body would still be regarded as breast cancer. The growth of some breast cancer can be stimulated by hormones such as estrogen due to the presence of an estrogen receptor (ER) on the cell surface (Osborne *et al.*, 1980). The growth of ER-positive (ER+) breast cancer can be suppressed by the presence of estrogen-antagonist. Therefore, ER has become a promising therapeutic target in breast cancer treatment. Due to the absence of a therapeutic target, ER-negative (ER-) breast cancer is often more invasive and difficult to treat (Osborne *et al.*, 1980), thus requiring more attention.

A number of breast cancer cell lines have been established as models for breast tumors. The first breast cancer cell line, BT-20, was established in 1958. This cell line is derived from the primary tumor of an invasive ductal carcinoma from a 74 year old Caucasian female (Lasfargues & Ozzello, 1958). However, culturing breast cancer cell lines from primary tumors has been largely unsuccessful due to technical difficulties. Therefore, most of the available breast cancer cell lines are derived from metastatic tumors, mainly from pleural effusions (Lacroix & Leclercq, 2004). Pleural effusions are the accumulation of excess fluids in the pleural space of the lungs caused by metastatic breast cancer (Cailleau *et al.*, 1974). The most widely used breast cancer cell lines in research settings are MCF-7, T-47D and MDA-MB-231, which were established in the late 1970s (Lacroix & Leclercq, 2004). All three cell lines were issued from metastatic pleural effusions of tumors of invasive ductal carcinoma (Lacroix & Leclercq, 2004). However, they each have unique morphological and growth characteristics and model different types of breast tumors. For instance, MCF-7, derived from a 69 year old Caucasian female, is ER+ which renders it a popular model for hormone responsive tumors (Soule *et al.*, 1973). Conversely, MDA-MB-231, derived from a 51 year old Caucasian female, is ER- thus is commonly used as a model for hormone non-responsive tumors (Cailleau *et al.*, 1974). Other commonly used breast cancer cell lines in breast cancer research include BT-474 and SK-BR-3 (Lacroix & Leclercq, 2004). Examples of breast cancer cell lines are presented in Table 1.1.

1.1.3 Treatments

Current treatments for breast cancer include surgery, radiation therapy, hormone therapy, and chemotherapy. For breast tumors that are localized (i.e. non-invasive breast cancer), surgery is the most common treatment (King & Primrose, 2009). Surgical removal of just the lump is known as lumpectomy, whereas removal of the whole breast is known as mastectomy (King & Primrose, 2009). Radiation therapy is usually performed after surgery, especially for those that have just had the lump removed (Falk, 2006). Radiation therapy uses high-energy X-rays or gamma rays to directly kill cancer cells at the tumor site or post-surgery tumor site, thereby preventing the growth of cancer cells that may have escaped the surgery (Falk, 2006). Surgery and radiation therapy are examples of local therapy, which intend to treat tumors at the site without affecting the rest of the body (Falk, 2006; King & Primrose, 2009). Hormone therapy and chemotherapy are considered systemic therapies. Systemic therapy involves the administration of drugs into the bloodstream, which intends to treat tumors (e.g. invasive breast cancer) that have spread to the lymph nodes or metastasized to distant sites (e.g. lung) (Parnell & Woll, 2003; Stokes & Chan, 2003). Hormone therapy uses drugs to either block the hormone receptor or the production of the hormone to effectively suppress the growth of ER+ breast cancers (Stokes & Chan, 2003). However, ER- breast cancers lack the advantage for such therapeutic targets, thus are usually more invasive and harder to treat. Chemotherapy using cytotoxic drugs that destroy cancer cells by inducing cell death or inhibiting cell proliferation is often used to treat ER- breast cancers (Parnell & Woll, 2003).

Despite early diagnosis and advances in cancer therapeutics, the incidence and mortality of breast cancer remains high in Canada. Therefore, the development of novel and effective therapeutic strategies against breast cancer, such as the induction of cancer cell death through apoptosis, are much needed.

1.2 Apoptosis

Apoptosis is a form of cell death, which follows a genetically encoded sequence of events leading to the self-destruction of the cells without triggering inflammation (Saikumar *et al.*, 1999). Apoptosis is involved in eliminating old, unnecessary and damaged cells, thereby playing a crucial role in embryological development, morphogenesis of life, cell population control, and development of diseases (Saikumar *et al.*, 1999). For example, the sculpting of fetal organs during embryological development is due to apoptosis of the cells that are produced in excess (Meier *et al.*, 2000). Maturation of the adult brain also relies on apoptosis to remove the neurons that were produced during the earlier stage (Hutchins & Barger, 1998). One of the most important physiological processes that apoptosis is involved in is to remove severely damaged DNA that can no longer be repaired, thereby preventing malignant transformation (Fadeel *et al.*, 1999b). Defective or dysregulated apoptosis may lead to uncontrolled malignant cell proliferation contributing to cancer development and growth (Fadeel *et al.*, 1999b).

Apoptosis can be triggered by various intra- and extra-cellular stimuli, such as ligation of cell surface death receptors, absence of cell survival signals, genetic damage,

oxidative stress, starvation, as well as treatment with chemotherapeutic drugs, etc. (Saikumar *et al.*, 1999). Despite the fact that apoptosis can be triggered by such diverse stimuli, the apoptotic machinery is induced via two main pathways involving the activation of the death receptors or the mitochondria. The pathway involving the activation of the death receptors is known as the extrinsic or receptor-apoptotic pathway (Sartorius *et al.*, 2001). The pathway involving the mitochondria is known as the intrinsic or mitochondrial apoptotic pathway (Loeffler & Kroemer, 2000). Upon activation of the pathways by stimuli, the apoptotic signals trigger a cascade of apoptotic regulators such as the Bcl-2 family proteins, ultimately leading to the activation of caspase-3 (Saikumar *et al.*, 1999). Activated caspase-3 then catalyzes the cleavage of DNA resulting in DNA fragmentation, the characteristic feature of apoptotic cell death (Earnshaw *et al.*, 1999; Saraste & Pulkki, 2000). Therefore, caspase-3 activity and DNA fragmentation are often used as indicators for apoptosis.

1.2.1 Morphological features of apoptosis

Upon induction of apoptosis, cells shrink in size which is accompanied by rounding of the cell shape and loss of connection with neighbouring cells. The shrinkage and rounding of the cell is due to chromatin condensation resulting from DNA fragmentation, the classical hallmark of apoptosis (Saikumar *et al.*, 1999; Saraste & Pulkki, 2000; Figure 1.1). This leads to collapse of the nucleus and blebbing or ‘budding’ of the plasma membrane and the resultant buds are later broken down into membrane-enclosed ‘apoptotic bodies’. These apoptotic bodies, containing DNA fragments, collapsed nucleus and cell debris, are then engulfed by phagocytic cells such as

macrophages without triggering inflammatory responses (Saikumar *et al.*, 1999; Saraste & Pulkki, 2000).

1.2.2 Caspases

Caspases are a family of 14 cysteine-dependent aspartate (Asp)-specific proteases (Denault & Salvesen, 2002). Their proteolytic activity depends on a critical cysteine-residue, which specifically cleaves their substrate after Asp residues. Caspases are organized in a cascade: the initiator caspases (i.e. caspase-8 and -9) and the executioner caspases (i.e. caspase-3 and -6).

Caspases are normally present as procaspases, the inactive form of the enzymes. Initiator procaspases (i.e. procaspase-9) are activated by binding to specific caspase activator proteins (e.g. cytochrome c is an activator of procaspase-9) (Earnshaw *et al.*, 1999). Upon activation, the initiator caspase activates the executioner procaspases (e.g. procaspase-3) through proteolytic cleavage. Activation of executioner caspases represents the commitment of the cell to apoptosis as executioner caspases, such as caspase-3, proteolytically cleave a specific subset of cytosolic and nuclear substrates (i.e. protein kinases, cytoskeletal proteins and DNA repair proteins) that are critical to cell survival (Earnshaw *et al.*, 1999; Saraste & Pulkki, 2000). One of the substrates of caspase-3 is the deoxyribonuclease (DNase) inhibitor. Since DNase catalyzes DNA degradation, activation of caspase-3 activates DNase, ultimately resulting in DNA fragmentation and apoptotic cell death (Earnshaw *et al.*, 1999; Saraste & Pulkki, 2000).

1.2.3 Caspase-3 activation

Caspase-3, an executor of apoptosis, can be activated through both the receptor-apoptotic pathway and the mitochondrial apoptotic pathway.

The receptor-apoptotic pathway. Activation of the receptor-apoptotic pathway can be triggered by the binding of the extracellular death ligand (i.e. tumor necrosis factor; TNF) to the death receptors (i.e. tumor necrosis factor receptor; TNFR) on the plasma membrane (Sartorius *et al.*, 2001; Figure 1.2). The binding of death ligand to the death receptor on the cell surface triggers the formation of the death-inducing signalling complex (DISC). DISC subsequently catalyzes the conversion of procaspase-8 to caspase-8, an initiator caspase. Activation of caspase-8 in turn catalyzes the conversion of procaspase-3 to caspase-3. The activated executioner caspase-3 then catalyzes proteolytic cleavage to a specific subset of substrates, including DNase, leading to DNA fragmentation and ultimately apoptotic cell death (Saraste & Pulkki, 2000; Sartorius *et al.*, 2001).

The mitochondrial apoptotic pathway. Caspase-3 can also be activated by intrinsic stimuli through the mitochondrial apoptotic pathway (Loeffler & Kroemer, 2000; Figure 1.3). In the mitochondrial apoptotic pathway, stimuli from inside the cell, such as genetic damage and oxidative stress, generate a death signal to the mitochondria which triggers the rupture or permeabilization of the mitochondrial outer membrane. As a result, cytochrome c is released from the mitochondrial intra-membrane space into the cytosol. Upon entering the cytosol, cytochrome c promotes the activation of caspase-9, an initiator

caspase, from procaspase-9. Caspase-9 then catalyzes the conversion of procaspase-3 to caspase-3. Activated executioner caspase-3 subsequently leads to DNA fragmentation and ultimately apoptotic cell death as described previously (Loeffler & Kroemer, 2000; Saraste & Pulkki, 2000).

1.2.4 Mediators of the mitochondrial apoptotic pathway

The release of cytochrome c due to mitochondrial outer membrane permeabilization is the key event in the mitochondrial apoptotic pathway as it activates the subsequent caspase-apoptotic cascade (Loeffler & Kroemer, 2000). Mitochondrial outer membrane permeabilization constitutes an early event in the mitochondrial apoptotic pathway and can be triggered by an overload of intracellular Ca^{2+} and Bcl-2 family proteins (Gross *et al.*, 1999; Orrenius *et al.*, 2003; Figure 1.4).

Intracellular Ca^{2+} . Certain conditions, such as ischemia, promote cellular uptake of Ca^{2+} from the extracellular spaces and/or release of Ca^{2+} from the intracellular Ca^{2+} stores which causes cytosolic Ca^{2+} levels to increase and intracellular Ca^{2+} overload (Hajnóczky *et al.*, 2003; Orrenius *et al.*, 2003). In response to this intracellular Ca^{2+} overload, there is an increased influx of Ca^{2+} from the cytosol into the mitochondria, which serve as the intracellular Ca^{2+} buffers. When mitochondrial Ca^{2+} is overloaded, it triggers the opening of the permeability transition pore (PTP), a large non-selective mitochondrial conductance channel that allows molecules to pass freely (Crompton, 1999; Figure 1.4). The opening of the mitochondrial PTP releases Ca^{2+} back into the cytosol and causing the loss of the mitochondrial membrane potential (MMP). Mitochondrial

PTP opening induces the swelling of the mitochondrial matrix, which causes the rupture or permeabilization of the mitochondrial outer membrane. As a result, cytochrome c is released from the mitochondrial intra-membrane space into the cytosol, and subsequently activates the caspase-apoptotic cascade (Green & Kroemer, 2004; Gross *et al.*, 1999; Orrenius *et al.*, 2003). A more detailed discussion on how Ca^{2+} mediates caspase-3 activation will be presented later in Section 1.4 Calcium Signalling and Apoptosis.

Bcl-2 family. Mitochondrial outer membrane permeabilization can be induced by the pro-apoptotic Bcl-2 proteins (Gross *et al.*, 1999; Figure 1.4). The Bcl-2 family proteins play an important role in modulating the integrity of the mitochondrial outer membrane. The Bcl-2 family consists of both pro-apoptotic proteins (Bax and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-X_L). Under normal conditions, the pro-apoptotic Bax and Bak are located in the cytosol (Eskes *et al.*, 2000). In the presence of apoptotic stimuli, the stimuli trigger the expression of Bax and Bak and their incorporation into the mitochondrial outer membrane. Bax and Bak are incorporated into the mitochondrial outer membrane either by directly inserting into the outer membrane thereby forming pores in the membrane or by forming a large protein-permeable channel (i.e. Bax channel) (Antonsson *et al.*, 2000; Eskes *et al.*, 2000). As a result, the mitochondrial outer membrane becomes permeable allowing cytochrome c to be released from the mitochondrial intra-membrane space into the cytosol, leading to the activation of the caspase-apoptotic cascade. Conversely, anti-apoptotic Bcl-2 and Bcl-X_L reside in the mitochondrial outer membrane and preserve the integrity of the membrane by inhibiting Bax and Bak, thereby preventing the activation of the apoptotic cascade (Antonsson *et al.*,

1997). Therefore, the ratio of pro-/anti-apoptotic Bcl-family proteins is critical in deciding cell survival or death.

1.2.5 Apoptosis in cancer development and treatment

Apoptosis plays a critical role in the elimination of potentially malignant cells therefore defective or inhibited apoptosis is a major causative factor in the development and progression of cancer (Saikumar *et al.*, 1999). Restoration of the defective apoptotic pathway or induction of apoptosis has been the focus of many cancer therapies (Kasibhatla & Tseng, 2003; Wong, 2011). Further, cancer cells can develop resistance to apoptosis due to an altered ratio between the pro- and anti-apoptotic proteins in the Bcl-2 family and impairment of caspase function (Krajewski *et al.*, 1999; Wong, 2011).

Altered ratio between the pro- and anti-apoptotic proteins in Bcl-2 family. The Bcl-2 family proteins play an important role in mediating the mitochondrial apoptotic pathway (Gross *et al.*, 1999). A lower pro-/anti-apoptotic Bcl-2 protein ratio results in less apoptotic cell death which in turn promotes survival of malignant cells. A lower pro-/anti-apoptotic Bcl-2 protein ratio can be achieved through reduced expression of the pro-apoptotic proteins (Bax), an over expression of the anti-apoptotic proteins (Bcl-2 and Bcl-X_L), or a combination of both (Cory & Adams, 2002; Gross *et al.*, 1999). This has been observed in a variety of cancer types such as lymphocytic leukaemia (Pepper *et al.*, 1997) and breast cancer (Krajewski *et al.*, 1995a). For instance, Bax expression level is lower in breast tumors than in normal breast tissues (Krajewski *et al.*, 1995a). This lower Bax expression level is associated with reduced malignant cell death and is correlated

with a poorer prognosis and lower survival rate due to faster tumor progression and failure of the cells to respond to chemotherapy (Krajewski *et al.*, 1995a).

Conversely, over-expression of anti-apoptotic proteins of the Bcl-2 family inhibits apoptosis in cancer cells (Cory & Adams, 2002). For example, cancer cell death has been observed to be prevented by over-expression of Bcl-2 in the primary tumors of breast cancer patients (Gee *et al.*, 1994; Krajewski *et al.*, 1995a). In contrast, an increased Bcl-2 expression through transfection consistently inhibits apoptosis in breast cancer cell lines (i.e. MCF-7), confirming that the anti-apoptotic Bcl-2 is capable of suppressing breast cancer cell death (Pratt *et al.*, 1998). Similarly, Bcl-X_L expression is elevated in malignant breast cancer tissues compared to that in the adjacent normal breast tissues (Krajewski *et al.*, 1999). Bcl-X_L over-expression has also been reported to inhibit drug-induced apoptosis in cancer cells such as FL5.12 cells, a prolymphocytic cell line, (Minn *et al.*, 1995).

Bcl-2 family proteins have been considered as a promising target for restoring apoptosis. Cancer treatment strategies targeting the Bcl-2 family proteins have been developed to inhibit the expression of the anti-apoptotic proteins in the Bcl-2 family (Bcl-2 and Bcl-X_L), thereby inducing apoptosis (Kasibhatla & Tseng, 2003; Wong, 2011). For instance, inhibiting Bcl-2 expression using Bcl-2 antisense oligonucleotides results in the catalytic degradation of Bcl-2 mRNA resulting in decreased expression. One of the Bcl-2 antisense oligonucleotides is Oblimersen sodium, which is the first chemotherapeutic agent designed to target a specific member of the Bcl-2 family (Kasibhatla & Tseng,

2003; Wong, 2011). Oblimersen sodium has been used to treat several types of cancer, including breast cancer, chronic lymphocytic leukemia and B-cell lymphoma (Abou-Nassar & Brown, 2010; Rai *et al.*, 2008). Moreover, several small molecule inhibitors have also been designed to directly inhibit the anti-apoptotic proteins in the Bcl-2 family (Kang & Reynolds, 2009). For example, ABT-737, a small molecule inhibitor, has been shown to reduce the tumor size of breast cancer and lymphoma (Oltersdorf *et al.*, 2005).

Reduced activity of caspases. Caspases play a pivotal role in the regulation and execution of apoptosis. Therefore, lower caspase activity can reduce apoptotic cell death, favouring malignant cell survival. Reduced caspase activity can be achieved through inhibition of caspase activation or down-regulation of caspase expression (Lavrik, *et al.*, 2005; Wong, 2011). For instance, a down-regulation of caspase-3 mRNA expression has been observed in breast tumors (Devarajan *et al.*, 2002). Several strategies have been developed to treat cancer by restoring caspase activity. These include the use of chemotherapeutic agents to directly activate caspases or to induce their gene expression, thereby restoring apoptosis (Wong, 2011). One of such example is apoptin, which is a chemotherapeutic drug designed to directly activate caspase-9 and -3 resulting in a selective induction of apoptosis in malignant cells of lymphoid, dermal, epidermal, endothelial, and smooth-muscle cells. (Danen-Van *et al.*, 1997; Rohn & Notebornl, 2004).

Targeting members of the Bcl-2 family and caspase family are two common strategies used in cancer treatment presently. Novel agents that are able to induce

apoptosis by targeting other players in the apoptotic pathway have also been perceived as potential novel anti-cancer therapies.

1.3 Zinc

Zinc is an essential trace mineral for humans. The Recommended Dietary Allowance (RDA) for zinc in adults is 11 and 8 mg/day for men and women, respectively. The Tolerable Upper Intake Level (UL) of zinc is 40 mg/day; however incidences of zinc toxicity are rarely reported (Institute of Medicine, 2001). Zinc deficiency in humans is also rarely reported, but symptoms of zinc deficiency include growth retardation, poor reproductive performance, immune dysfunction, cognitive impairment, anorexia and delayed wound healing (Chasapic *et al.*, 2012).

1.3.1 Food sources and bioavailability

Zinc in food is usually complexed with proteins and zinc content varies widely depending on the source. Animal sources are often high in zinc content (Groff & Gropper, 2000). For example, red meat such as ground beef is very rich in zinc (3.9-4.1 mg zinc/100g). Organ meat such as liver also contains high amount of zinc (3.1-3.9 mg zinc/100g). Other animal sources, such as cheese, contain moderate amounts of zinc (2.8-3.2 mg zinc/100g; Groff & Gropper, 2000). Seafood is also a rich source of zinc, especially oysters (17-91 mg zinc/100g). In contrast, plant sources such as fruits (<0.1 mg zinc/100g) and vegetables (0.1-0.7 mg zinc/100g) are poor sources of zinc (Groff & Gropper, 2000). Whole grain cereal such as whole wheat bread is a good plant source of zinc (1 mg zinc/100g). However, refined cereal such as white flour becomes a poor

source of zinc (0.6-0.8 mg/100g) (Groff & Gropper, 2000). Zinc content of selected foods is summarized in Table 1.2.

The bioavailability of zinc is influenced by the presence of zinc enhancers or inhibitors. For instance, ligands such as amino acids (i.e. histidine, cysteine, lysine and glycine) and products of protein digestion (i.e. tripeptides) are zinc enhancers and may increase the absorption or bioavailability of zinc (Groff & Gropper, 2000). This explains why animal sources, often high in protein content, are considered rich sources of zinc. The presence of zinc inhibitors such as phytate, oxalate and fiber decrease the absorption or bioavailability of zinc. Phytate is found in legumes and cereals such as maize and bran, which forms an insoluble complex with zinc (Groff & Gropper, 2000). However, zinc absorption may be enhanced by fermentation, which reduces the phytate content and releases zinc from the insoluble zinc-phytate complex (Groff & Gropper, 2000). Oxalate, found in vegetables such as spinach, also binds to zinc and inhibits its absorption. Therefore, zinc from plant sources, especially vegetables and fruits, tend to have lower bioavailability than zinc from animal sources due to the presence of zinc inhibitors such as phytate, oxalate and fiber (Groff & Gropper, 2000; Solomons & Cousins, 1984). Furthermore, the co-ingestion of non-heme iron greater than that of zinc (≥ 20 mg) may reduce zinc absorption (Whittaker, 1998). This is because non-heme iron and zinc share the same transporter (divalent mineral transporter 1; DMT1) for zinc uptake at the brush border membrane (Whittaker, 1998). Food processing also affects the bioavailability of zinc (Labuza, 1982). For instance, minerals such as zinc are concentrated in the bran and germ of grain kernels. Therefore, milling of cereals results in the loss of zinc due to the

removal of the bran and germ (Groff & Gropper, 2000; Labuza, 1982). This explains why higher zinc content is observed in whole wheat bread compared to white bread.

1.3.2 Physiological functions

Zinc plays a structural, catalytic and co-activator role in more than 300 zinc metalloenzymes that are involved in a wide range of physiological functions, including DNA and RNA synthesis, cell growth, protein synthesis, energy metabolism, cellular antioxidant defense, brain development, bone formation, and the immune system function (Sandstead, 2003; Stephanidou *et al.*, 2006). For example, zinc is catalytically required for the activities of DNA and RNA polymerase (Prasad & Oberleas, 1974) and is directly involved as a cofactor for Cu/Zn superoxide dismutase (Cu/Zn SOD), a cellular antioxidant enzyme that removes the superoxide anion radical (Tapiero & Tew, 2003).

There are two classes of zinc-proteins: metallothionein (MT) and zinc-finger proteins. MT is a low molecular weight cysteine-rich protein that is capable of binding zinc (Zn^{2+}) and acts as an intracellular zinc storage site (Tapiero & Tew, 2003). MT also plays an important role in the cellular antioxidant defense system by chelating oxidatively active metals and by scavenging hydroxyl radicals via its cysteine residues. In fact, the ability of MT to scavenge hydroxyl radicals is 300 times greater than that of glutathione (GSH), the most abundant cellular antioxidant in the cytosol (Sato, 1992). In zinc-finger proteins, zinc is responsible for the formation of the zinc-finger motif. Zinc-finger proteins are gene-regulatory proteins, which are directly involved in the expression and regulation of genes involved in numerous cellular processes such as cell proliferation and

cell death (Dreosti, 2001). One of the most profound functions of zinc is as a regulator of apoptosis (Truong-Tran *et al.*, 2001).

1.3.3 Zinc homeostasis

Whole body zinc homeostasis. The human body typically contains 2 - 3 g of zinc (Plum *et al.*, 2010). There is no known true storage site for zinc in the body. Zinc homeostasis at the whole body level is mainly achieved at the level of absorption and excretion. Dietary zinc absorption is inversely related to dietary zinc intake while zinc excretion is altered in response to body zinc content (Solomons & Cousin, 1984). Excessive dietary zinc intake reduces zinc absorption and increases zinc excretion.

Nearly 90 % of the body's zinc is found in the muscle and bone (Wastney *et al.*, 1986). A high level of zinc is found in the skin and liver with a moderate amount of zinc in the pancreas, kidney, heart, prostate, gastrointestinal tract, lungs and brain (Bentley & Grubb, 1991; He *et al.*, 1991; Llobet *et al.*, 1988). The normal zinc concentration in interstitial fluid or plasma is approximately 2 – 5 μM . In fact, the majority of body zinc (95-99%) is found intracellularly with only a trace amount found in the plasma (Cousins, 1997; Franklin & Costello, 2009).

Cellular zinc homeostasis. Normal intracellular zinc concentration is approximately 100 – 500 μM , in which 50% of zinc is localized in the cytosol, 30 – 40% is in the nucleus, and the remaining intracellular zinc is associated with membranes or presents as free ionic zinc (Zn^{2+} ; Stefanidou *et al.*, 2006). Intracellular zinc is either

tightly bound to proteins, which is known as the non-exchangeable pool of zinc, loosely bound to proteins, or as free Zn^{2+} . Loosely bound zinc and free Zn^{2+} are collectively known as the labile intracellular pool of zinc (LIPZ; Wu, 2003). LIPZ is metabolically important, and the abundance of LIPZ is generally in the fM – pM range of many cells (Coyle *et al.*, 1994; Zalewski *et al.*, 1993). A lower abundance of LIPZ is associated with impaired DNA synthesis and cell proliferation in 3T3 cells (Paski & Xu, 2001). Furthermore, a lower abundance of LIPZ has also been shown to increase apoptosis in leukaemic lymphocytes (Zalewski *et al.*, 1993) and in MDA-MB-231 cells (Wu, 2003).

Cellular zinc concentration is under tight regulation involving a complex interplay of proteins such as the ZRT, IRT-like protein family (ZIPs), the zinc transporter family (ZnTs), and zinc binding proteins such as MT (Kambe *et al.*, 2004). ZIPs are a family of zinc importers involved in zinc influx from the extracellular space into the cytosol or zinc release from the subcellular organelles (i.e. endoplasmic reticulum, Golgi, and lysosomes) into the cytosol. On the other hand, ZnTs are a family of zinc exporters involved in zinc efflux from the cytosol into the extracellular space or into the subcellular organelles. MT acts as a zinc buffer protein and plays an important role in maintaining intracellular zinc homeostasis during times of intracellular zinc overload (Kambe *et al.*, 2004; Murakami & Hirano, 2008).

1.3.4 Zinc as an apoptotic regulator

Zinc and apoptosis in animals. Zinc is considered a regulator for apoptosis. Elmes (1977) first reported that dietary zinc deficiency induces apoptosis in the small

intestinal crypts of rats. Yanagisawa and Wada (2001) observed increased apoptosis as indicated by DNA fragmentation in the thymus, testis, kidney, liver and skin in rats fed a zinc-free diet compared to the control rats fed a zinc-standard diet (20 mg Zn/kg diet). In addition, these authors also observed atrophy of the thymus and testis, which could be attributed to the increased apoptotic cell death in these tissues. Similarly, King *et al.* (2002) observed thymus atrophy and enhanced DNA fragmentation, an indicator of apoptosis, in thymocytes of rats fed a zinc-deficient diet (0.5 mg Zn/kg diet) compared to rats fed a zinc-sufficient diet (30 mg Zn/kg diet). Chickens fed a zinc-deficient diet (10 mg Zn/kg diet) results in increased apoptosis in the growth plate chondrocytes compared to those fed an adequate zinc diet (68 mg Zn/kg diet; Wang *et al.*, 2002).

Zinc and apoptosis in cultured cells. Studies *in vitro* also provide strong evidence supporting the role of zinc in apoptosis. Exposure of cells to low-zinc medium promotes apoptosis in a number of cell types. For instance, culturing human leukaemia Jurkat T-cells, rat fibroblasts 3T3 cells and human neuroblastoma IMR-32 cells in a low-zinc medium (1.5 μ M Zn) results in increased apoptosis as indicated by DNA fragmentation compared to cells cultured in a zinc-supplemented medium (15 μ M Zn; Verstraeten *et al.*, 2004). Similarly, increased apoptosis is also noted in human lymphoid and human myeloid HL-60 cells cultured in a zinc free medium (0 μ M Zn) compared to cells cultured in a zinc-replenished medium (50 μ M Zn; Martin *et al.*, 1991). Zinc depletion using zinc chelators can also induce apoptosis. In our lab, we have previously shown that depletion of cellular zinc with TPEN, a membrane permeable intracellular zinc chelator, induces apoptosis in three human breast cancer lines, including MCF-

7(ER-), T-47D(ER-) and MDA-MB-231(ER+), in which MDA-MB-231 cells are the most responsive to TPEN-induced apoptosis (Wu, 2003; Xu & Purtzki, 2010a). In addition, TPEN (1 – 100 μ M) has also been shown to induce apoptosis in a variety of cell types, including human lymphocytes (Kolenko *et al.*, 2001; Treves *et al.*, 1994), rat and human thymocytes (McCabe *et al.*, 1993), human monocytic leukemia THP-1 cells (Cao *et al.*, 2001), primary sheep (Truong-Tran *et al.*, 2000b) and human (Carter *et al.*, 2002) airway epithelium cells, human malignant airway epithelium A549 and NCI-H292 cells (Truong-Tran *et al.*, 2000b), human retinal pigment epithelium cells (Hyun *et al.*, 2001), Bowes human melanoma cells (Rudolf & Cervinka, 2004), human keratinocyte HaCaT cells (Wilson *et al.*, 2006), neuroblastoma-glioma NG108 cells (Adler *et al.*, 1999) and mouse osteoblastic MC3T3-E1 cells (Guo *et al.*, 2012). This chelator-induced apoptosis can be prevented by zinc replenishment. For example, TPEN-induced zinc depletion induces apoptosis as indicated by an elevated caspase-3 activity and DNA fragmentation in rat and human thymocytes (McCabe *et al.*, 1993) and human retinal pigment epithelium cells (Hyun *et al.*, 2001). This TPEN-induced apoptosis can be prevented by adding equimolar concentrations of zinc (Hyun *et al.*, 2001; McCabe *et al.*, 1993).

It has been demonstrated that zinc supplementation can protect cells against apoptosis induced by a variety of cell death stimuli. For example, Zn^{2+} at 25 - 50 μ M has been shown to suppress H_2O_2 -induced apoptosis in primary sheep airway epithelium cells (Truong-Tran *et al.*, 2000b) and osteoblastic MC3T3-E1 cells (Liang *et al.*, 2012). Lipopolysaccharide is an endotoxin produced by the Gram-negative bacteria that can elicit strong immune responses in humans through the apoptotic cascade. It has recently

been shown that Zn^{2+} (10 μ M) protects sheep pulmonary artery endothelial cells from lipopolysaccharide-induced apoptosis (Thambiyaya *et al.*, 2012). Furthermore, the cytopathic effect of the influenza virus occurs primarily through induction of apoptotic cell death. Addition of Zn^{2+} (150 μ M) inhibits the influenza-induced apoptosis in HeLa cells (Srivastava *et al.*, 2009). Collectively, available evidence shows that zinc deprivation achieved through feeding a zinc-deficient diet to animals, culturing cells in low-zinc medium, or depleting intracellular zinc using zinc chelator, induces apoptosis in a variety of tissue and cell types, whereas adequate zinc and zinc supplementation can inhibit apoptosis.

Labile intracellular pool of zinc (LIPZ) and apoptosis. Zalewski and co-workers (1993) first showed an inverse relationship between the abundance of the LIPZ and apoptosis. In rat thymocyte and human leukemia lymphocytes, TPEN treatment (0 - 100 μ M) results in a reduced abundance of the LIPZ from 30 - 7 pM/ 10^6 cells along with increased apoptosis (Zalewski *et al.*, 1993). Conversely, an increase abundance of the LIPZ using Zn^{2+} ($ZnSO_4$) or zinc ionophore (sodium pyrithione) reduces apoptosis (Zalewski *et al.*, 1993). Similarly, TPEN treatment (25 μ M) results in reduced abundance of the LIPZ and increased apoptosis (indicated by casapse-3 activation and DNA fragmentation) in primary airway epithelium and malignant human airway epithelium cell lines (Truong-Tran *et al.*, 2000b). Collectively, these studies have provided strong evidence linking the abundance of the LIPZ to apoptosis. In our lab, we have previously observed that after TPEN treatment, the abundance of the LIPZ is significantly reduced while total cellular zinc content remains unchanged (Wu, 2003; Xu & Purtzki, 2010a).

Therefore, it is clear that the LIPZ, not the total intracellular zinc content, plays an important role in the regulation of apoptosis.

Involvement of the mitochondria. Previous evidence has suggested a possible involvement of the mitochondria in zinc depletion-induced apoptosis. For instance, intracellular zinc depletion with TPEN (15 μM) triggers the release of cytochrome c from the mitochondria into the cytosol and subsequent caspase activation and DNA fragmentation in human peripheral blood T lymphocytes (Kolenko *et al.*, 2001). Moreover, exposure of cells to low-zinc medium (0.5 μM) reduces total intracellular zinc content, causes a loss of MMP, and triggers caspase-3 activation in human leukemia HL-60 cells (Duffy *et al.*, 2001). Similarly, the loss of MMP followed by caspase-3 activation has been observed in Bowes human melanoma cells treated with TPEN (25 – 100 μM) (Rudolf & Cervinka, 2004). More recently, intracellular zinc depletion using TPEN (5 μM) has been shown to induce cytochrome c release and increased apoptosis as indicated by caspase-9 and -3 activation and DNA fragmentation along with an increased expression of Bax, a pro-apoptotic Bcl-2 proteins, in mouse osteoblastic MC3T3-E1 cells (Guo *et al.*, 2012). The pro-apoptotic Bax is an upstream mediator in the mitochondrial apoptotic pathway which triggers the permeabilization of the mitochondrial outer membrane, allowing a subsequent release of cytochrome c (Gross *et al.*, 1999). Together, these observations suggest the involvement of the mitochondria in zinc depletion-induced apoptosis. However, it is not clear how zinc depletion triggers mitochondria dependent-apoptosis. As discussed earlier, in human breast cancer MDA-MB-231 cells, TPEN-induced zinc depletion induces apoptosis (Wu, 2003; Xu & Purtzki, 2010a). Interestingly,

this TPEN-induced zinc depletion triggers a sustained increase in the concentration of intracellular Ca^{2+} over a period of 6 h in MDA-MB-231 cells (Xu & Purtzki, 2010b). Intracellular Ca^{2+} overload is a known upstream mediator of the mitochondrial apoptotic pathway (Orrenius *et al.*, 2003). Therefore, the elevated concentration of intracellular Ca^{2+} might be an important mediator in TPEN-induced apoptosis in MDA-MB-231 cells.

1.4 Calcium Signalling and Apoptosis

Perturbation of Ca^{2+} homeostasis appears to be a common mediator of apoptosis induced by various agents such as UV-irradiation, reactive oxygen species, and cytotoxic drugs (e.g. doxorubicin, cadmium; Hajnoczky *et al.*, 2003). Intracellular Ca^{2+} overload or elevated cytosolic Ca^{2+} functions as a crucial mediator of apoptosis by triggering the mitochondrial apoptotic pathway (Hajnoczky *et al.*, 2006; Orrenius *et al.*, 2003).

1.4.1 Intracellular Ca^{2+} homeostasis

In physiological conditions, cytosolic Ca^{2+} concentration is maintained at a very low level (~100 nM) compared to extracellular fluid (~1 mM; Hajnoczky *et al.*, 2003). Ca^{2+} levels in the mitochondria are similar to that in the cytosol (~100 nM), whereas in the ER, Ca^{2+} is maintained at a higher level (~1 mM), as it serves as the intracellular calcium storage site. However, upon stimulation (i.e. oxidative stress or certain drugs) or under certain pathological conditions (i.e. ischemia), cytosolic Ca^{2+} level can be elevated, reaching $\geq 1\mu\text{M}$ (Hajnoczky *et al.*, 2006). Such cytosolic Ca^{2+} overload serves as an important signal in initiating apoptotic events. This increase in cytosolic Ca^{2+} can be due

to increased Ca^{2+} entry from the extracellular spaces, increased release from the internal ER stores (Hajnoczky *et al.*, 2003; Orrenius *et al.*, 2003), or a combination of both.

Ca^{2+} entry from the extracellular space is primarily mediated through activation of plasma membrane calcium channels, such as receptor-operated channels (ROC), voltage-operated channels (VOC) and store-operated channels (SOC; Orrenius *et al.*, 2003; Figure 1.5). Ca^{2+} release from the internal ER store is either through phospholipase C (PLC) – coupled inositol trisphosphate receptors (InsP_3R) or through ryanodine receptors (RyR; Berridge *et al.*, 2000; Figure 1.5). Ca^{2+} release through InsP_3R is initiated when PLC cleaves phosphatidylinositol bisphosphate (PtdInsP_2) into diacyl glycerol (DAG) and inositol trisphosphate (InsP_3) at the plasma membrane (Berridge, 1993). InsP_3 then diffuses into the cytosol and binds to InsP_3R on the ER to allow Ca^{2+} to be released. Ca^{2+} can also be released directly from the ER through RyR (Orrenius *et al.*, 2003).

Mitochondria serve as intracellular Ca^{2+} buffers. Therefore, a rise in the cytosolic Ca^{2+} can lead to an increased influx of Ca^{2+} into the mitochondria. Influx of Ca^{2+} into the mitochondria is primarily mediated through the mitochondrial uniporter (UP; Hajnoczky *et al.*, 2006; Orrenius *et al.*, 2003), which has been identified as a Ca^{2+} selective ion channel (Kirichok *et al.*, 2004; Figure 1.5). However, a prolonged elevation of cytosolic Ca^{2+} can lead to mitochondrial Ca^{2+} overload. Mitochondrial Ca^{2+} overload in turn triggers the opening of a large mitochondrial conductance channel, known as the PTP. The opening of the mitochondrial PTP serves as an efflux route for the mitochondrial Ca^{2+} during Ca^{2+} overload, resulting in the release of Ca^{2+} back into the cytosol

(Hajnóczky *et al.*, 2006; Orrenius *et al.*, 2003). The mitochondrial PTP is located at the contact site between the inner and the outer mitochondrial membrane (Crompton, 1999). The exact composition of the PTP remains to be defined, but it is believed to be a large protein complex comprised of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocater (ANT) and the cyclophilin D (Halestrap *et al.*, 1998). Moreover, mitochondrial PTP opening induced by mitochondrial matrix Ca^{2+} is through interaction with the cyclophilin D (Basso *et al.* 2005).

1.4.2 Ca^{2+} -mediated mitochondrial apoptotic pathway

Mitochondrial Ca^{2+} overload triggers the opening of the PTP releasing Ca^{2+} back into the cytosol. This opening of the PTP reduces the mitochondrial membrane potential (Green & Kroemer, 2004; Orrenius *et al.*, 2003). PTP traverses both the inner and outer mitochondrial membrane. Therefore, mitochondrial PTP opening allows water and solutes (as small as ≤ 1.5 KD) to enter the mitochondrial matrix (Bernardi, 1999; Pfeiffer *et al.*, 2001), which results in swelling of the mitochondrial matrix. Mitochondrial matrix swelling subsequently leads to the rupture or permeabilization of the mitochondrial outer membrane. As a result, cytochrome c is released from the mitochondrial intra-membranous space into the cytosol (Green & Kroemer, 2004; Orrenius *et al.*, 2003). Cytochrome c release into the cytosol activates the initiator caspase-9, which in turn activates the executioner caspase-3. Caspase-3 then catalyzes the activation of a specific subset of substrates including DNase, ultimately resulting in DNA fragmentation and apoptotic cell death (Orrenius *et al.*, 2003; Saraste & Pulkki, 2000; Figure 1.6). Therefore,

Ca^{2+} is an important mediator in triggering the mitochondria dependent-apoptotic pathway.

1.5 Summary

In summary, breast cancer remains a major health threat to Canadian women. Cancer cells are characterized by uncontrolled cell proliferation and impaired apoptosis, also known as programmed cell death (Saikumar *et al.*, 1999). Therefore, induction of apoptosis has been used as a strategy in cancer treatment (Kasibhatla & Tseng, 2003; Wong, 2011). Zinc is considered a regulator of apoptosis, however the mechanisms involved remains to be elucidated (Truong-Tran *et al.*, 2001). Zinc depletion achieved through feeding a zinc deficient diet to animals, culturing cells in low-zinc medium, or using zinc chelators (i.e. membrane permeable intracellular zinc chelator TPEN), induces apoptosis in animals and in numerous types of cells (Clegg *et al.*, 2005; Sunderman, 1995), including human breast cancer MDA-MB-231 cells (Wu, 2003; Xu & Purtzki, 2010a). We have consistently shown that TPEN-induced zinc depletion promotes apoptosis in MDA-MB-231 cells (Wu, 2003; Xu & Purtzki, 2010a). This zinc depletion-induced apoptosis is accompanied by a sustained elevation in cellular Ca^{2+} content (Xu & Purtzki, 2010b). Ca^{2+} is a known mediator of the mitochondrial apoptotic pathway (Orrenius *et al.*, 2003). Thus, it is possible that Ca^{2+} plays an important role in mediating the zinc depletion-induced apoptosis in MDA-MB-231 cells.

1.6 Hypothesis

The hypothesis for my thesis research was that zinc depletion-induced apoptosis is mediated by Ca^{2+} through the mitochondrial-apoptotic pathway in human breast cancer MDA-MB-231 cells (Figure 1.7).

1.7 Overall Objective and Specific Aims

The overall objective of my thesis research project was to investigate the role of intracellular Ca^{2+} and its involvement of the mitochondria in zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells.

The specific aims were:

- 1) To assess the effect of intracellular Ca^{2+} chelation on zinc depletion-induced apoptosis, as indicated by casapse-9 and -3 activity and DNA fragmentation, in MDA-MB-231 cells.
- 2) To assess the effect of intracellular Ca^{2+} chelation on zinc depletion-induced mitochondrial dysfunctions, as indicated by mitochondrial membrane potential and cytosolic cytochrome c, in MDA-MB-231 cells.

Table 1.1: Examples of breast cancer cell line

Cell line	Cancer type	Tumor origin	Estrogen receptor (ER)	Tumorigenic in mice	Reference
BT-20	Invasive ductal carcinoma	Primary	–	Yes	Lasfargues & Ozzello, 1958
BT-474	Invasive ductal carcinoma	Primary	+	Yes	Lasfargues <i>et al.</i> , 1978
MCF-7	Invasive ductal carcinoma	Metastasis (pleural effusion)	+	Yes (with estrogen)	Soule, 1973
MDA-MB-231	Invasive ductal carcinoma	Metastasis (pleural effusion)	–	Yes	Cailleau <i>et al.</i> , 1974
MDA-MB-330	Invasive lobular carcinoma	Metastasis (pleural effusion)	–	No	Cailleau <i>et al.</i> , 1978
SK-BR-3	Invasive ductal carcinoma	Metastasis (pleural effusion)	–	No	Engel & Young, 1978
T-47D	Invasive ductal carcinoma	Metastasis (pleural effusion)	+	Yes (with estrogen)	Keydar <i>et al.</i> , 1979

Table 1.2: Zinc content of selected foods (Groff & Gropper, 2000)

Food	Zinc (mg/100g)
Oysters	17-91
Crabmeat	3.8-4.3
Shrimp	1.1
Liver	3.1-3.9
Beef, ground	3.9-4.1
Pork	1.6-2.1
Chicken	1.0-2.0
Cheese	2.8-3.2
Eggs	1.1
Bread (whole wheat)	1.0
Bread (white)	0.6-0.8
Vegetables	0.1-0.7
Fruits	<0.1

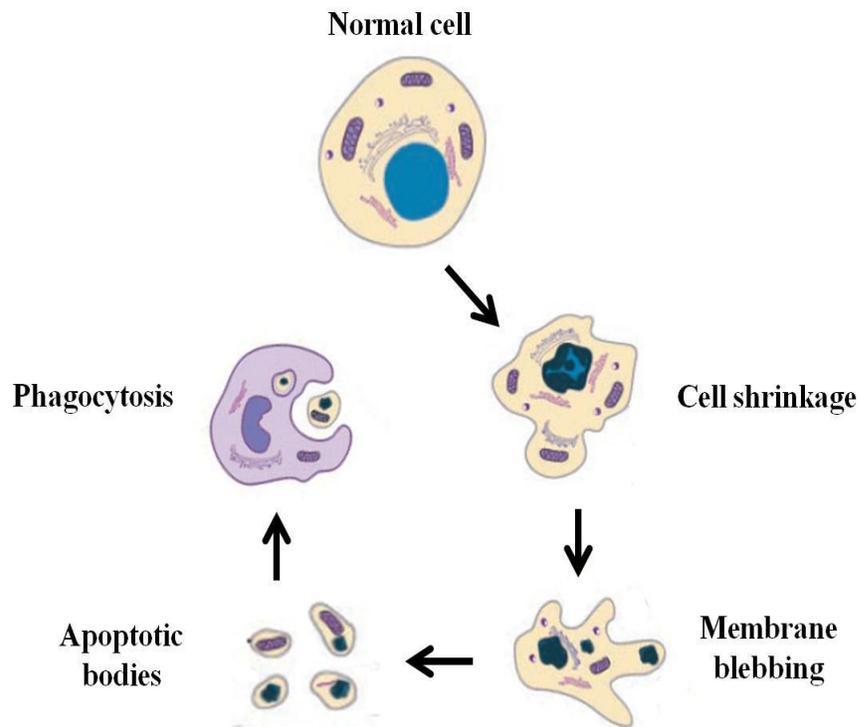


Figure 1.1: Morphological features of apoptosis. Apoptosis includes shrinkage of cells, membrane blebbing, and the formation of apoptotic bodies. Apoptotic bodies are then phagocytosed without triggering inflammation. (Modified from Saikumar *et al.*, 1999)

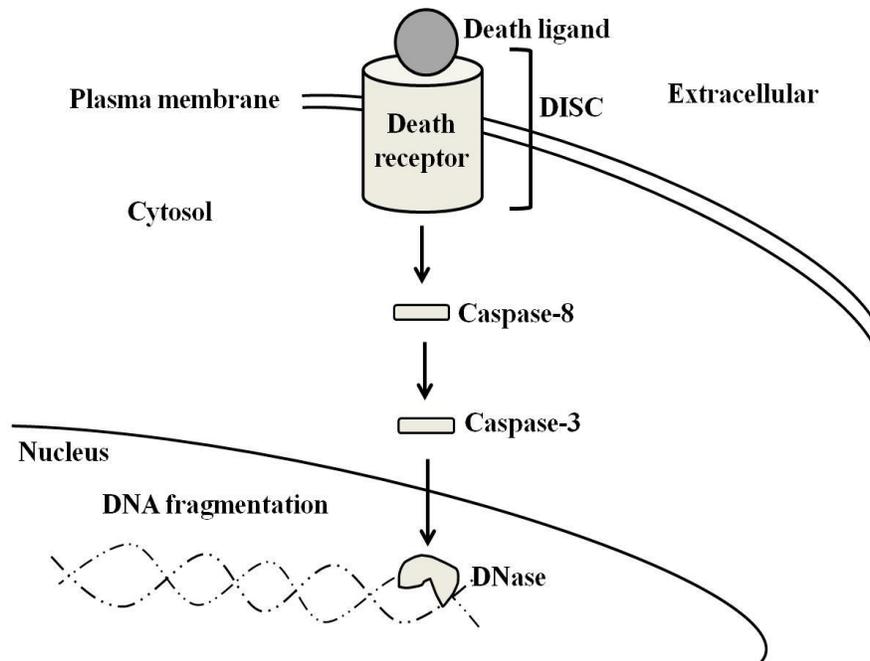


Figure 1.2: The receptor-apoptotic pathway. Activation of the receptor-apoptotic pathway is triggered by binding of the extracellular death ligand to the death receptor on the plasma membrane, thereby forming the death-inducing signaling complex (DISC). DISC activates caspase-8 and subsequently caspase-3, ultimately leading to DNA fragmentation and apoptotic cell death (Saraste & Pulkki, 2000; Sartorius *et al.*, 2001).

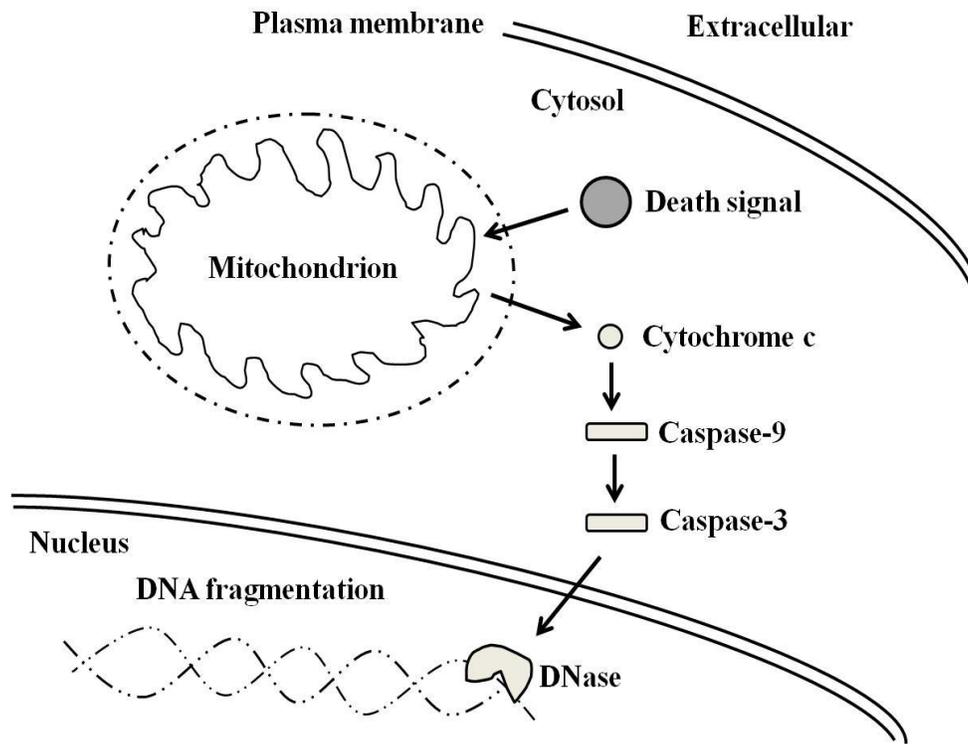


Figure 1.3: The mitochondrial-apoptotic pathway. Activation of the mitochondrial-apoptotic pathway is triggered by the death signal that causes mitochondrial outer membrane permeabilization. As a result, cytochrome c is released from the mitochondria into the cytosol. Cytochrome c activates caspase-9 and subsequently caspase-3, ultimately leading to DNA fragmentation and apoptotic cell death (Loeffler & Kroemer, 2000; Saraste & Pulkki, 2000).

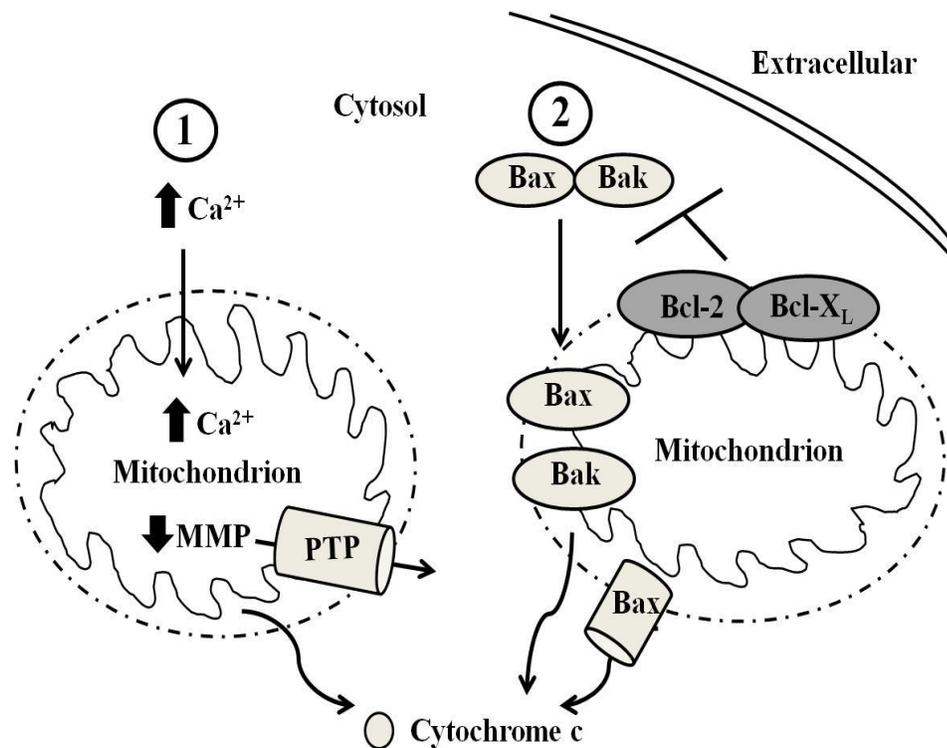


Figure 1.4: Mechanisms causing mitochondrial outer membrane permeabilization. (1) The first mechanism is mediated by an overload of intracellular Ca^{2+} , which induces the opening of the mitochondrial permeability transition pore (PTP). Opening of the PTP reduces the mitochondrial membrane potential (MMP), leading to mitochondrial outer membrane permeabilization and subsequent cytochrome c release. (2) The second mechanism is mediated by the pro-apoptotic Bcl-2 proteins (Bax and Bak). Under normal conditions, Bax and Bak are located in the cytosol and are inhibited by the anti-apoptotic Bcl-2 and Bcl- X_L. Apoptotic stimuli trigger the translocation and incorporation of Bax and Bak into the mitochondrial outer membrane, leading to mitochondrial outer membrane permeabilization and subsequent cytochrome c release (Gross *et al.*, 1999; Orrenius *et al.*, 2003).

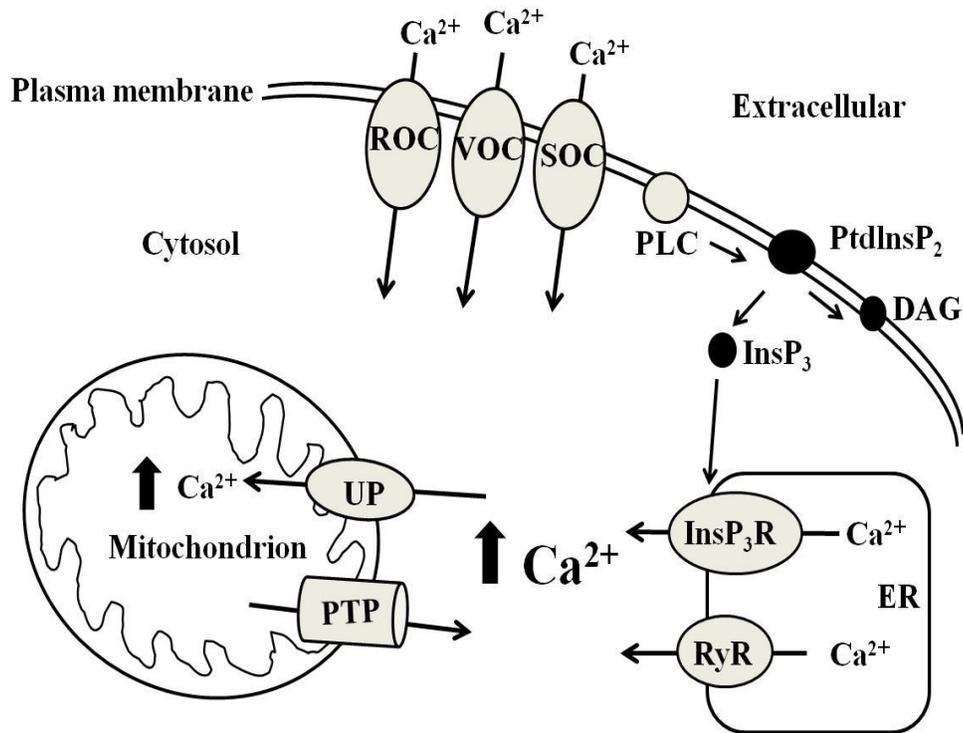


Figure 1.5: Intracellular Ca²⁺ homeostasis. An elevated cytosolic Ca²⁺ level can result from Ca²⁺ entry from the extracellular spaces through the plasma membrane Ca²⁺ channels such as receptor-operated channel (ROC), voltage-operated channel (VOC), or store-operated channel (SOC). Ca²⁺ can also be released from the internal ER stores via the phospholipase C (PLC)-coupled inositol trisphosphate receptors (InsP₃R) or ryanodine receptors (RyR). This increase in cytosolic Ca²⁺ level triggers Ca²⁺ to be taken up by the mitochondria through the uniporter (UP). Mitochondrial Ca²⁺ overload then triggers the opening of the permeability transition pore (PTP), allowing Ca²⁺ to be released back into the cytosol (Orrenius *et al.*, 2003).

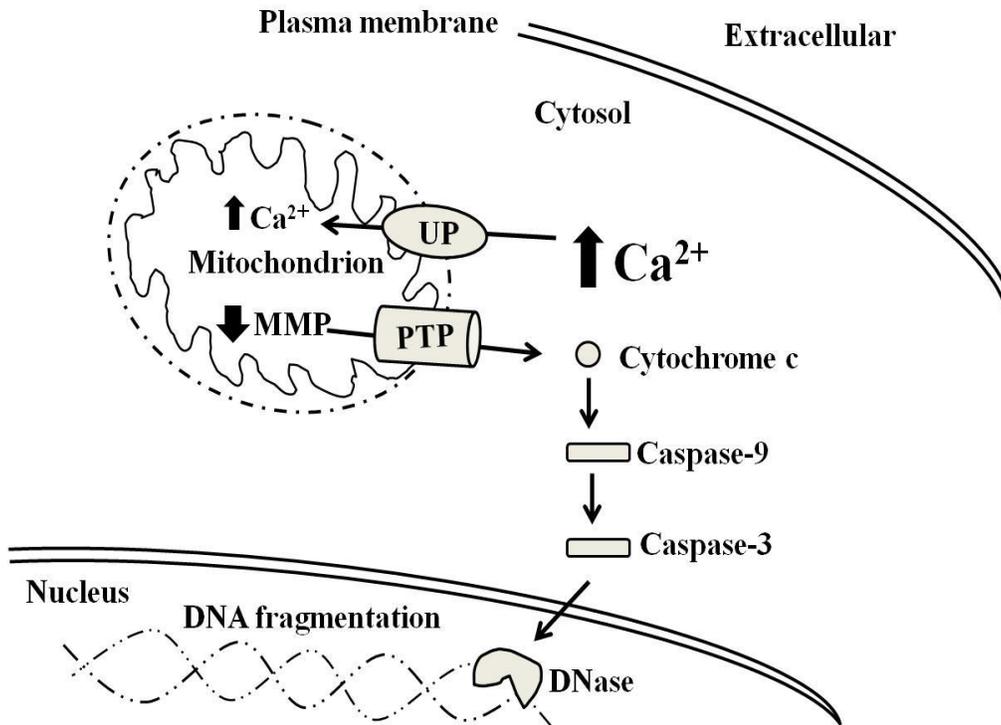


Figure 1.6: Ca²⁺-mediated mitochondrial apoptotic pathway. The opening of mitochondrial permeability transition pore (PTP) triggered by the cytosolic Ca²⁺ influx leads to the loss of mitochondrial membrane potential (MMP). As a result, cytochrome c is released from the mitochondria into the cytosol. Cytochrome c activates caspase-9 and subsequently caspase-3, ultimately leading to DNA fragmentation and apoptotic cell death (Orrenius *et al.*, 2003; Saraste & Pulkki, 2000).

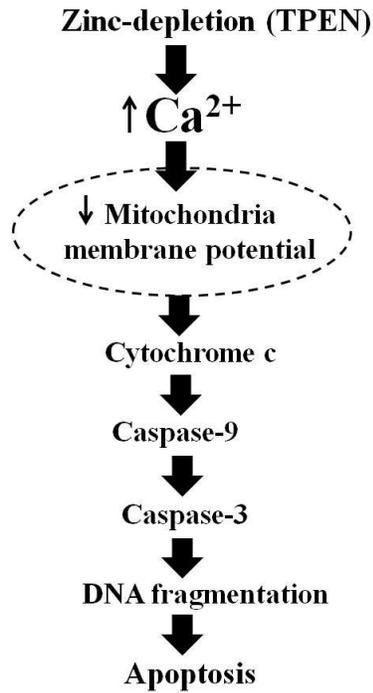


Figure 1.7: Hypothetical model of zinc depletion-induced apoptosis in MDA-MB-231. Zinc depletion induced by TPEN results in an elevated intracellular Ca^{2+} level, which triggers mitochondrial dysfunctions including the loss of mitochondrial membrane potential (MMP) and release of cytochrome c. Cytochrome c activates caspase-9 and subsequently caspase-3, ultimately resulting in DNA fragmentation and apoptotic cell death.

Chapter 2. Zinc Depletion Induced Apoptosis Through Ca²⁺-Dependent Mitochondrial Apoptotic Pathway in Human Breast Cancer MDA-MB-231 Cells

2.1 Introduction

Despite the development of earlier diagnoses and aggressive therapeutics, breast cancer remains the most common cancer and the second leading cause of death among Canadian women (Canadian Cancer Society, 2012). Therefore, the need for the development of novel therapeutic agents against breast cancer remains an important goal.

Apoptosis plays a critical role in the elimination of potentially malignant cells; therefore, defective or inhibited apoptosis is a major causative factor in the development and progression of cancer (Fadeel *et al.*, 1999b; Saikumar *et al.*, 1999). Bcl-2 is a family of apoptotic regulatory proteins consists of both pro- (Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-X_L) proteins. The ratio between the pro- and anti-apoptotic proteins determines whether the cell undergoes apoptosis, in which a higher pro/anti-apoptotic proteins ratio would favor apoptosis (Gross *et al.*, 1999). The pro-/anti-apoptotic protein ratio (Bax: Bcl-2 ratio) tends to be lower in cancerous cells than in non-cancerous cells (Kasibhatla & Tseng, 2003; Krajewski *et al.*, 1999). Furthermore, the activity of caspases, a family of proteases that plays a pivotal role in the regulation and execution of apoptosis, is also reduced in cancer cells (Lavrik *et al.*, 2005; Wong, 2011). A lower pro-/anti-apoptotic protein ratio and reduced caspase activity prevents cancer cells from undergoing apoptosis, thus promoting cancer cell survival (Gross *et al.*, 1999; Lavrik *et al.*, 2005). Inevitably, both restoration of the defective apoptotic pathway and induction

of apoptosis have been used as strategies in cancer treatment to kill malignant cells (Kasibhatla & Tseng, 2003; Wong, 2011).

Zinc is considered an apoptotic regulator (Truong-Tran *et al.*, 2001). Cellular zinc depletion, induced by culturing cells in low zinc medium or using cell membrane permeable zinc chelators such as TPEN, has been shown to induce apoptotic cell death in a variety of cell lines such as rat and human thymocytes (Clegg *et al.*, 2005; Sunderman, 1995). However, little is known about the effects of zinc depletion in breast cancer. We have previously observed that TPEN induces apoptotic cell death in human breast cancer MDA-MB-231 cells (Wu, 2003; Xu & Purtzki, 2010a). Interestingly, this TPEN-induced apoptosis is accompanied by a sustained elevation of intracellular Ca^{2+} (Xu & Purtzki, 2010b). An elevated intracellular Ca^{2+} can mediate apoptosis via the mitochondrial apoptotic pathway (Orrenius *et al.*, 2003). During cytosolic Ca^{2+} overload, mitochondria play a major role in buffering the intracellular Ca^{2+} . However, a prolonged elevation of cytosolic Ca^{2+} leads to mitochondrial Ca^{2+} overload, which triggers the opening of a large mitochondrial conductance channel known as the PTP (Crompton, 1999; Hajnoczky *et al.*, 2006). The opening of the PTP allows mitochondrial Ca^{2+} to be released back into the cytosol, which is accompanied by a decrease in mitochondrial membrane potential. As a result, cytochrome c is released from the mitochondrial intra-membrane space into the cytosol where it activates caspase-9 and subsequently caspase-3. Once caspase-3 is activated, the cell is committed to apoptosis (Green & Kroemer, 2004). However, we do not know whether Ca^{2+} mediates the zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells.

I hypothesized that zinc depletion-induced apoptosis is mediated by Ca^{2+} through the mitochondrial-apoptotic pathway in human breast cancer MDA-MB-231 cells. The overall objective of this study was to investigate the role of intracellular Ca^{2+} and its involvement of the mitochondria in zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells. The specific aims for this study were 1) to assess the effect of intracellular Ca^{2+} chelation on zinc depletion-induced apoptosis, as indicated by caspase-9 and -3 activity and DNA fragmentation, in MDA-MB-231 cells, and 2) to assess the effect of intracellular Ca^{2+} chelation on zinc depletion-induced mitochondrial dysfunctions, as indicated by mitochondrial membrane potential and cytosolic cytochrome c, in MDA-MB-231 cells.

2.2 Materials and Methods

2.2.1 Cell culture system

MDA-MB-231, a metastatic human breast cancer cell line (American Type Culture Collection; Manassas, Virginia), was routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, New York), d-glucose (4,500 mg/L), L-glutamine (2 mM), sodium pyruvate (110 mg/L), sodium bicarbonate (1,500 mg/L) and penicillin/streptomycin (50,000 Units/L penicillin and 50,000 $\mu\text{g/L}$ streptomycin) at 37°C, 5% CO_2 . Throughout the rest of the thesis, this medium is referred to as the "growth medium." To start an experiment, the cells were grown in the growth medium in a 6-well plate, a 96-well plate, or a 10 cm culture dish for three days or until 80 – 90 % confluence prior to TPEN treatment.

2.2.2 Induction of zinc depletion

The stock solution (20 mM) of an intracellular zinc chelator, TPEN (Catalogue No. 4413; Sigma, St. Louis, MO), was dissolved in DMSO (Shumaker *et al.*, 1998). The working solution was prepared by diluting the stock solution with DMSO. To deplete intracellular zinc, the TPEN working solution was added to the cell culture (0.5 % v/v) with a final concentration of 20 μ M TPEN. Equal volume of DMSO was added to the cell culture as the control.

2.2.3 Zinc replenishment

A zinc stock solution (50 mM) was prepared by dissolving ZnSO₄ (Catalogue No. Z-0251; Sigma, St. Louis, MO) in ddH₂O. The working solution was prepared by diluting the stock solution with ddH₂O. To replenish zinc, the zinc working solution was added to cell culture (0.5 % v/v) with a final concentration of 5, 10, 20 or 40 μ M. An equal volume of ddH₂O was added to the cell culture as the control.

2.2.4 Cytosolic Ca²⁺ chelation

The stock solution (20 mM) of a cytosolic Ca²⁺ chelator, BAPTA-AM (Catalogue No. A-1076; Sigma, St. Louis, MO), was dissolved in DMSO (Strayer *et al.*, 1999). The working solution was prepared by diluting the stock solution with DMSO. To chelate the cytosolic Ca²⁺, the BAPTA-AM working solution was added to the cell culture (0.5 % v/v) with a final concentration of 10 or 20 μ M. DMSO alone was added to the cell culture at the same volume as the control.

2.2.5 Caspase-9 and -3 activities

Caspase-9 and -3 were both used as indicators for apoptosis. Caspase-9 and -3 activities were assessed using fluorogenic substrate Ac-LEHD-AFC (Catalogue No. 218756; Calbiochem, Darmstadt, Germany) and Ac-DEVD-AFC (Catalogue No. 264151; Calbiochem, Darmstadt, Germany), respectively as described (Kohler *et al.*, 2002). MDA-MB-231 cells were cultured in the growth medium with an initial density of 1.0×10^5 cells/well in a 6-well plate for 3 days followed by treating the cells with TPEN to deplete intracellular zinc. There were two TPEN treatment regimens: 1) TPEN (20 μ M) alone for 0, 1, 2, 3, 4, 5 or 6 h and 2) TPEN plus 0, 10, or 20 μ M BAPTA-AM for 6 h. At the end of the treatment period, cells were harvested by rinsing once with warm PBS (37° C) followed by trypsinization with 250 μ l of warm 0.25 % Trypsin-EDTA. After the cells were detached, the trypsin was neutralized with an equal volume of the growth medium. The cell suspension was transferred to a 1.7 ml micro-centrifuge tube and centrifuged at 300 x g for 5 min at 4° C, followed by aspiration of the supernatant. The cell pellet was then resuspended in 500 μ l of cold PBS (4° C) and counted using a particle counter (Z1 Particle Counter, Beckman Coulter, Fullerton, CA) with a cutoff point set at 8 μ m. The cells were re-pelleted by centrifugation (300 x g; 5 min; 4° C). After aspirating the supernatant, the cell pellet was resuspended in a caspase extraction buffer (50 μ L/ 10^6 cells; 50 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, 1.6 mM CHAPS, 0.1 % triton-X-100, 5.0 mM DTT, and 1 % protease inhibitors (Catalogue No. P-8340; Sigma, St. Louis, MO); pH 7.4). The resuspended cells were placed on ice for 30 min and vortexed every 10 min to lyse the cells. The cell lysate was then centrifuged at 14,000 x g for 10 min at

4°C. The resultant supernatant was collected and either used fresh or stored at -80°C up to 2 days before analysis.

To determine the caspase activity, a reaction mixture containing the supernatant (50 µg of protein), 20 µM caspase-9 or -3 substrate and caspase reaction buffer (pH 7.4; 50 mM HEPES, 100 mM NaCl, 10 µM EDTA, 1.6 mM CHAPS, 10 % glycerol, and 10 mM DTT) was added at 100 µl/well in a 96-well plate. The plate was then placed in the microplate reader (SpectraMAX GEMINI XS, Molecular Devices, Sunnyvale, CA) that was pre-heated to 30°C. The fluorescence intensity was monitored using the kinetic mode over a period of 180 min with an interval of 10 min. The excitation and emission wavelengths were 400 nm and 505 nm, respectively. The caspase activity was expressed in relative fluorescent units (RFU). Protein concentration of the supernatant collected was determined using the DC protein kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

2.2.6 DNA fragmentation

DNA fragmentation was assessed by propidium iodide (PI; Catalogue No. 81845; Sigma, St. Louis, MO) -staining flow cytometry (Riccardi & Nicoletti, 2006). MDA-MB-231 cells were cultured in the growth medium for 3 days as described earlier with an initial density of 1.0×10^5 cells/well in a 6-well plate. At the end of the cultured period, cells were treated with TPEN (20 µM) alone for 0, 3, 6, 12, 24, 48 or 72 h, or TPEN plus 0, 10 or 20 µM BAPTA for 48 or 72 h. At the end of each TPEN treatment period, cells were harvested, counted and re-pelleted as described above. After aspirating the

supernatant, the cell pellet was fixed by re-suspension in 50 μ l cold PBS (4°C) containing 450 μ l of cold ethanol (-20°C; 70%, v/v) and left at -20°C overnight. After fixation, the cells were pelleted by centrifugation (400 g, 5 min, 4°C) followed by washing once with cold PBS (4°C) and re-pelleting by centrifugation (8,000 g, 5 min, 4°C). The cell pellet was then re-suspended in DNA staining solution (200 μ g/ml RNase A and 20 μ g/ml PI in PBS; pH 7.4) at 1 ml/ 10^6 cells. Finally, the cell suspension was transferred to a 5 ml Falcon tube and incubated in the dark at room temperature for 30 min. The intensity of PI-fluorescence was determined by flow cytometry (CellQuest, Becton-Dickinson, Franklin Lakes, NJ). The excitation and emission wavelengths were 488 nm and 610 nm, respectively. A total of 10,000 cells were counted per TPEN treatment regimen. The percentage of cells with fragmented DNA content or apoptosis was determined using FlowJo 8.7 analytical software (Tree Star, Inc., San Carlos, CA).

2.2.7 Intracellular Ca²⁺ content

Intracellular Ca²⁺ content was determined using Fura-2-AM (Catalogue No. F-1201; Sigma, St. Louis, MO), a cell membrane permeable fluorogenic Ca²⁺ indicator (Robinson *et al.*, 2004). MDA-MB-231 cells were cultured in the growth medium as described earlier with an initial density of 1.0×10^4 cells/well in a 96-well plate. At the end of the 3-day culture period, the growth medium was carefully aspirated and the cells were stained with Fura-2 in Fura-2 loading buffer (100 μ L/well). The Fura-2 loading buffer (pH 7.3) consisted of 8 μ M Fura-2, 5.9 mM KCl, 1.4 mM MgCl₂, 10 mM HEPES, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 140 mM NaCl, 11.5 mM glucose, 1.8 mM CaCl₂ and 3 mg/ml bovine serum albumin (BSA). The cells were stained in the dark at 37°C for 30

min followed by rinsing the cells once with warm PBS (37°C). After washing, the cells were treated with TPEN in a physiological salt solution (200 µL/well). The cells were treated with either TPEN (20 µM) alone for 0, 1, 2, 3, 4, 5 or 6 h, or TPEN plus 0, 10, or 20 µM BAPTA-AM for 6 h. The physiological salt solution (pH 7.3) consisted of 5.9 mM KCl, 1.4 mM MgCl₂, 10 mM HEPES, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 140 mM NaCl, 11.5 mM glucose, 1.8 mM CaCl₂ and 3.6 µM zinc (ZnSO₄). The intensity of the fluorescence was determined at an alternative excitation wavelengths 340 or 380 nm, and the emission wavelength 510 nm using a fluorescence spectrofluorometer (SpectraMAX GEMINI XS, Molecular Devices, Sunnyvale, CA). Binding of Fura-2 to Ca²⁺ ions increases the fluorescence intensity when it is excited at 340 nm and decreases the fluorescence intensity when it is excited at 380 nm (Robinson *et al.*, 2004). Thus, intracellular Ca²⁺ level was expressed as 340/380 nm ratio allowing a more accurate measure of intracellular Ca²⁺. The fluorescence intensity from the samples was subtracted from the reagent blank, which contained cells without Fura-2 staining.

2.2.8 Mitochondrial membrane potential

MMP was determined using 3, 3'-dihexyloxacarbo-cyanine iodide (DiOC₆; Catalogue No. D-2730; Invitrogen, Carlsbad, CA), a cell permeable, positively charged fluorescent dye that selectively accumulates in the mitochondria due to the negatively charged mitochondrial inner membrane (Hertveldt *et al.*, 1997). Briefly, MDA-MB-231 cells were cultured in the growth medium as described earlier with an initial seeding density of 1.0 x 10⁵ cells/well in a 6-well plate. At the end of 3-day culture period, cells were treated with either TPEN (20 µM) alone for 0, 1, 2, 3, 6, 12, 24 or 48 h, or TPEN

plus 0, 10, or 20 μM BAPTA-AM for 24 h. At the end of the each treatment period, cells were harvested, counted and re-pelleted as described above. After aspirating the supernatant, the cells were suspended in PBS (pH 7.4, $1 \text{ mL}/10^6$ cells) containing 80 nM DiOC₆. The cell suspension was transferred to a 5 ml Falcon tube and incubated in the dark at 37°C for 20 min then at room temperature for 10 min. The intensity of DiOC₆-dependent fluorescence was determined by flow cytometry (CellQuest, Becton-Dickinson, Franklin Lakes, NJ). The excitation and emission wavelengths were 488 nm and 501 nm, respectively. A total of 25,000 cells were counted per TPEN treatment regimen. The percentage of cells with normal and reduced MMP was determined using FlowJo 8.7 analytical software (Tree Star, Inc., San Carlos, CA).

2.2.9 Mitochondrial cytochrome c release

Cytochrome c release from the mitochondria was assessed by measuring the abundance of cytosolic cytochrome c using Western blotting (Dave *et al.*, 2008).

The cytosolic fraction was prepared using a cytosol/mitochondria fractionation kit (Catalogue No. K256-25; BioVision, Mountain View, CA) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were cultured in the growth medium for 3 days as described earlier with an initial density of 1.0×10^6 cells/10 cm culture dish. At the end of the culture period, cells were treated with TPEN (20 μM) with or without BAPTA-AM (10 μM) for 24 h. At the end of the TPEN- treatment period, cells were harvested and counted as described above. The cells were re-pelleted by centrifugation (600 x g; 5 min; 4°C). After aspirating the supernatant, the cell pellet was

resuspended in the cytosol extraction buffer containing 0.1% DTT and 0.2 % protease inhibitor at 1 ml/5x10⁷ cells and incubated on ice for 10 min. The cells were lysed and sheared by passing the cell suspension through a 1 mL syringe that was fitted with a 27 ¼ gauge needle (40 passages). The cell lysate was then centrifuged at 700 x g for 10 min at 4°C. The resultant supernatant, containing both the cytosolic and mitochondrial fractions, was transferred to a 1.7 ml microcentrifuge tube whereas the cell pellet, containing the nuclear fractions and unbroken cells, was discarded. The supernatant was further centrifuged at 10,000 x g for 30 min at 4°C. The resultant supernatant, which contained the cytosolic fraction of the cell was collected and stored at -80°C until analysis. Protein concentration of the cytosolic fraction was determined using a DC protein kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

The abundance of cytochrome c in the cytosol was determined by Western blot. Briefly, cytosolic protein (100 µg/lane) was separated on a SDS-polyacrylamide electrophoresis gel at a constant voltage (175 V) for 60 min for both stacking gel (5% acrylamide) and separating gel (12% acrylamide, 1.5mm) and then electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA) at a constant voltage (25 V) for 50 min followed by increasing the voltage to 50 V for another 50 min in the transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). After the transfer, the membrane was incubated in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.05 % Tween 20) containing 2.5% skim milk powder for 75 min. The membrane was then incubated with polyclonal rabbit anti-human cytochrome c IgG (Sc-7159; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) in TTBS containing 2.5% skim milk powder at 4°C

overnight. After overnight incubation, the membrane was washed with TTBS 3 times with 10 min/wash. The membrane was then incubated with horseradish peroxidase-conjugated bovine anti-rabbit IgG (Sc-2370; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 dilution) in TTBS buffer containing 1% skim milk powder for 60 min. The membrane was subsequently washed with TTBS three times (10 min/wash). The presence of cytochrome c protein was visualized using enhanced chemiluminescence (ECL) kit (Catalogue No. 32106; Thermo Scientific, Rockford, IL, USA) by following the manufacturer's instructions. Briefly, detection reagent containing the chemiluminescent substrate was added onto the membrane and incubated for 1 min at room temperature. The membrane was then exposed using ChemiDOC™ MP (Bio-Rad, Hercules, CA) for 180 second. All blocking, incubation with the 2nd IgG, washing, and detection procedures were performed at room temperature. The intensity of the band was quantified with Image™ Lab 4.0 software (Bio-Rad, Hercules, CA) and α -tubulin was used as the loading control. The abundance of α -tubulin protein was assessed using the monoclonal mouse anti-human α -tubulin IgG2a (Sc-5286; Santa Cruz Biotechnology, Santa Cruz, CA; 1:300 dilution) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG2a (Sc-2061; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 dilution) as the secondary antibody using the same blocking, incubation, washing, and detection procedures described above. The intensity of the cytochrome c band was normalized on the intensity of the corresponding α -tubulin band.

2.2.10 Statistical analysis

The data were analyzed by one-way ANOVA for treatment effects followed by Tukey's honest test for the significant differences among the means (GraphPad Prism, version 5; San Diego, CA; $p < 0.05$).

2.3 Results

2.3.1 Zinc prevented TPEN-induced apoptosis

TPEN-induced apoptosis was assessed by caspase-9 and -3 activity, and DNA fragmentation.

TPEN increased caspase-9 and -3 activity. TPEN exposure for up to 2 h had no effect on caspase-9 activity in MDA-MB-231 cells (Fig. 2.1A). Caspase-9 activity was increased by 3 fold after 3 h of TPEN exposure compared to control (0 h of TPEN exposure; $p < 0.05$). This TPEN-induced increase in caspase-9 activity was continued for another 3 h ($p < 0.05$) reaching 7 fold increase after 6 h of TPEN exposure compared to control (0 h of TEPN exposure; $p < 0.05$). Similarly, caspase-3 activity was increased 3 fold after 3 h of TPEN exposure and continued for another 3 h reaching a 10 fold increase after 6 h of TPEN exposure compared to control (0 h of TPEN exposure; $p < 0.05$; Fig. 2.1B).

Zinc reduced TPEN-induced increase in caspase-3 activity. Caspase-3 activity was increased by 3 fold after 3 h of TPEN exposure in MDA-MB-231 cells compared to control (DMSO only; Fig. 2.2A; $p < 0.05$). When the cells were treated with TPEN + 5

μM zinc, caspase-3 activity was reduced by 40 % compared to the cells treated with TPEN alone ($p < 0.05$). In the presence of 10 μM zinc, caspase-3 activity was reduced by 67 and 44% compared to cells treated with TPEN alone and cells treated with TPEN + 5 μM zinc, respectively ($p < 0.05$). The activity of caspase-3 in cells treated with TPEN + 10 μM zinc was the same as in the control. A further increase in zinc to 20 and 40 μM had no effect on caspase-3 activity, compared to cells treated with TPEN + 10 μM zinc.

Zinc suppressed TPEN-induced DNA fragmentation. Cells with fragmented DNA were not detected in cells treated with TPEN for up to 6 h (Fig. 2.3B). Cells with fragmented DNA were detected (1%) after 6 h of TPEN exposure. The proportion of cells with fragmented DNA was increased to (2%) after 12 h of TPEN exposure, reaching 6% after 24 h of TPEN treatment. Thereafter, the proportion of cells with fragmented DNA rose steeply, reaching 46% after 72 h of TPEN exposure. However, in the presence of zinc (10 - 40 μM), the proportion of cells with fragmented DNA was reduced by 99% after 72 h of TPEN exposure (Fig. 2.4).

2.3.2 TPEN increased intracellular Ca^{2+} content

Intracellular Ca^{2+} remained unchanged for up to 2 h of TPEN exposure in MDA-MB-231 cells compared to control (DMSO only; Fig. 2.5). After 3 h of TPEN exposure, intracellular Ca^{2+} content was significantly elevated compared to control (13%; $p < 0.05$) and remained elevated for another 3 h.

2.3.3 Ca²⁺ chelation with BAPTA-AM reduced intracellular Ca²⁺ content and suppressed TPEN-induced caspase-9 and -3 activity and DNA fragmentation

Exposing MDA-MB-231 cells to TPEN alone for 6 h resulted in a 340/380 ratio (intracellular Ca²⁺) of 5.3 (Fig. 2.6). Addition of BAPTA-AM at 10 and 20 μM reduced the intracellular Ca²⁺ content by 20 and 40%, respectively, compared to cells treated with TPEN alone ($p < 0.05$; Fig. 2.6). Furthermore, addition of BAPTA-AM at 10 and 20 μM also suppressed caspase-9 activity by 39 and 66 %, respectively, compared to cells treated with TPEN alone ($p < 0.05$; Fig. 2.7A). Similarly, addition of BAPTA-AM at 10 and 20 μM also suppressed caspase-3 activity by 57 and 78%, respectively, compared to cells treated with TPEN alone ($p < 0.05$; Fig. 2.7B). Addition of BAPTA-AM at 10 and 20 μM also reduced the proportion of cells with fragmented DNA by 31 and 58%, respectively, compared to cells treated with TPEN alone for 48 h ($p < 0.05$; Fig. 2.8A) and by 10 and 43 %, respectively, compared to cells treated with TPEN alone for 72 h ($p < 0.05$; Fig. 2.8B).

2.3.4 Chelation of intracellular Ca²⁺ with BAPTA-AM protected cells from TPEN-induced mitochondrial membrane potential loss

In the absence of TPEN (0 h), MMP was maintained in 87% of the MDA-MB-231 cells (Fig. 2.9). The proportion of cells with normal MMP remained unchanged by exposing the cells to TPEN alone for up to 2 h (Fig. 2.9B). However, the proportion of cells with normal MMP was reduced to 77% after exposing the cells to TPEN for 3 h. This TPEN-induced decrease in the proportion of cells with normal MMP was accelerated reaching 65% after exposing the cells to TPEN for 6 h. The decrease

continued, but at a slower rate, for 18 more hours reaching 44% after exposing the cells to TPEN for 24 h. Interestingly, the proportion of cells with normal MMP increased to 88%, which is similar to the level in the control, after 48 h of TPEN exposure.

The proportion of cells with normal MMP was not affected by BAPTA-AM alone at 10 and 20 μM compared to the control (DMSO only; Fig. 2.10). Exposing the cells to TPEN alone reduced the proportion of cells with normal MMP by 62% compared to control. Treatment with TPEN plus BAPTA-AM at 10 and 20 μM increased the proportion of cells with normal MMP by 0.94 and 1.15 fold compared to TPEN alone, respectively, indicating that BAPTA-AM protected the cells from TPEN-induced loss of MMP (Fig. 2.10).

2.3.5 Chelation of intracellular Ca^{2+} with BAPTA-AM suppressed TPEN-induced mitochondrial cytochrome c release

Cytochrome c was not detected in the cytosolic fraction of untreated (DMSO only) and BAPTA-AM-treated MDA-MB-231 cells (Fig. 2.11A). Treatment with TPEN alone for 24 h resulted in the presence of cytochrome c in the cytosolic fraction, indicating the release of cytochrome c from the mitochondria into the cytosol. However, co-treatment of TPEN with BAPTA-AM suppressed the abundance of cytochrome c in the cytosolic fraction, indicating that BAPTA-AM suppressed TPEN-induced cytochrome c release (Fig. 2.11B).

2.4 Discussion

2.4.1 Zinc depletion with TPEN induced apoptosis.

In the current study, TPEN treatment resulted in a time-dependent increase in the activity of caspase-9 and -3, and the proportion of cells with fragmented DNA in MDA-MB-231 cells, indicating increased apoptosis in these cells. This TPEN-induced apoptosis is consistent with previous observations. Zalewski *et al.* (1993) showed that treating lymphocytes with TPEN at 5 – 20 μM overnight induces a dose-dependent apoptosis. Similarly, TPEN has also been shown to induce apoptosis in a variety of cell types, including human monocytic leukemia THP-1 cells (Cao *et al.*, 2001), primary sheep (Truong-Tran *et al.*, 2000b) and human (Carter *et al.*, 2002) airway epithelium cells, and human malignant airway epithelium A549 and NCI-H292 cells (Truong-Tran *et al.*, 2000b). Consistent with the results reported in this study, Xu and Purtzki (2010a) previously demonstrated a sustained increase in the activity of caspase-9 and -3 for a period of 12 h in MDA-MB-21 cells treated with TPEN (20 μM).

TPEN is a membrane permeable intracellular chelator with a very high affinity for zinc and a much weaker affinity for other metals such as Ca^{2+} and Mg^{2+} (Shumaker *et al.*, 1998). For this reason, TPEN is considered an intracellular Zn^{2+} chelator. To affirm the TPEN-induced apoptosis was a consequence of TPEN-induced depletion of intracellular zinc, MDA-MB-231 cells were treated with TPEN in the presence of equal concentration of zinc. In the presence of zinc, both TPEN-induced increase in caspase-3 activity and increase in the proportion of cells with fragmented DNA were suppressed. Similar effects of zinc replenishment (2 – 100 μM) on TPEN-induced apoptosis have been

reported previously in rat and human thymocytes, neuroblastoma-glioma cells, and human retinal pigment epithelium (Adler *et al.*, 1999; Hyun *et al.*, 2001; McCabe *et al.*, 1993). It is noteworthy that zinc replenishment suppressed TPEN-induced caspase-3 activation down to the baseline after 3 h of treatment, which further indicated that zinc depletion triggers caspase-3 activation during the early phase of apoptosis. Similar findings have been reported in other cell types. For example, in human peripheral blood T lymphocytes, caspase-9 and -3 activations are observed just after 2 h exposure with TPEN (15 μ M), whereas DNA fragmentation is evident after 4 h of TPEN-treatment (Kolenko *et al.*, 2001). Furthermore, exposure of human retinal pigment epithelium to TPEN (2 μ M) induces caspase-3 activation after 24 h and DNA fragmentation after 48 h, while addition of equimolar zinc completely reverses the TPEN-induced apoptotic events (Hyun *et al.*, 2001). Thus, it is apparent that TPEN induced a depletion of intracellular zinc, which in turn induced apoptosis, in MDA-MB-231 cells.

The mechanisms whereby zinc protects cells from TPEN-induced apoptosis are unknown. It could be explained by the potential inhibitory effect of zinc on caspase-3 (Perry *et al.*, 1997; Thambiayya *et al.*, 2012). A proportion of caspase-3 normally exists in the active form but is held in check by intracellular inhibitors such as zinc (Chimienti *et al.*, 2001; Fanzo *et al.*, 2002; Perry *et al.*, 1997). Therefore, the absence of zinc would release the inhibition and trigger the broad activation of caspase-3. Perry *et al.* (1997) first reported that zinc functions as a potent inhibitor to caspase-3. They showed that zinc at mid-nM to low- μ M range inhibits the activity of purified recombinant caspase-3. In contrast, when an apoptotic cell extract is used, a higher concentration of zinc is required

to achieve the same level of inhibition of caspase-3 activity observed using purified recombinant caspase-3. It is important to point out that this zinc-mediated inhibition of caspase-3 is observed in a 'purified' experimental system. Its applicability to inhibit caspase-3 in the whole-cell systems or at the whole-body level remains to be demonstrated. In fact, zinc exhibits no inhibitory effect on caspase-3 activity when an *E. coli* lysate containing recombinant caspase-3 is used (Takahashi *et al.*, 1996). Furthermore, the concentrations of zinc required to inhibit caspase-3 activity demonstrated in the Perry's study (1997) are quite high compared to the abundance of the LIPZ, which is in the range of pM- to fM. Therefore, caution must be exercised regarding the function of zinc as a caspase-3 inhibitor in the whole-cell systems and at the whole-body level. Further investigation is warranted.

Apoptosis can be induced by the mitochondrial apoptotic pathway or by the receptor apoptotic pathway (Loeffler & Kroemer, 2000; Sartorius *et al.*, 2001). One of the characteristics of the mitochondrial apoptotic pathway is that caspase-3 is activated by caspase-9 via the mitochondria (Loeffler & Kroemer, 2000). In contrast, in the receptor apoptotic pathway, caspase-3 is activated by caspase-8 via the receptor on the plasma membrane (Sartorius *et al.*, 2001). The observation that both caspase-9 and -3 activities were increased in the current study suggested the involvement of the mitochondrial apoptotic pathway in TPEN-induced apoptosis in MDA-MB-231 cells.

2.4.2 Zinc depletion induced apoptosis through the Ca²⁺-mediated mitochondrial apoptotic pathway.

One of the triggers of the mitochondrial apoptotic pathway is an elevated cytosolic Ca²⁺ level (Orrenius *et al.*, 2003). In this study, we have observed that TPEN treatment resulted in a sustained increase in the level of intracellular Ca²⁺ in MDA-MB-231 cells. Importantly, diminishing the elevated intracellular Ca²⁺ level with BAPTA-AM, an intracellular Ca²⁺ chelator, suppressed TPEN-induced increase in caspase-9 and 3 activity and DNA fragmentation, suggesting that Ca²⁺ served as a mediator in zinc depletion-induced apoptosis in MDA-MB-231 cells. Such perturbation of intracellular Ca²⁺ homeostasis is involved in apoptosis induced by a wide variety of stimuli, including UV-irradiation, reactive oxygen species or cytotoxic drugs (e.g. doxorubicin, cadmium) (Hajnoczky *et al.*, 2003).

It is well established that reduced MMP is a critical cellular event in Ca²⁺ mediated apoptosis through the mitochondrial apoptotic pathway (Hajnoczky *et al.*, 2006; Orrenius *et al.*, 2003). In this study, exposing the cells to TPEN alone reduced the proportion of cells with normal MMP, indicating that TPEN reduced the MMP in these cells. In contrast, exposing the cells to TPEN in the presence of 10 µM of BAPTA-AM increased the proportion of cells with normal MMP by 94% compared to cells exposed to TPEN alone. Increase the concentration of BAPTA-AM from 10 µM to 20 µM further increased the proportion of cells with normal MMP by 11% compared to cells exposed to TPEN + 10 µM BAPTA-AM. BAPTA-AM is an intracellular Ca²⁺ chelator. Ca²⁺ is a known mediator of the mitochondrial apoptotic pathway (Orrenius *et al.*, 2003). In the

events where cytosolic Ca^{2+} is overloaded, the mitochondria serve as buffers of the increased Ca^{2+} . Prolonged cytosolic Ca^{2+} overload can result in mitochondrial Ca^{2+} overload. Mitochondria Ca^{2+} overload then triggers the opening of the PTP releasing Ca^{2+} back into the cytosol (Crompton, 1999; Hajnoczky *et al.*, 2006). This opening of the PTP reduces the MMP due to mitochondrial matrix swelling (Green and Kroemer 2004; Orrenius *et al.*, 2003). Thus, BAPTA-AM concentration-dependent reversal of MMP confirmed that the elevated intracellular Ca^{2+} was an upstream trigger of the mitochondrial apoptotic pathway. Similar findings have been reported previously. For instance, exposing human peripheral blood T lymphocytes to TPEN (15 μM) results in the release of cytochrome c followed by caspase-9 and -3 activation and DNA fragmentation (Kolenko *et al.*, 2001). Cytochrome c released subsequent to mitochondrial outer membrane permeabilization is considered as the key event in the mitochondrial apoptotic pathway as it in turn activates the downstream caspases. However, the trigger to mitochondrial outer membrane permeabilization in Kolenko's study (2001) is unclear. Mitochondrial outer membrane permeabilization can be triggered by two main mechanisms: 1) the opening of PTP induced by Ca^{2+} as discussed earlier and 2) the increase expression of pro-apoptotic Bcl-2 proteins (Bax; Gross *et al.*, 1999; Orrenius *et al.*, 2003). A more recent study suggested that the pro-apoptotic Bax is responsible for triggering mitochondrial outer membrane permeabilization, which is followed by cytochrome c release, caspase activation and DNA fragmentation in mouse osteoblastic MC3T3-E1 cells exposed to TPEN (5 μM ; Guo *et al.*, 2012). However, Xu and Purtzki (2010a) showed that the abundance of Bax protein is not affected by TPEN (20 μM) in MDA-MB-231 cells. It is likely that the pro-apoptotic Bax is not responsible for

mitochondrial outer membrane permeabilization in MDA-MB-231 cells exposed to TPEN. In the current study, exposing the cells with TPEN in the presence of 10 μM BAPTA-AM suppressed TPEN-induced cytochrome c release, indicating that Ca^{2+} was the trigger to mitochondrial outer membrane permeabilization. Therefore, TPEN-induced apoptosis in MDA-MB-231 cells was through the Ca^{2+} -mediated mitochondrial apoptotic pathway.

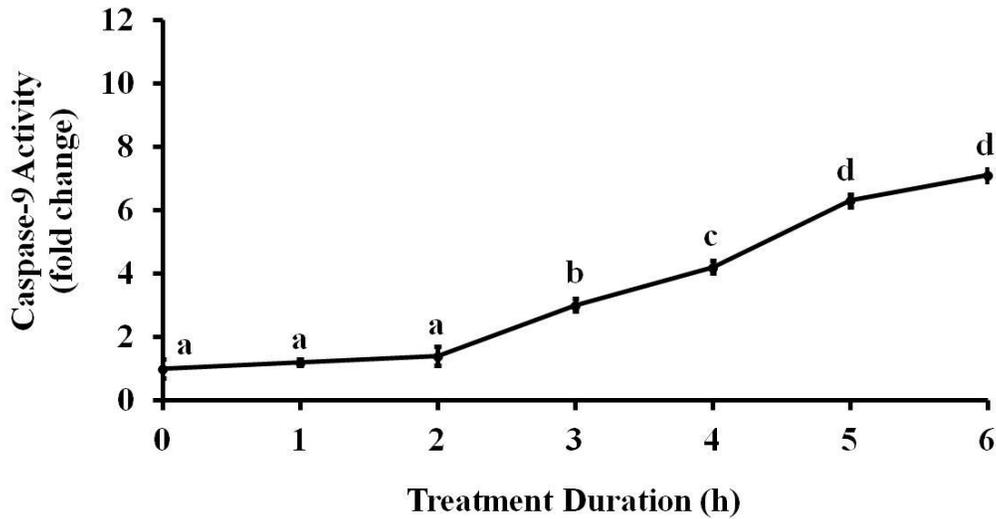
The proportion of cells with normal MMP was reduced after up to 24 h of TPEN exposure; however, it was raised back to the control level after 48 h of TPEN exposure. This apparent inconsistency was due to the disappearing of the proportion of cells with reduced MMP as these cells have already gone through apoptotic cell death. This notion is supported by the observation that a drastic increase of DNA fragmentation was apparent after 48 h of TPEN exposure compared to after 24 of TPEN exposure.

2.4.3 Summary

In summary, in MDA-MB-231 cells, cellular zinc depletion by TPEN induced a sustained intracellular Ca^{2+} elevation. The elevated intracellular Ca^{2+} triggered the loss of MMP, followed by the release of mitochondrial cytochrome c into the cytosol. Cytochrome c in the cytosol then activated caspase-9, an initiator caspase, which subsequently activated caspase-3, an executioner caspase. Caspase-3 then activated DNase, which ultimately resulted in DNA fragmentation and apoptotic cell death (Fig. 2.12A). Therefore, zinc depletion induced apoptosis through the Ca^{2+} -mediated mitochondrial apoptotic pathway in human breast cancer MDA-MB-231 cells.

Furthermore, it is believed that this intracellular Ca^{2+} entered the mitochondria and triggered the subsequent apoptotic cascade during the first 2 h of TPEN exposure. This assumption was indicated by a rise in intracellular Ca^{2+} (due to release of buffer Ca^{2+} from the mitochondria), loss of MMP and activation of caspase-9 and -3 all observed after 2 h of TPEN-treatment. The loss of MMP continued to augment until 24 h of TPEN exposure. The activation of caspase-9 and -3 continued until 6 h of TPEN-exposure in the current study but is indicated by the previous study to continue activation until 24 h of TPEN-treatment (Xu & Purtzki, 2010a). Cytochrome c release was observed after 24 h of TPEN exposure, but the exact time the release was initiated remained to be elucidated. DNA fragmentation occurred greatly after 24 h of TPEN exposure, indicating that the cells were entering the cell death phase (Fig. 2.12B).

(A)



(B)

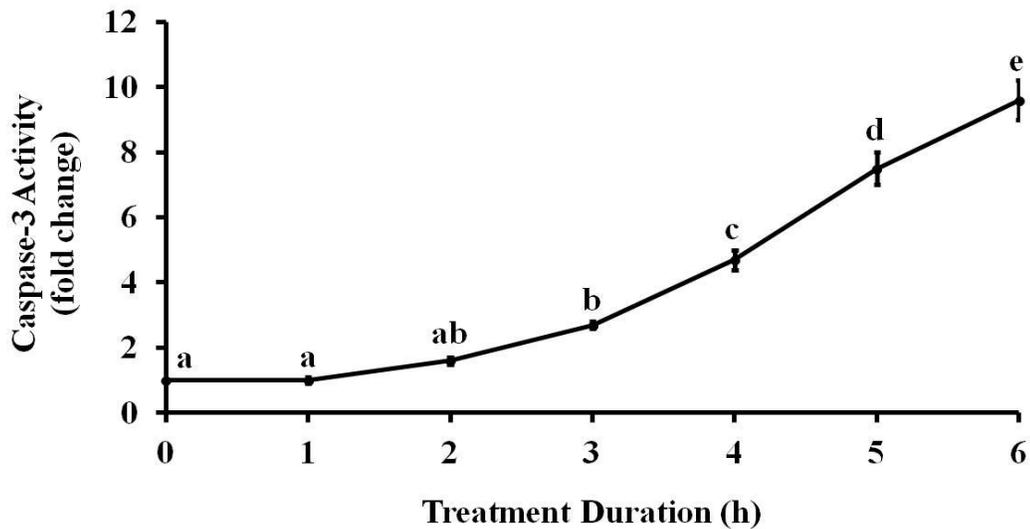
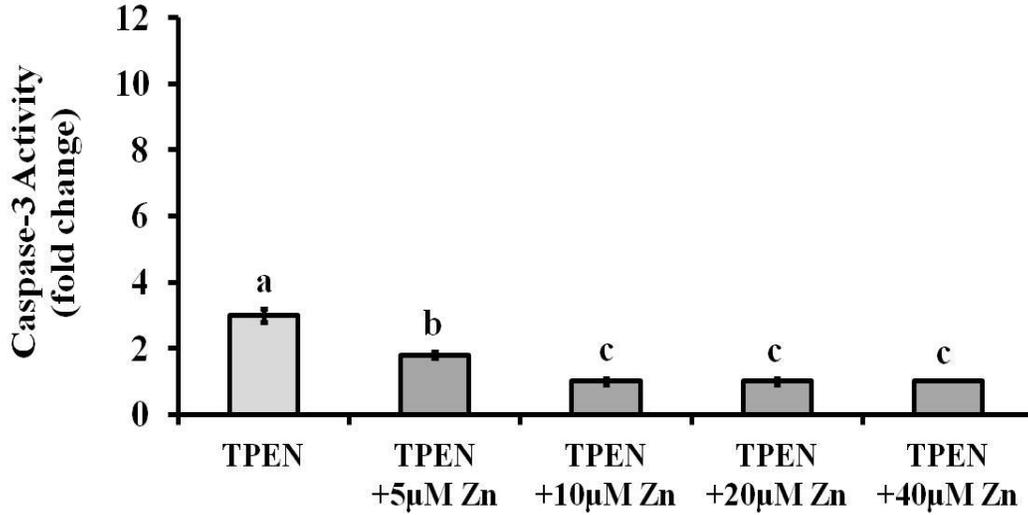


Figure 2.1: TPEN increased caspase-9 and -3 activity in MDA-MB-231 cells. Cells were treated with TPEN (20 μ M) for 0, 1, 2, 3, 4, 5 or 6 h. The activity of caspase-9 (A) and -3 (B) were determined by fluorogenic caspase substrate using a fluorescence microplate reader. Fold change was calculated by the treatment values divided by the control value (0 h). Values represent mean \pm SEM ($n = 3$; the experiment was repeated independently once). Means with different letters are significantly different ($p < 0.05$).

(A)



(B)

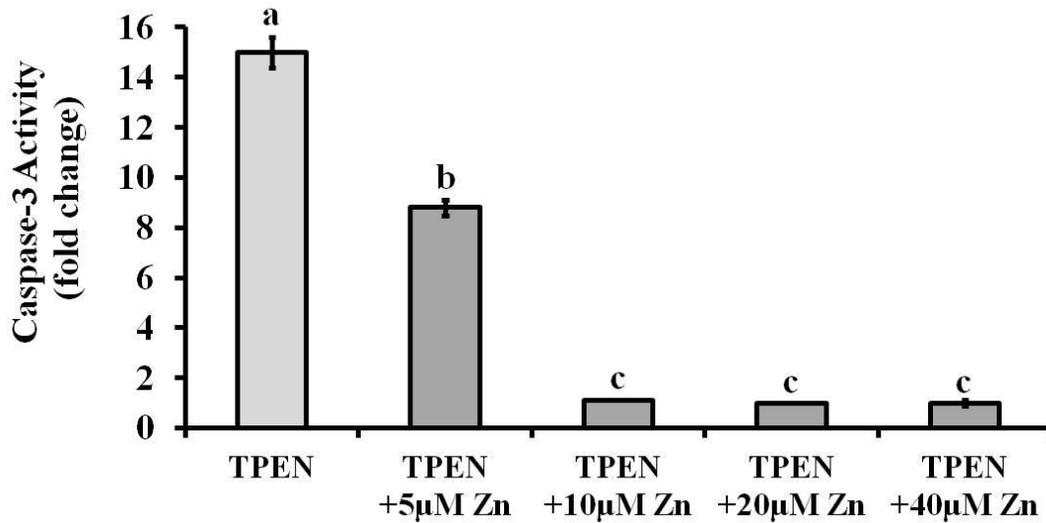
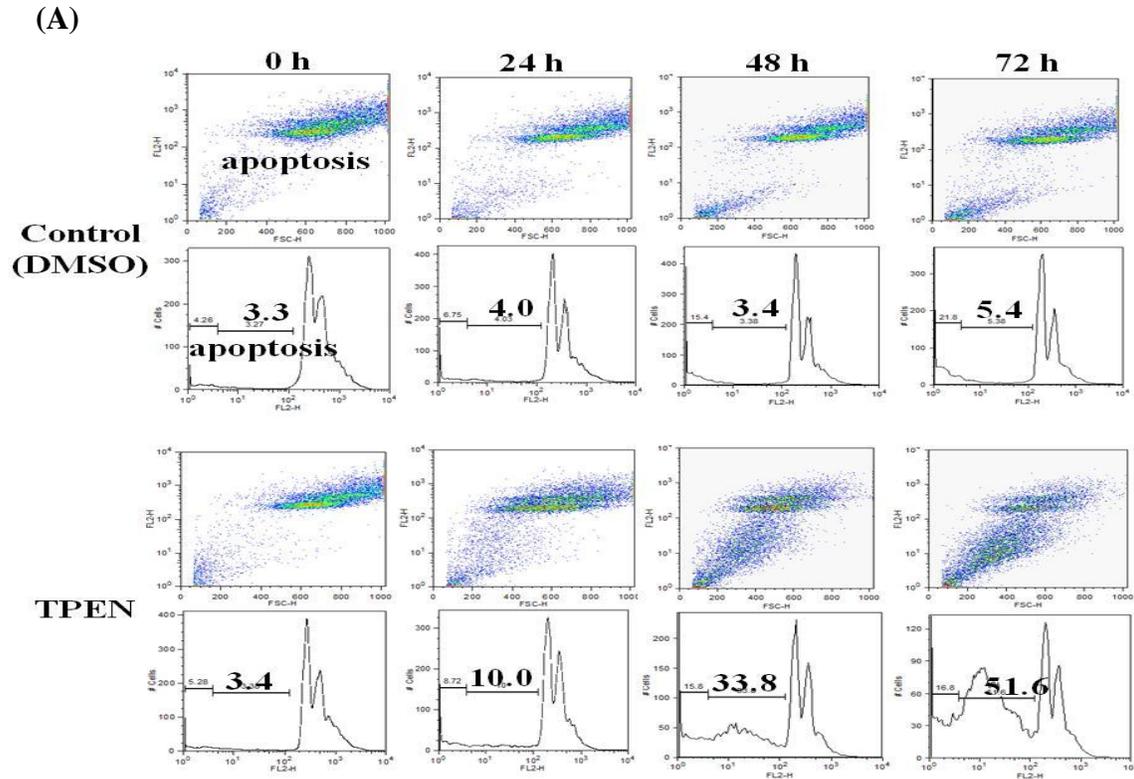


Figure 2.2: Zinc replenishment suppressed TPEN-induced increase in caspase-3 activity in MDA-MB-231 cells. Cells were treated with TPEN (20 μ M) plus zinc (0, 5, 10, 20 or 40 μ M) for 3 (A) or 6 (B) h. Cells treated with DMSO only served as the control. Caspase-3 activity was determined by fluorogenic caspase substrate using a fluorescence microplate reader. Fold change was calculated by the TPEN treatment values divided by the control value. Values are mean \pm SEM ($n = 3$; the experiment was repeated independently once). Means with different letters are significantly different ($p < 0.05$).



(B)

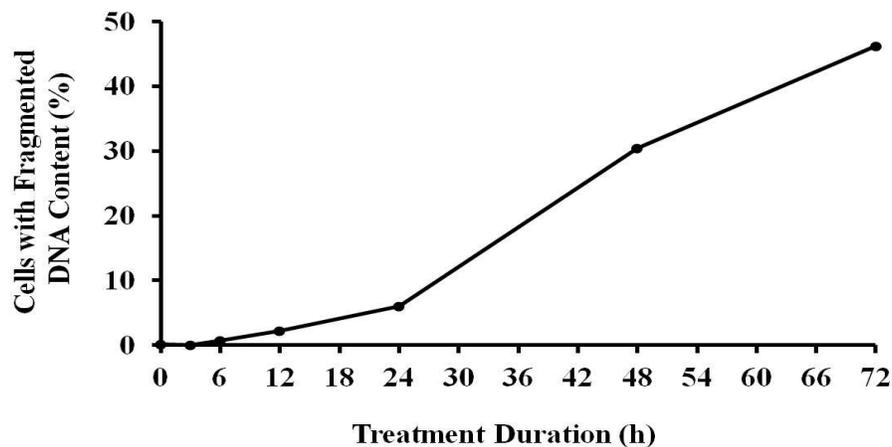
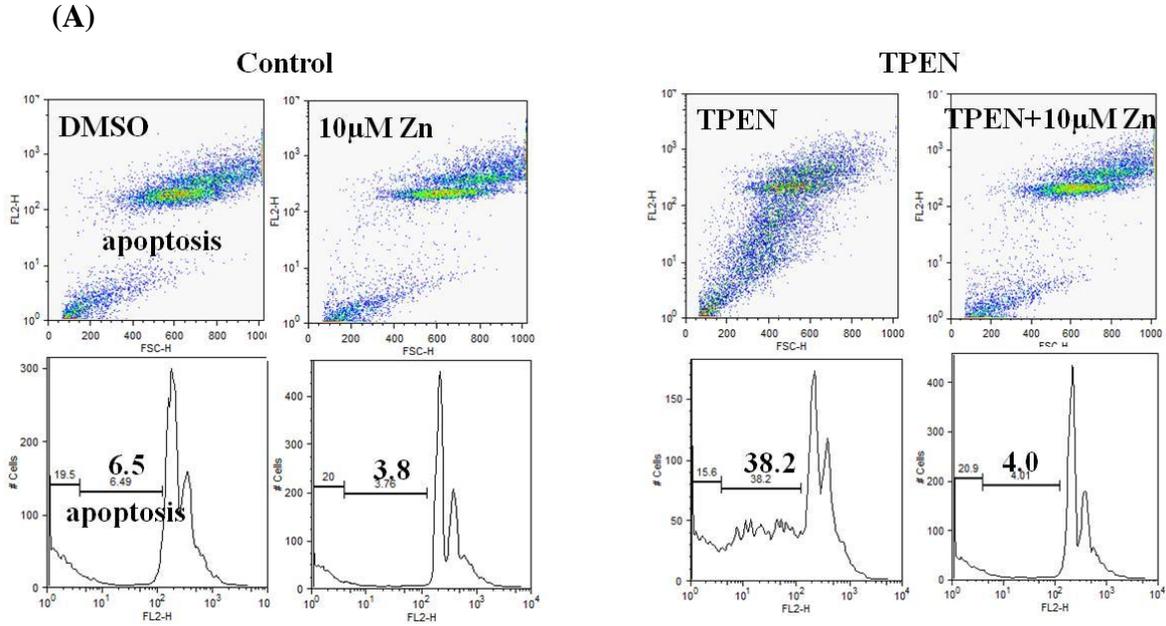


Figure 2.3: TPEN induced DNA fragmentation in MDA-MB-231 cells. Cells were treated with TPEN (20 μ M) for 0, 3, 6, 12, 24, 48 and 72 h. Cells treated with DMSO only served as the corresponding controls. DNA content was analyzed by PI -staining flow cytometry. (A) Representative of flow cytometry data. Dot plots (upper panel) and histograms (lower panel) of PI-labeled cells. In the histograms, the area to the left of the first peak represents cells with fragmented DNA (apoptosis). (B) Scatter plot of flow cytometry data. Values were calculated by the TPEN treatment values subtracted by the corresponding control values. The experiment was repeated independently once.



(B)

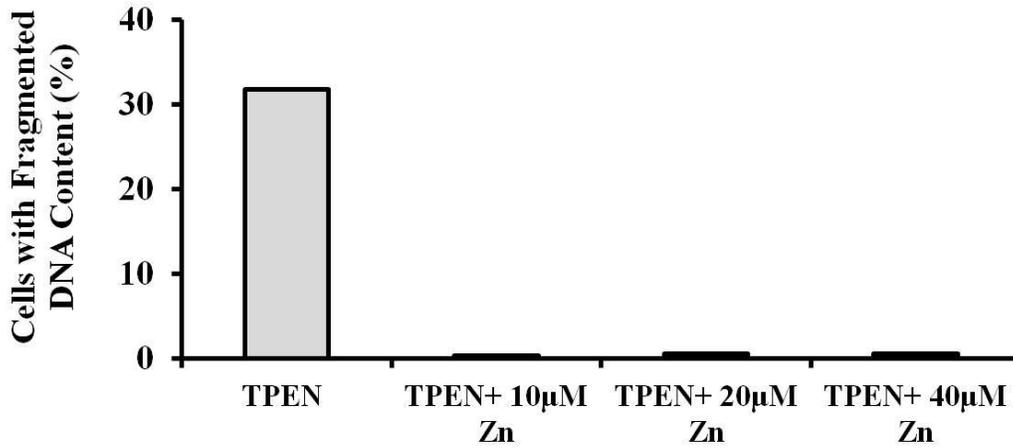


Figure 2.4: Zinc replenishment suppressed TPEN-induced DNA fragmentation in MDA-MB-231 cells. Cells were treated with TPEN (20 μM) plus zinc (0, 10, 20 or 40 μM) for 72 h. Cells treated with DMSO or zinc alone served as the corresponding controls. DNA content was analyzed by PI-staining flow cytometry. Flow cytometry data (A) are presented in bar graph (B). Values were calculated by the TPEN treatment values subtracted by the corresponding control values. The experiment was repeated independently once.

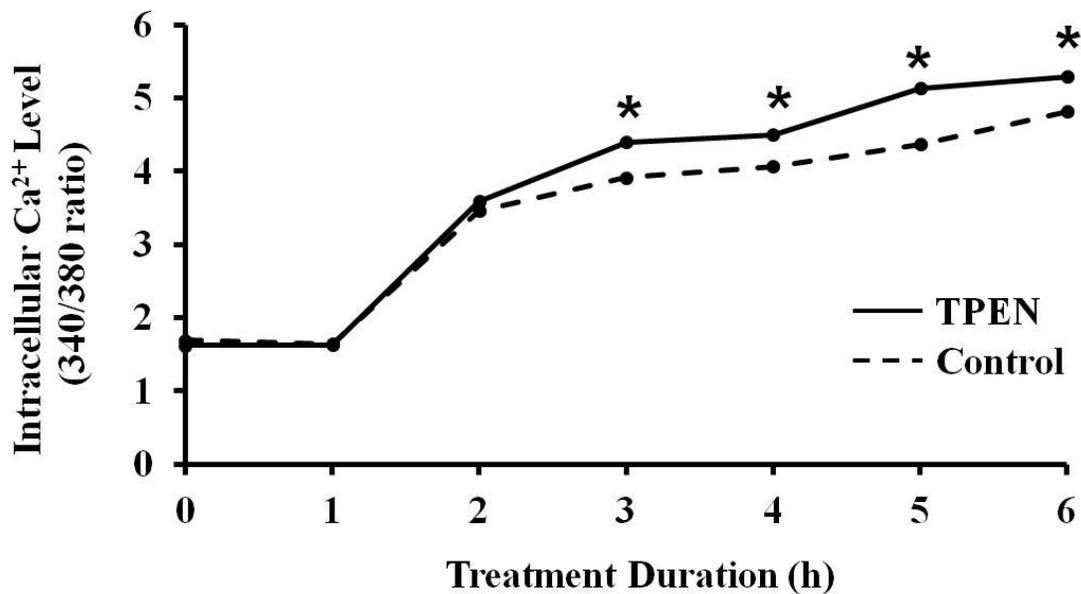


Figure 2.5: TPEN induced intracellular Ca^{2+} elevation in MDA-MB-231 cells. Cells were treated with DMSO (control) or TPEN (20 μM) for 0, 1, 2, 3, 4, 5 and 6 h. Intracellular Ca^{2+} was determined by the intensity of Fura-2-dependent fluorescence using fluorescence microplate reader. Values represent mean \pm SEM ($n = 6$; the experiment was repeated independently once). Asterisks indicate a significant difference between the control and TPEN-treated cells at each time point ($p < 0.05$).

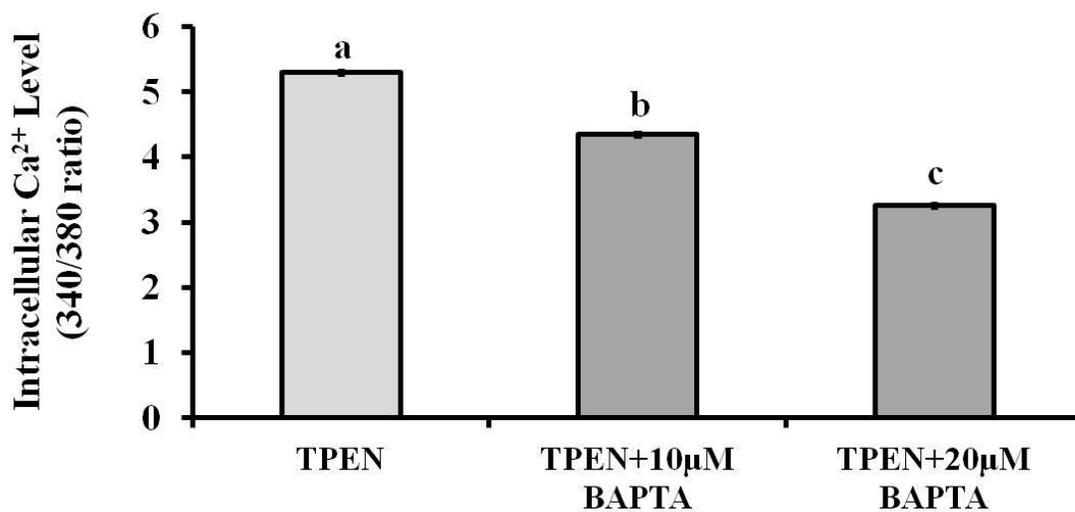


Figure 2.6: Intracellular Ca²⁺ chelation with BAPTA-AM suppressed TPEN-induced intracellular Ca²⁺ elevation in MDA-MB-231 cells. Cells were treated with TPEN (20 µM) plus BAPTA-AM (0, 10 or 20 µM) for 6 h. Intracellular Ca²⁺ was determined by the intensity of Fura-2-dependent fluorescence intensity using fluorescence microplate reader. Values are means ± SEM (n = 6; the experiment was repeated independently once). Means with different letters are significantly different (*p* < 0.05).

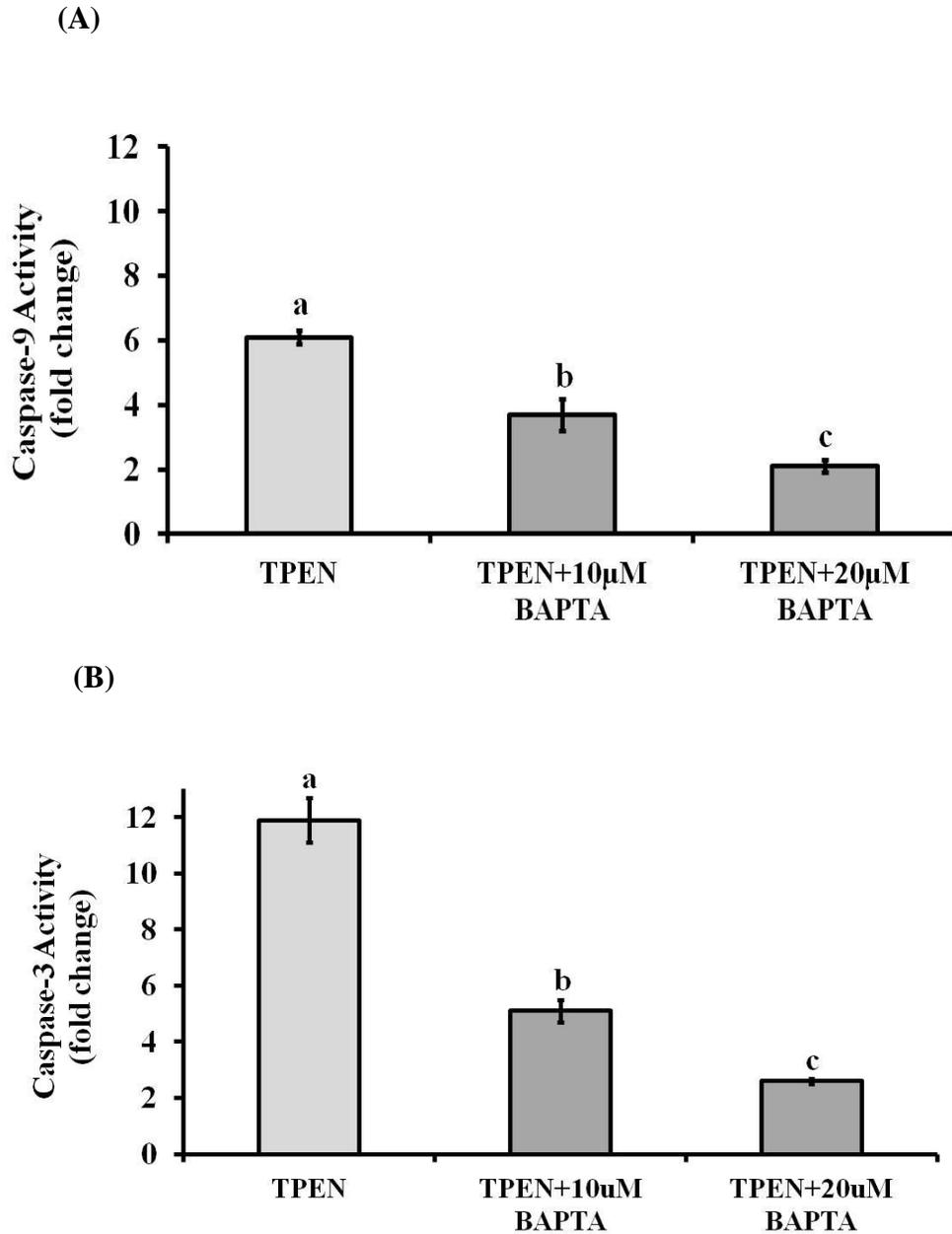


Figure 2.7: Intracellular Ca^{2+} chelation with BAPTA-AM suppressed TPEN-induced increase in caspase-9 and -3 activity in MDA-MB-231 cells. Cells were treated with TPEN (20 μM) plus BAPTA-AM (0, 10 or 20 μM) for 6 h. Cells treated with DMSO or BAPTA-AM alone served as the corresponding controls. The activity of caspase-9 (A) and -3 (B) was determined by fluorogenic caspase substrate using a fluorescence microplate reader. Fold change was calculated by the TPEN treatment values divided by the corresponding control values. Values are mean \pm SEM (n = 3; the experiment was repeated independently once). Means with different letters are significantly different ($p < 0.05$).

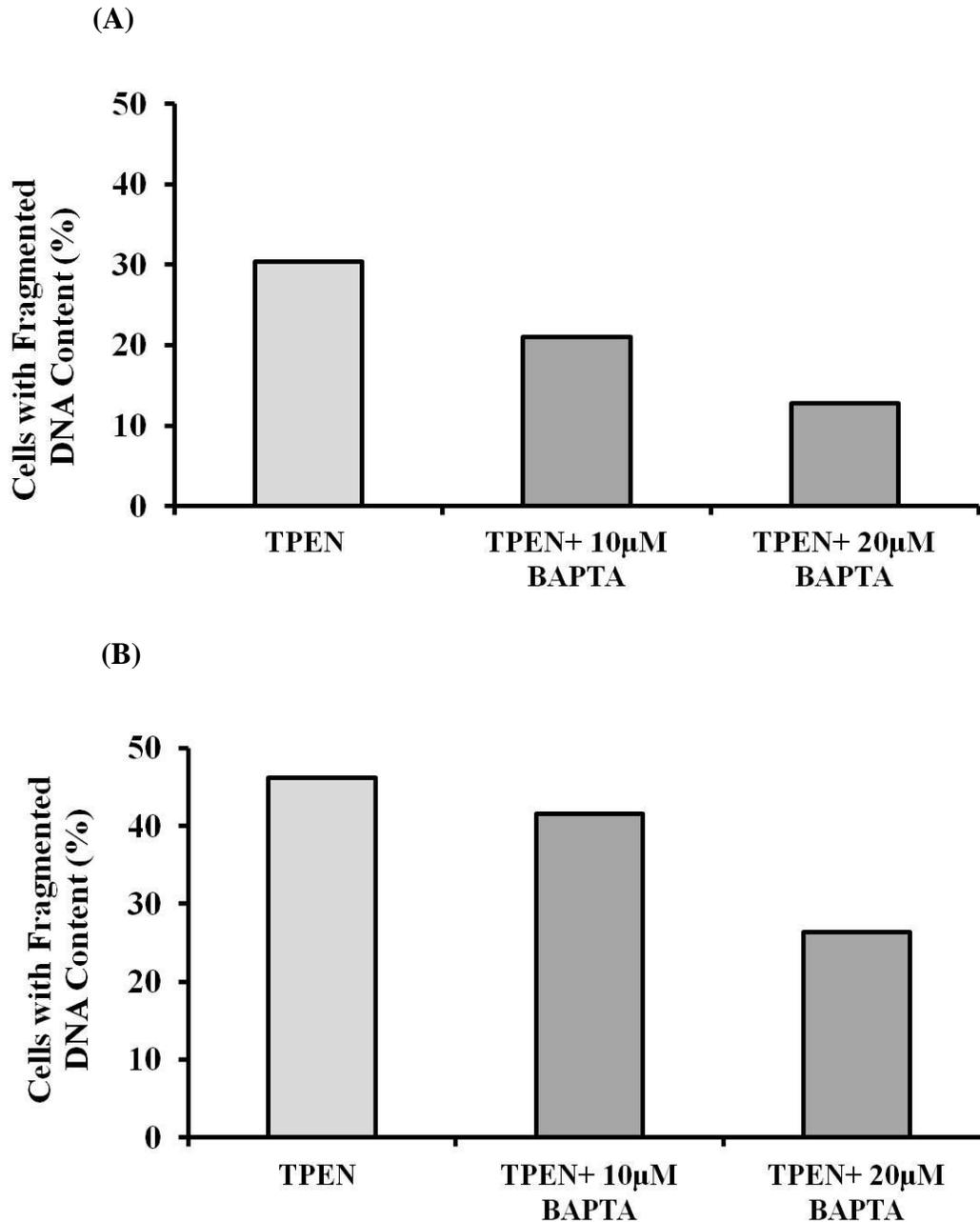
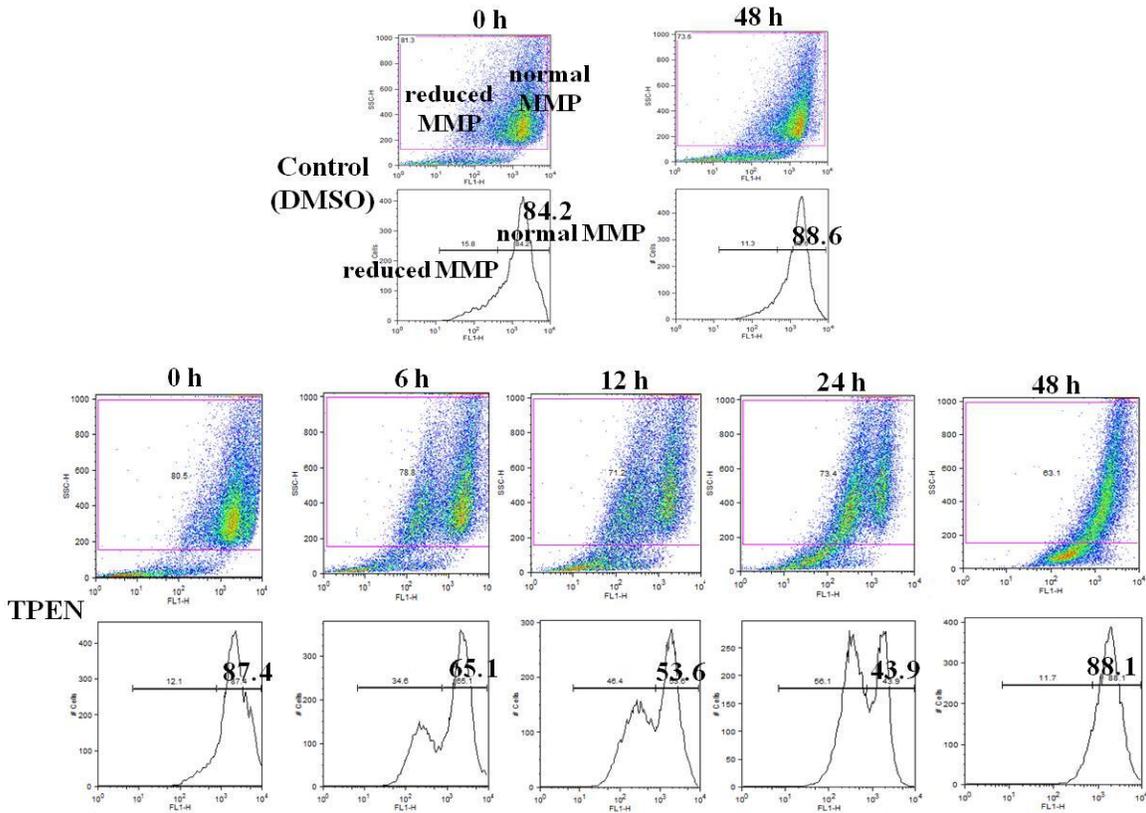


Figure 2.8: Intracellular Ca^{2+} chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation. Cells were treated with TPEN (20 μM) plus BAPTA-AM (0, 10 or 20 μM) for 48 (A) and 72 (B) h. Cells treated with DMSO or BAPTA-AM alone served as the corresponding controls. DNA content was analyzed by PI-staining flow cytometry. Values were calculated by the TPEN treatment values subtracted by the corresponding control values. The experiment was repeated independently once.

(A)



(B)

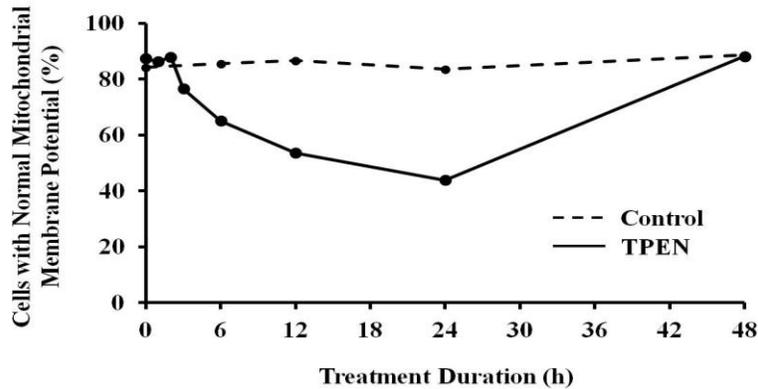


Figure 2.9: TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells. Cells were treated with TPEN (20 μ M) for 0, 1, 2, 3, 6, 12, 24 or 48 h. Cells treated with DMSO only served as the control. MMP was determined using DiOC₆ dye-staining flow cytometry. (A) Representative of flow cytometry data. Dot plots (upper panel) and histograms (lower panel) of DiOC₆-labeled cells. In the histograms, the right and left peaks are cells with normal and reduced MMP, respectively. (B) Scatter plot of flow cytometry data. The experiment was repeated independently once.

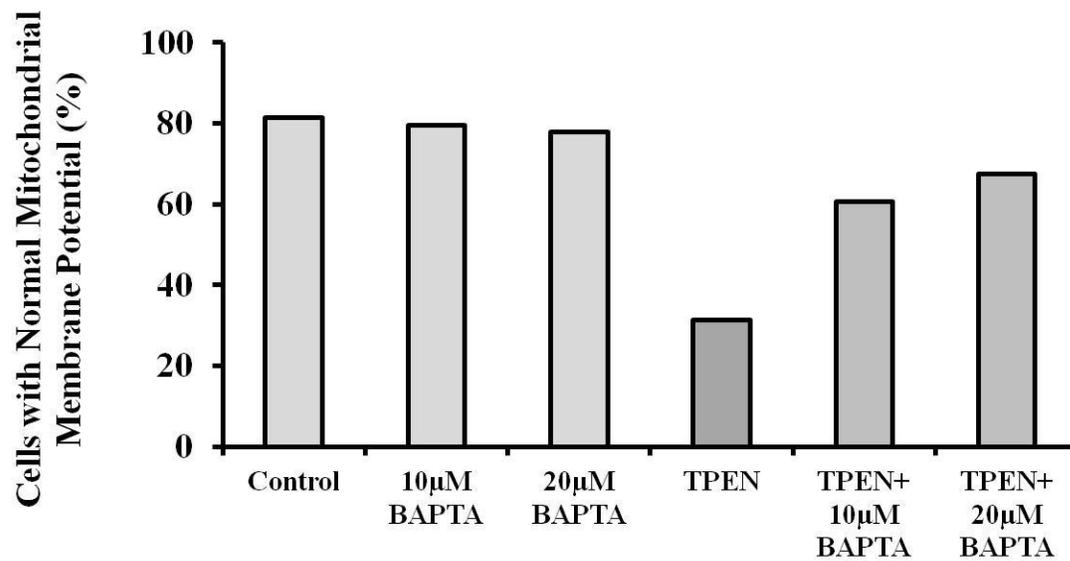
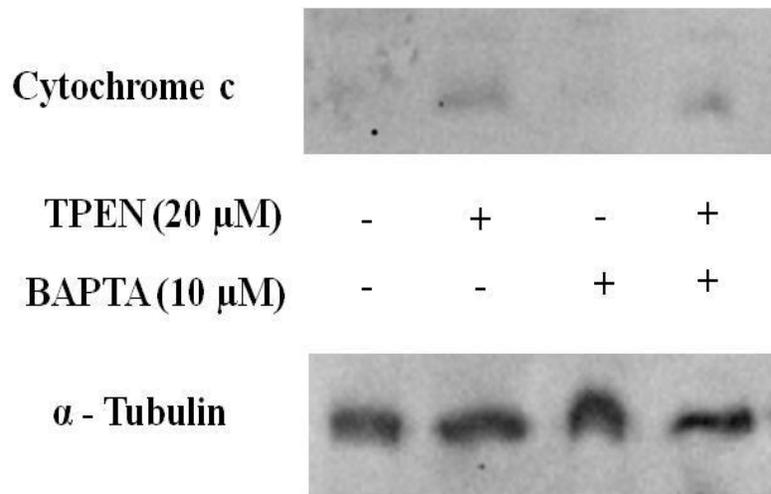


Figure 2.10: Intracellular Ca^{2+} chelation with BAPTA-AM prevented TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells. Cells were treated with DMSO only (Control), BAPTA-AM alone (10 or 20 μM), TPEN alone (20 μM), or TPEN plus BAPTA-AM (10 or 20 μM) for 24 h. MMP was determined by DiOC₆ dye-staining flow cytometry. The experiment was repeated independently once.

(A)



(B)

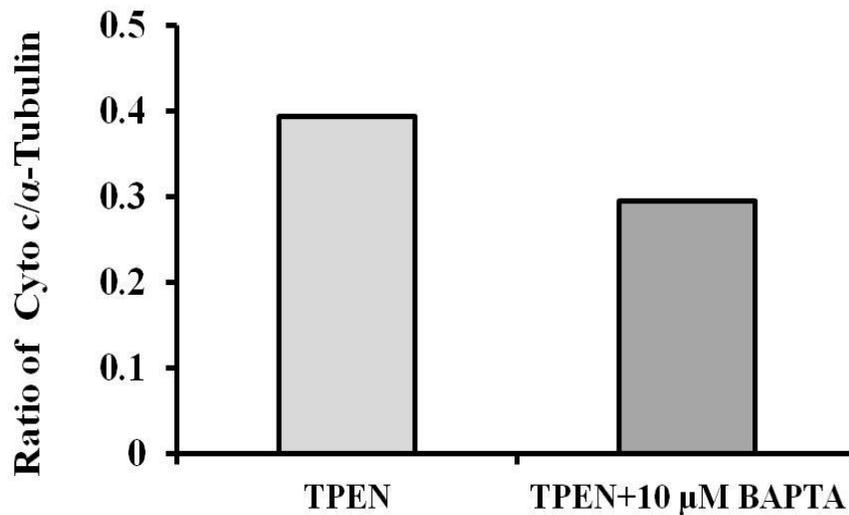
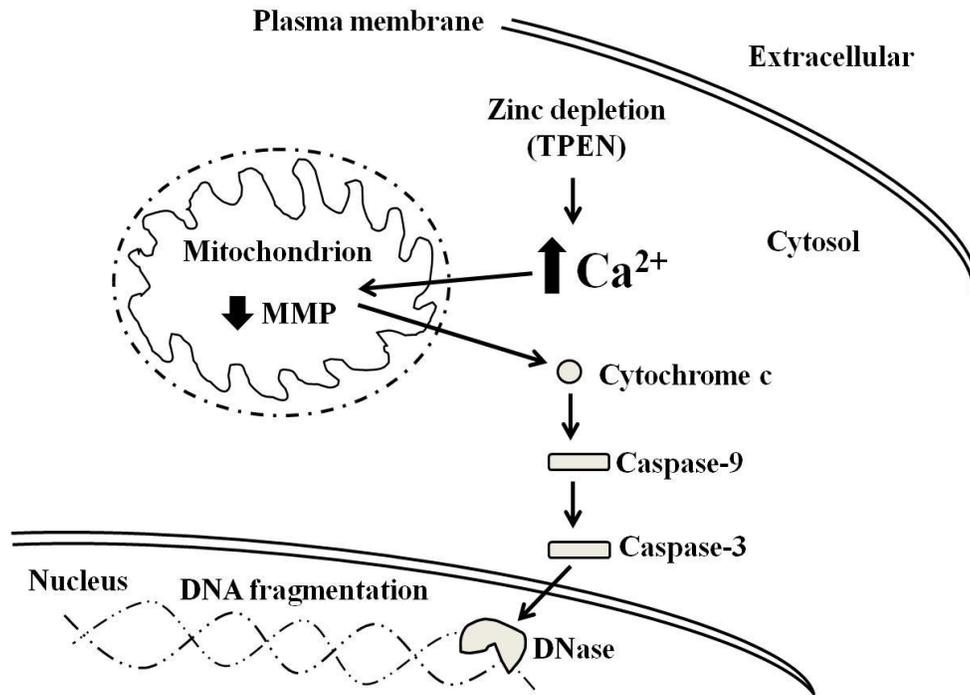


Figure 2.11: Intracellular Ca^{2+} chelation with BAPTA-AM suppressed TPEN-induced release of mitochondrial cytochrome c into the cytosol. Cells were treated with DMSO only, TPEN alone (20 μ M), BAPTA-AM alone (10 μ M), or TPEN plus BAPTA-AM (10 μ M) for 24 h. Cytochrome c protein in the cytosolic fraction was determined by Western blot. α -Tubulin was used as the loading control. Western blot results (A) were analyzed for ratio of cytochrome c/ α -Tubulin (B). The experiment was repeated independently once.

(A)



(B)

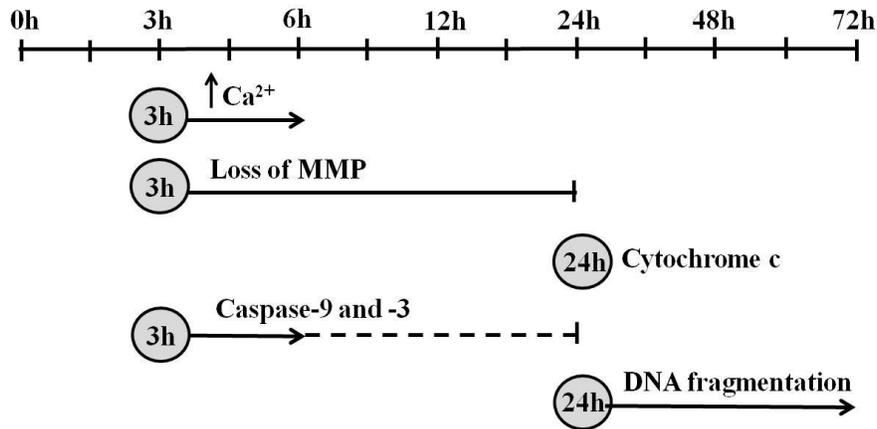


Figure 2.12: Zinc depletion induced apoptosis through the Ca^{2+} -mediated mitochondrial apoptotic pathway in MDA-MB-231 cells. (A) TPEN induced cellular zinc depletion and resulted in an elevated intracellular Ca^{2+} level. This increase in intracellular Ca^{2+} triggered Ca^{2+} to be taken up by the mitochondria, leading to mitochondrial membrane potential (MMP) loss and cytochrome c release. Cytochrome c activated caspase-9 and subsequently caspase-3, which ultimately resulted in DNA fragmentation and apoptotic cell death. (B) Timeline of the events involved.

Chapter 3. General Discussion, Limitations, and Future Directions

3.1 General Discussion

Apoptosis, also known as programmed cell death, plays a critical role in the elimination of damaged or potentially malignant cells (Fadeel *et al.*, 1999b; Saikumar *et al.*, 1999). Defective or inhibited apoptosis is a major factor in the development and progression of cancer, thus restoring the defective apoptotic pathway or inducing apoptosis has been used as a strategy in cancer therapy (Kasibhatla & Tseng, 2003; Wong, 2011). Zinc is considered a regulator of apoptosis (Truong-Tran *et al.*, 2001). Cellular zinc depletion has been shown to induce apoptosis in a number of cell types (Clegg *et al.*, 2005; Sunderman, 1995). However, mechanisms by which zinc functions as an apoptotic regulator remains largely unknown. In this study, we have demonstrated that, in human breast cancer MDA-MB-231 cells, cellular zinc depletion induced apoptosis through the Ca^{2+} -mediated mitochondrial-apoptotic pathway. To the best of my knowledge, this was the first study that showed zinc depletion-induced apoptosis to be mediated through the Ca^{2+} -dependent mitochondrial apoptotic pathway.

In this study, depletion of intracellular zinc by TPEN resulted in an elevated intracellular Ca^{2+} level in human breast cancer MDA-MB-231 cells. An elevated intracellular Ca^{2+} level can be due to Ca^{2+} entry from the extracellular spaces or release from the internal ER stores (Hajnoczky *et al.*, 2003; Orrenius *et al.*, 2003). Ca^{2+} entry from the extracellular spaces is through the plasma membrane Ca^{2+} channels such as VOC, ROC or SOC. The type of plasma membrane Ca^{2+} channel expressed in MDA-

MB-231 cells is the VOC (Taylor *et al.*, 2008). Ca^{2+} release from the internal ER stores is either by RyR or PLC-coupled InsP_3R . ER Ca^{2+} release via InsP_3R is initiated when PLC cleaves PtdInsP_2 into DAG and InsP_3 at the plasma membrane. InsP_3 then diffuses across the cytosol and binds to InsP_3R on the ER to allow Ca^{2+} to be released (Berridge *et al.*, 2000; Orrenius *et al.*, 2003). More work, not included in this thesis, has been conducted to investigate the possible Ca^{2+} sources for the elevated intracellular Ca^{2+} involved in zinc depletion-induced apoptosis, part of which was presented at a conference in 2011. These experiments suggest that the Ca^{2+} needed to mediate zinc depletion-induced apoptosis is from both extra- and intracellular sources (Lin & Xu, 2011). Ca^{2+} from extracellular spaces enters the cells through the plasma membrane VOC while Ca^{2+} from the internal ER stores is released through the RyR (Lin & Xu, 2011). Mitochondria serve as important intracellular Ca^{2+} buffers. An increase in cytosolic Ca^{2+} triggers uptake of Ca^{2+} into the mitochondria through the UP (Hajnóczky *et al.*, 2006; Orrenius *et al.*, 2003). Prolonged elevation of intracellular Ca^{2+} leads to mitochondrial Ca^{2+} overload, which in turn triggers the opening of PTP. Mitochondrial PTP opening leads to mitochondria swelling as it allows water and solutes to pass freely into the matrix while serving as a mitochondrial Ca^{2+} efflux route (Bernardi, 1999; Pfeiffer *et al.*, 2001). Swelling of the mitochondria due to PTP opening results in the loss of MMP, which is followed by the rupture or permeabilization of the mitochondrial outer membrane (Green & Kroemer, 2004; Orrenius *et al.*, 2003). As a result, cytochrome c is released from the mitochondrial intra-membrane space into the cytosol, which triggers the subsequent caspase-apoptotic cascade (Orrenius *et al.*, 2003; Saraste & Pulkki, 2000). Additional work, not included in this thesis, has been done to investigate the involvement of mitochondrial UP and PTP in

mediating zinc depletion-induced apoptosis in MDA-MB-231 cells. We have demonstrated in this study that MMP loss followed by mitochondrial cytochrome c release was mediated by Ca^{2+} in zinc depletion-induced apoptosis in MDA-MB-231 cells. This line of evidence confirmed the involvement of the Ca^{2+} -dependent mitochondrial apoptotic cascade. Cytochrome c further activated the initiator caspase-9, which subsequently activated the executioner caspase-3. Caspase-3 then activated DNase, which ultimately resulted in DNA fragmentation and apoptotic cell death as was observed in this study (Fig. 3.1).

3.2 Limitations

There are several limitations to this study. The first limitation is the use of an *in vitro* system with one single breast cancer cell line. The human breast cancer MDA-MB-231 cell line is derived from pleural effusion or lung metastasis (Cailleau *et al.*, 1974). The genetic makeup and phenotypes (such as morphological and growth characteristics) differ from other human breast cancer cell lines such as MCF-7, T-47D and MDA-MB-330, etc. Furthermore, being a cell line, it lacks the complexity seen at the whole tissue level and the whole body level. Thus, validation of the observations obtained from the current study using other human breast cancer cell lines and at the whole body level, such as an experimental rodent model, is absolutely critical.

The second limitation is that the mitochondrial Ca^{2+} level was not assessed in this study. It is established that in the presence of apoptotic stimuli, there is an increase in the influx of Ca^{2+} from the extracellular spaces into the cytosol, leading to mitochondrial

Ca^{2+} overload (Hajnóczky *et al.*, 2006; Orrenius *et al.*, 2003). Mitochondrial Ca^{2+} overload then triggers the subsequent apoptotic cascade, resulting in apoptotic cell death. In this study, cytosolic Ca^{2+} levels started to rise after 2 h of TPEN exposure and continued for another 4 h. Coinciding with the rise of intracellular Ca^{2+} , the activity of caspase-9 and -3 also started to increase after 2 h of TPEN exposure. In contrast, in the presence of BAPTA-AM, an intracellular Ca^{2+} chelator, the rise of cytosolic Ca^{2+} levels, and the activation of caspase-9 and -3 were abolished. Furthermore, TPEN exposure reduced the MMP along with a leakage of cytochrome c into the cytosol while the presence of BAPTA-AM prevented TPEN-induced MMP loss and cytochrome c leakage. Thus, these observations strongly suggested that TPEN-induced apoptosis was Ca^{2+} -dependent and involved the mitochondria. However, monitoring changes of the mitochondrial Ca^{2+} in response to TPEN exposure would provide more direct evidence showing that mitochondrial Ca^{2+} overload is the trigger to TPEN-induced apoptosis in MDA-MB-231 cells.

The third limitation is the lack of assessment of the abundance of cytochrome c in the mitochondria in response to TPEN exposure. In normal conditions, cytochrome c remains inside the mitochondria (Loeffler & Kroemer, 2000). The presence of apoptotic stimulus triggers the release of cytochrome c from the mitochondria into the cytosol, which subsequently activates the apoptotic cascade (Loeffler & Kroemer, 2000). In this study, cytochrome c release from the mitochondria into the cytosol was assessed by measuring the abundance of cytochrome c in the cytosol only. Monitoring changes in the abundance of cytochrome c in the mitochondria would provide more direct evidence for

the involvement of mitochondria in releasing cytochrome c into the cytosol in response to TPEN-induced apoptosis in MDA-MB-231 cells.

3.3 Future Directions

Further investigations are needed to delineate the upstream causes of the elevated intracellular Ca^{2+} levels involved in zinc depletion-induced apoptosis in MDA-MB-231 cells. Oxidative stress has been shown to be involved in zinc depletion-induced apoptosis in a number of cell types, including rat glioma cells, human lung fibroblasts, human airway epithelial cells and human melanoma cells (Carter *et al.*, 2002; Ho & Ames, 2002; Ho *et al.*, 2003; Rudolf & Cervinka, 2004). Nevertheless, oxidative stress has also been well demonstrated to induce intracellular Ca^{2+} elevation in diverse cell types, such as smooth muscle cells and hepatocytes (Ermak & Davies, 2001; Feissner *et al.*, 2009). Oxidative stress induces intracellular Ca^{2+} elevation by modulating the activity of Ca^{2+} transporter or channels. For instance, reactive oxygen species such as O_2^- and H_2O_2 have been shown to directly activate ER-InsP₃R and RyR, thereby releasing Ca^{2+} from the ER stores into the cytosol (Brookes *et al.*, 2004; Ermak & Davies, 2001; Feissner *et al.*, 2009). Therefore, it is possible that oxidative stress may be the cause of the intracellular Ca^{2+} elevation involved in zinc depletion-induced apoptosis in MDA-MB-231 cells. However, this speculation needs to be experimentally confirmed.

Furthermore, it appears that two distinct mechanisms might be responsible for the release of cytochrome c due to mitochondrial outer membrane permeabilization, which is the key step in the mitochondrial apoptotic pathway. The two mechanisms include: 1)

Ca²⁺-induced mitochondrial PTP opening and 2) an altered ratio of the Bcl-2 proteins (Gross *et al.*, 1999; Orrenius *et al.*, 2003). These two mechanisms might in fact be closely interrelated. For instance, anti-apoptotic Bcl-2 has been shown to prevent Ca²⁺-induced mitochondrial PTP opening and cytochrome c release (Kowaltowski *et al.*, 2000), as well as increase the overall mitochondrial Ca²⁺ buffering capacity (Murphy *et al.*, 1996; Zhu *et al.*, 1999). Conversely, pro-apoptotic Bax has been shown to promote Ca²⁺-induced mitochondrial PTP opening and cytochrome c release (Gogvadze *et al.*, 2001). Bax has also been demonstrated to stimulate mitochondrial Ca²⁺ uptake by directly binding to the mitochondrial UP (Shimizu *et al.*, 1999). However, there is currently no evidence supporting the role of Ca²⁺ in regulating the expression or translocation of the Bcl-2 family proteins during apoptosis. Studying the involvement of these two mechanisms will provide a better understanding of zinc depletion-induced apoptosis in MDA-MB-231 cells.

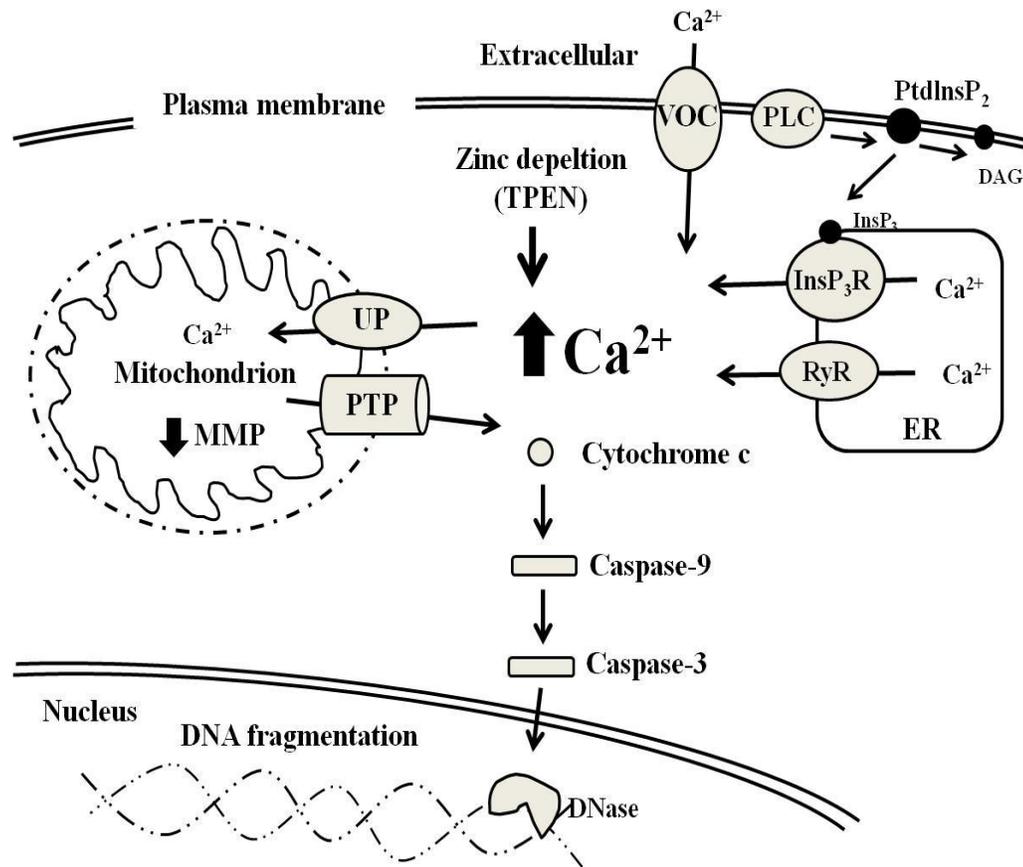


Figure 3.1: Involvement of Ca^{2+} in mediating zinc depletion-induced apoptosis in MDA-MB-231 cells. Zinc depletion induced by TPEN results in an elevated intracellular Ca^{2+} level. This Ca^{2+} is from Ca^{2+} entering from the extracellular spaces through the voltage operated channel (VOC), or Ca^{2+} releasing from the internal ER stores through the phospholipase C (PLC)-coupled inositol trisphosphate receptors ($InsP_3R$) or ryanodine receptors (RyR). This increase in intracellular Ca^{2+} triggers Ca^{2+} to be taken up by the mitochondria through the uniporter (UP). Mitochondrial Ca^{2+} overload then induces the opening of the permeability transition pore (PTP), which leads to mitochondrial membrane potential loss and cytochrome c release. Cytochrome c then activates caspase-9 and subsequently caspase-3, ultimately resulting in DNA fragmentation and apoptotic cell death (Orrenius *et al.*, 2003; Saraste & Pulkki, 2000).

References

- Abou-Nassar, K. & Brown, J. R. (2010). Novel agents for the treatment of chronic lymphocytic leukaemia. *Clinical Advances in Hematology and Oncology*, 8(12), 886-895.
- Adler, M., Shafer, H., Hamilton, T. & Petrali, J. P. (1999). Cytotoxic actions of the heavy metal chelator TPEN on NG108-15 neuroblastoma-glioma cells. *Neurotoxicology*, 20, 571–582.
- Aguas, F., Martins, A., Gomes, T. P., Sousa, M. D. & Silva, D. P. (2005). Prophylaxis approach to a-symptomatic post-menopausal women: Breast cancer. *Maturitas*, 52(Suppl1), 23-31.
- Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I. *et al.* (1997). Inhibition of Bax channel-forming activity by Bcl-2. *Science*, 277, 370-372.
- Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. & Martinou, J. C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochemical Journal*, 345(2), 271-8.
- Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A. & Bernardi, P. (2005). Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *Journal of Biological Chemistry*, 280, 18558–18561.
- Bentley, P.J. & Grubb, B.R. (1991). Experimental dietary hyperzincemia tissue disposition of excess zinc in rabbits. *Journal of Trace Elements in Medicine and Biology*, 8, 202-207.
- Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiological Reviews*, 79; 1127–1155.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, 361, 315-325.
- Berridge, M. J., Lipp, P. & Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nature Reviews Molecular Cell Biology*, 1, 11-21.
- Brookes, P.S., Yoon, Y., Robotham, J. L., Anders, M. W. & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *American Journal of Physiology-Cell Physiology*, 287, 817-833.
- Cailleau, R., Young, R., Olive, M. & Reeves, W. J. Jr. (1974). Breast tumor cell lines from pleural effusions. *Journal of the National Cancer Institution*, 53, 661-674.

- Cailleau, R., Olivé, M. & Cruciger, Q. V. (1978). Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro*, 14, 911–915.
- Canadian Cancer Society's Steering Committee on Cancer Statistics. (2012). Canadian Cancer Statistics 2012. *Toronto, ON: Canadian Cancer Society*, p.7-11.
- Cao, J., Bobo, J. A., Liuzzi, J. P. & Cousins, R. J. (2001). Effects of intracellular zinc depletion on metallothionein and ZIP2 transporter expression and apoptosis. *Journal of Leukocyte Biology*, 70(4), 559-66.
- Carter, J. E., Truong-Tran, A. Q., Grosser, D., Ho, L., Ruffin, R. E. & Zalewski, P. D. (2002). Involvement of redox events in caspase activation in zinc-depleted airway epithelial cells. *Biochemical and Biophysical Research Communications*, 297, 1062 – 1070.
- Chasapic, C. T., Loutsidou, A. C., Spiliopoulou, C. A. & Stefanidou, M. E. (2012). Zinc and human health: an update. *Archives of Toxicology*, 86, 521-534.
- Chimienti, F., Seve, M., Richard, S., Mathieu, J. & Favier, A. (2001). Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. *Biochemical Pharmacology*, 62, 51 – 62.
- Clegg, M. S., Hanna, L. A., Niles, B. J., Momma, T. Y. & Keen, C. L. (2005). Zinc deficiency-induced cell death. *Life*, 57(10), 661-669.
- Cory, S. & Adams, J. M. (2002). The Bcl-2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer*, 2, 647-656.
- Cousins, R. J. (1997). Zinc. *Present Knowledge in Nutrition*, 29, 293-306.
- Coyle, P., Zalewski, P. D. & Philcox, J. C. (1994). Measurement of zinc in hepatocytes by using a fluorescence probe, zinquin: relationship to metallothionein and intracellular zinc. *Biochemical Journal*, 303, 781-786.
- Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochemical Journal*, 341, 233-249.
- Danen-Van Oorschot, A. A., Fischer, D. F., Grimbergen, J. M., Klein, B., Zhuang, S., Falkenburg, J. H., *et al.* (1997). Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proceeding of the National Academy of Sciences of USA*, 94, 5843–5847.
- Dave, Z., Byfield, M. & Bossy-Wetzel, E. (2008). Assessing mitochondrial outer membrane permeabilization during apoptosis. *Methods*, 46, 319-323.

- Denault, J. B. & Salvesen, G. S. (2002). Caspases: keys in the ignition of cell death. *Chemical Reviews*, 102(12), 4489-500.
- Devarajan, E., Sahin, A. A., Chen, J. S., Krishnamurthy, R. R., Aggarwal, N., Brun, A. M., *et al.* (2002). Downregulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene*, 21(57), 8843-8851.
- Dreosti, I. V. (2001). Zinc and the gene. *Mutation Research*, 475, 161-167.
- Duffy, J. Y., Miller, C. M., Rutschilling, G. L., Ridder, G. M., Clegg, M. S., Keen, C. L. & Daston, G. P. (2001). A decrease in intracellular zinc level precedes the detection of early indicators of apoptosis in HL-60 cells. *Apoptosis*, 6, 161 – 172.
- Dumitrescu, R. G. & Cotarla, I. (2005). Understanding breast cancer risk: where do we stand in 2005? *Journal of Cellular and Molecular Medicine*, 9, 208-21.
- Earnshaw, W. C., Martins, L. M. & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual Review of Biochemistry*, 68, 383-424.
- Elmes, M. (1997). Apoptosis in the small intestine of zinc-deficient and fasted rats. *Journal of Pathology*, 123, 219-223.
- Engel, L. W. & Young, N. A. (1978). Human breast carcinoma in continuous culture: a review. *Cancer Research*, 38, 4327–4339.
- Ermak, G. & Davies, K. J. A. (2001). Calcium and oxidative stress: from cell signalling to cell death. *Molecular Immunology*, 38, 713-721.
- Eskes, R., Desagher, S., Antonsson, B. & Martinou, J. C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Journal of Molecular Cell biology*, 20, 929-935.
- Fadeel, B., Orrenius, S. & Zhivotosky, B. (1999b). Apoptosis in human disease: a new skin for the old ceremony? *Biochemical and Biophysical Research Communications*, 266(3), 699-717.
- Falk, S. (2006) Principles of cancer treatment by radiotherapy. *Surgery (Oxford)*, 24(2), 62-65.
- Fanzo, J. C., Reaves, S. K., Cui, L., Zhu, L. & Lei, K. Y. (2002) p53 protein and p21 mRNA levels and caspase-3 activity are altered by zinc status in aortic endothelial cells. *American Journal of Physiology-Cell Physiology*, 283, C631 – 638.

- Feissner, R. F., Skalska, J., Gaum, W. E. & Sheu, S. S. (2009). Crosstalk signalling between mitochondrial Ca^{2+} and ROS. *Frontiers in Bioscience*, *14*, 1197-1218.
- Franklin, R.B. & Costello, L.C. (2009). The important role of the apoptotic effects of zinc in the development of cancers. *Journal of Cellular Biochemistry*, *106*, 750-757.
- Gee, J., Robertson, J., Ellis, I., Willsher, P., McClelland, R., Hoyle, H., Kyme, S., Finlay, P., Blamey, R. & Nicholson, R. (1994). Immunocytochemical localization of Bcl-2 protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *International Journal of Cancer*, *59*, 619-628.
- Gogvadze, V., Robertson, J. D., Zhivotovsky, B. & Orrenius, S. (2001). Cytochrome c release occurs via Ca^{2+} -dependent and Ca^{2+} -independent mechanisms that are regulated by Bax. *Journal of Biological Chemistry*, *276*, 19066-19071.
- Green, D.R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science*, *305*, 626–629.
- Groff, J. L. & Gropper, S. S. (2000). *Advanced Nutrition and Human Metabolism (3rd edition)*. Belmont, CA: Wadsworth, 419-428.
- Gross, A., McDonnell, J. M. & Korsmeyer, S. J. (1999). Bcl-2 family members and the mitochondria in apoptosis. *Genes & Development*, *12*, 1899-1911.
- Guo, B., Yang, M., Liang, D., Yang, L., Cao, J. & Zhang, L. (2012). Cell apoptosis induced by zinc deficiency in osteoblastic MC3T3-E1 cells via a mitochondrial-mediated pathway. *Molecular and Cellular Biochemistry*, *361*, 209-216.
- Hajnoczky, G., Davies, E. & Madesh, M. (2003). Calcium signalling and apoptosis. *Biochemical and Biophysical Research Communications*. *304*, 445-454.
- Hajnoczky, G., Csordas, G., Das, S., Garcia-Perez, C., Saotome, M., Roy, S.S. & Yi, M. (2006). Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca^{2+} uptake in apoptosis. *Cell Calcium*. *40*, 553-560.
- Halestrap, A. P., Kerr, P.M., Javadov, S. & Woodfield, K. Y. (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochimica et Biophysica Acta*, *1366*, 79-94.
- He, L.S., Yan, X.S. & Wu, D.C. (1991). Age-dependent variation of zinc-65 metabolism in LACA mice. *International Journal of Radiation Biology*, *60*, 907-916.

- Hertveldt, K., Philippe, J., Thierens, H., Cornelissen, M. Vral, A. & De Ridder, L. (1997). Flow cytometry as a quantitative and sensitive method to evaluate low dose radiation induced apoptosis *in vitro* in human peripheral blood lymphocytes. *International Journal of Radiation Biology*, 71(4), 429-433.
- Ho, E., & Ames, B. N. (2002). Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proceeding of the National Academy of Sciences USA*, 99, 16770 – 16775.
- Ho, E., Courtemanche, C., & Ames, B. N. (2003). Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *Journal of Nutrition*, 133, 2543 – 2548.
- Hutchins, J. B. & Barger, S. W. (1998). Why neurons die: cell death in the nervous system. *The Anatomical Record*, 253(3), 79-90.
- Hyun, H. J., Sohn, J. H., Ha, D. W., Ahn, Y.H., Koh, J. Y. & Yoon, Y.H. (2001). Depletion of intracellular zinc and copper with TPEN results in apoptosis of cultured human retinal pigment epithelial cells. *Investigative Ophthalmology & Visual Science*, 42(2), 460-465.
- Institute of Medicine. (2001). *Dietary Reference Intakes for Vitamins A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington, DC: National Academy Press.
- Johnson, K. C., Miller, A. B., Collishaw, N. E., Palmer, J. R., Hammond, S. K., Salmon, A. G., *et al.* (2011). Active smoking and secondhand smoke increase breast cancer risk: the report of the Canadian expert panel on tobacco smoke and breast cancer risk (2009). *Tobacco control*, 20(1), e2.
- Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R., & Nagao, M. (2004). Overview of mammalian zinc transporters. *Cellular and Molecular Life Sciences CMLS*, 61(1), 49-68.
- Kang, M. H. & Reynolds, C. P. (2009). Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clinical Cancer Research*, 15, 1126-1132.
- Kasibhatla, S. & Tseng, B. (2003). Why target apoptosis in cancer treatment. *Molecular Cancer Therapeutics*, 2, 573-580.
- Kenemans, P., Verstraeten, R. A. & Verheijen, R. H. M. (2004). Oncogenic pathways in hereditary and sporadic breast cancer. *Maturitas*, 49, 34-43.

- Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., *et al.* (1979). Establishment and characterization of a cell line of human breast carcinoma origin. *European Journal of Cancer*, 15, 659–670.
- King, L., Osati-Ashtiani, F. & Fraker, P. (2002). Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. *Journal of Nutrition*, 132, 974–979.
- King, A. T & Primrose, J. N. (2009). Principles of cancer treatment by surgery. *Surgery (Oxford)*, 27(4), 178-181.
- Kirichok, Y., Krapivinsky, G. & Clapham, D.E. (2004). The mitochondrial calcium uniporter is a highly selective ion channel. *Nature*, 427, 360-364.
- Kohler, C., Orrenius, S. & Zhivotovsky, B. (2002). Evaluation of caspase activity in apoptotic cells. *Journal of Immunological Methods*, 265, 97– 110.
- Kolenko, V. M., Uzzo, R. G., Dulin, N., Hauzman, E., Bukowski, R. & Finke, J. H. (2001). Mechanism of apoptosis induced by zinc-deficiency in peripheral blood T lymphocytes. *Apoptosis*, 6, 419–429.
- Kowaltowski, A. J., Vercesi, A. E. & Fiskum, G. (2000). Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. *Cell Death & Differentiation*, 7, 903-910.
- Krajewski, S., Blomqvist, C., Franssila, K., Krajewska, M., Wasenius, V. M., Niskanen, E. & Reed, J. C. (1995a). Reduced expression of pro-apoptotic gene Bax is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Research*, 55, 4471-4478.
- Krajewski, S., Krajewska, M., Turner, B. C., Pratt, C., Howard, B., Zapata, J. M., *et al.* (1999). Prognostic significance of apoptosis regulators in breast cancer. *Endocrine-Related Cancer*, 6, 29-40.
- Labuza, T. P. (1982). *Shelf-life Dating of Foods*, Food & Nutrition Press, Westport, CT.
- Lacroix, M. & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Research and Treatment*, 83, 249–289.
- Lasfargues, E. Y. & Ozzello, L. (1958). Cultivation of human breast carcinomas. *Journal of the National Cancer Institute*, 21, 1131-1147.

- Lasfargues, E. Y., Coutinho, W. G. & Redfield, E. S. (1978). Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *Journal of the National Cancer Institute*, 61, 967–978.
- Lavrik, I. N., Golks, A. & Krammer, P. H. (2005). Caspases: pharmacological manipulation of cell death. *Journal of Clinical Investigation*, 115, 2665-2672.
- Liang, D., Yang, M. Guo, B., Cao, J. Yang, L. Guo, X., *et al.* (2012). Zinc inhibits H(2)O(2)-induced MC3T3-E1 cells apoptosis via MAPK and PI3K/AKT pathways. *Biological Trace Element Research*, 148(3), 420-9.
- Lin, A. & Xu, Z. (2011). Blocking extracellular Ca²⁺ influx and release of endoplasmic reticulum Ca²⁺ resulted in a concentration-and duration-dependent suppression of zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells. *Canadian Nutrition Society Annual Meeting*. (Abstracts).
- Llobet, J.M., Domingo, J.L., Colomina, M.T., Mayayo, E. & Corbella, J. (1988). Subchronic oral toxicity of zinc in rats. *Bulletin of Environmental Contamination and Toxicology*, 41, 36-43.
- Loeffler, M. & Kroemer, G. (2000). The mitochondrion in cell death control: certainties and incognita. *Experimental Cell Research*, 256(1), 19-26.
- MacDonald, R. S. (2000). The role of zinc in growth and cell proliferation. *Journal of Nutrition*, 130, 1500S-1508S.
- Martin, S. J., Mazdai, G., Strain, J. J., Cotter, T. G. & Hannigan, B. M. (1991). Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. *Clinical & Experimental Immunology*, 83, 338–343.
- McCabe, M. J. Jr., Jiang, S. A. & Orrenius, S. (1993). Chelation of intracellular zinc triggers apoptosis in mature thymocytes. *Laboratory Investigation*, 69, 101–110.
- Meier, P., Finch, A. & Evan, G. (2000). Apoptosis in development. *Nature*, 407(6805), 796-801.
- Minn, A. J., Rudin, C. M., Boise, L. H. & Thompson, C. B. (1995). Expression of Bcl-XL can confer a multidrug resistance phenotype. *Blood*, 86, 1903-1910.
- Murakami, M. & Hirano, T. (2008). Intracellular zinc homeostasis and zinc signaling. *Cancer Science*, 99(8), 1515-1522.
- Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E. & Fiskum, G. (1996). Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proceedings of the National Academy of Sciences USA*, 93, 9893-9898.

- Nelson, H. D., Humphrey, L. L., Nygren, P., Teutsch, S. M. & Allan, J. D. (2002). Postmenopausal hormone replacement therapy: scientific review. *Journal of the American Medical Association*, 288, 872-881.
- Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., et al. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*, 435(7042), 677-681.
- Orrenius, S., Zhivotovsky, B., & Nicotera, P. (2003). Regulation of cell death: the calcium-apoptosis link. *Nature Reviews Molecular Cell Biology*, 4, 552-565.
- Osborne, C. K., Yochmowitz, M. G., Knight, W. A. & McGuire, W. L. (1980). The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer*, 46, 2884-2888.
- Parnell, C. & Woll, P. J. (2003). Principles of cancer treatment by chemotherapy. *Surgery (Oxford)*, 21(11), 272-276.
- Paski, S. C. & Xu, Z. (2001). Labile intracellular zinc is associated with 3T3 cell growth. *Journal of Nutritional Biochemical*, 12, 655-661.
- Pepper, C., Hoy, T. & Bentley, D. P. (1997). Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. *British Journal of Cancer*, 76(7), 935-938.
- Perry, D. K., Smyth, M. J., Stennicke, H. R., Salvesen, G. S., Duriez, P., Poirier, G. G. & Hannun, Y. A. (1997). Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *Journal of Biological Chemistry*, 272, 18530 – 18533.
- Pfeiffer, D. R., Gunter, T. E., Eliseev, R., Broekemeier, K. M. & Gunter, K. K. (2001). Release of Ca²⁺ from mitochondria via the saturable mechanisms and the permeability transition. *International Union of Biochemistry and Molecular Biology /IUBMB Life*, 52, 205–212.
- Plum, L. M., Rink, L. & Haase, H. (2010). The essential toxin: impact of zinc on human health. *International Journal of Environmental Research and Public Health*, 7, 1342-1365.
- Prasad, A. S. & Oberleas, D. (1974). Thymidine kinase activity and incorporation of thymidine into DNA in zinc-deficient tissue. *Journal of Laboratory and Clinical Medicine*, 81, 634-639.

- Pratt, C., Krajewski, S., Menard, M., Krajewska, M., MacCleod, H. & Reed, J. (1998). Estrogen withdrawal-induced human breast cancer tumor regression in nude mice is prevented by Bcl-2. *Federation of European Biochemical Societies/ FEBS Letters*, 440, 403-408.
- Rai, K. R., Moore, J., Wu, J., Novick, S. C. & O'Brien, S. M. (2008). Effect of the addition of oblimersen (Bcl-2 antisense) to fludarabine/cyclophosphamide for relapsed/refractory chronic lymphocytic leukaemia (CLL) on survival in patients who achieve CR/nPR: Five-year follow-up from a randomized phase III study [abstract]. *Journal of Clinical Oncology*, 26, 7008.
- Reeder, J. G. & Vogel, V. G. (2008). Breast cancer prevention. *Cancer Treatment and Research*, 141, 149-64.
- Riccardi, C., & Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols*. 3, 1458-1461.
- Robinson, J. A., Jenkins, N. S., Holman, N. A., Roberts-Thomson, S. J. & Monteith, G. R. (2004). Ratiometric and nonratiometric Ca^{2+} indicators for the assessment of intracellular free Ca^{2+} in a breast cancer cell line using a fluorescence microplate reader. *Journal of Biochemical and Biophysical Methods*, 58(3), 227-237.
- Rohn, J. L. & Noteborn, M. H. (2004). The viral death effector Apoptin reveals tumour-specific processes. *Apoptosis*, 9, 315-322.
- Rudolf, E., & Cervinka, M. (2004). Depletion of endogenous zinc stores induces oxidative stress and cell death in human melanoma cells. *Acta Medica (Hradec Kralove)*, 47, 91-96.
- Saikumar, P., Dong, Z., Mikhaililov, V., Denton, M., Weinberg, J. M. & Venkatachalam, M. A. (1999). Apoptosis: definition, mechanisms, and relevance to disease. *American Journal of Medicine*, 107, 489-506.
- Sandstead, H.H. (2003). Zinc is essential for brain development and function. *The Journal of Trace Elements in Experimental Medicine*, 16, 165-173.
- Saraste, A. & Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular Research*, 45(3), 528-37.
- Sariego, J. (2010). Breast cancer in the young patient. *The American surgeon*, 76 (12), 1397-1401.
- Sato, M. (1992). Biological antioxidant defense system and metallothionein. *Japanese Journal of Toxicology and Environmental Health*, 38, 228-239.

- Sartorius, B., Schmitz, I. & Krammer, P. H. (2001). Molecular mechanisms of death-receptor-mediated apoptosis. *ChemBiochem*, 2(1), 20-9.
- Schanne, R. A. X., Kane, A. B., Young, E. E. & Farber, J. L. (1979). Calcium dependence of toxic cell death. *Science*, 206, 700-702.
- Shimizu, S., Narita, M. & Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, 399, 483-487.
- Shumaker, D. K., Vann, L. R., Goldberg, M. W., Allen, T. D. & Wilson, K. L. (1998). TPEN, a Zn²⁺/Fe²⁺ chelator with low affinity for Ca²⁺, inhibits lamin assembly, destabilizes nuclear architecture and may independently protect nuclei from apoptosis in vitro. *Cell Calcium*, 23, 151-164.
- Simpson, P. T., Reis-Filho, J. S., Gale, T. & Lakhani, S. R. (2005). Molecular evolution of breast cancer. *Journal of Pathology*, 205, 248-254.
- Singletary, S. E. (2003). Rating the risk factors for breast cancer. *Annals of Surgery*, 237, 474-482.
- Solomons, N. W. & Cousins, R. J. (1984). Zinc. In Solomons N. W., Rosenberg I. H. (eds), *Absorption and malabsorption of mineral nutrients*. Alan R. Liss, New York, pp 125-197.
- Soule, H. D., Vazquez, J., Long, A., Albert, S. & Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute*, 51, 1409-1416.
- Srivastava, V., Rawall, S., Vijayan, V. K. & Khanna, M. (2009). Influenza a virus induced apoptosis: inhibition of DNA laddering & caspase-3 activity by zinc supplementation in cultured HeLa cells. *Indian Journal of Medical Research*, 129(5), 579-86.
- Stefanidou, M., Maravelias, C., Dona, A., & Spiliopoulou, C. (2006). Zinc: a multipurpose trace element. *Archives of Toxicology*, 80, 1-9.
- Stokes, Z. & Chan, S. (2003). Principles of cancer treatment by hormone therapy. *Surgery (Oxford)*, 21(11), 280-283.
- Strayer, D. S., Hoek, J. B., Thomas, A. P. & White, M. K. (1999). Cellular activation by Ca²⁺ release from stores in the endoplasmic reticulum but not by increased free Ca²⁺ in the cytosol. *Biochemical Journal*, 344(Pt 1), 39-46.
- Sunderman, F. W. JR. (1995). The influence of zinc on apoptosis. *Annals of Clinical and Laboratory Science*, 25(2), 134-142.

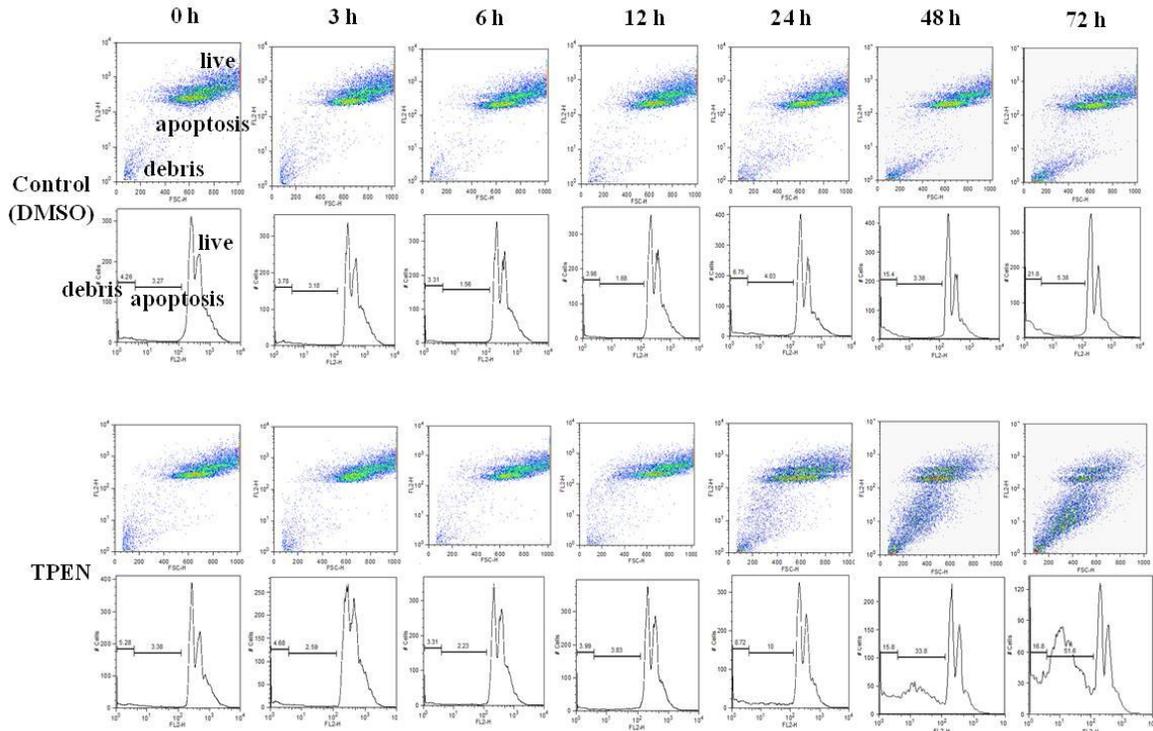
- Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D. *et al.* (1996). Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proceedings of the National Academy of Sciences USA*, 93(16), 8359-400.
- Tapiero, H., & Tew, K. D. (2003). Trace elements in human physiology and pathology: Zinc and metallothioneins. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, 57(9), 399-411.
- Taylor, J. T., Zeng, X.B., Pottle, J. E., Lee, K., Wang, A. R., Yi, S. G., et al. (2008). Calcium signalling and T-type calcium channels in cancer cell cycling. *World Journal of Gastroenterology*, 14(32), 4984-4991.
- Thambiayya, K., Wasserloos, K., Kagan, V. E., Stoyanovsky, D. & Pitt, B. R. (2012). A critical role for increase labile zinc in reducing sensitivity of cultured sheep pulmonary artery endothelial cells to LPS-induced apoptosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 302(12), L1287-95.
- Tretli, S. (1989). Height and weight in relation to breast cancer morbidity and mortality. A prospective study of 570,000 women in Norway. *International Journal of Cancer*, 44, 23-30.
- Treves, S., Trentini, P. L., Ascanelli, M., Bucci, G. & Di Virgilio, F. (1994). Apoptosis is dependent on intracellular zinc and independent of intracellular calcium in lymphocytes. *Experimental Cell Research*, 211, 339-343.
- Truong-Tran, A. Q., Ruffin, R. E. & Zalewski, P. D. (2000b). Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 279, L1172-L1183.
- Truong-Tran, A. Q., Carter, J., Ruffin, R. E. & Zalewski, P. D. (2001). The role of zinc in caspase activation and apoptotic cell death. *BioMetals*, 14, 315-330.
- Verstraeten, S. V., Zago, M. P., Mackenzie, G. G., Keen, C. L. & Oteiza, P. I. (2004). Influence of zinc deficiency on cell-membrane fluidity in Jurkat, 3T3 and IMR-32 cells. *Biochemical Journal*, 378, 579-587.
- Wang, X., Fosmire, G. J., Gay, C. V. & Leach, R. M. J. (2002). Short-term zinc deficiency inhibits chondrocyte proliferation and induces cell apoptosis in the epiphyseal growth plate of young chickens. *Journal of Nutrition*, 132, 665-673.

- Wastney, M.E., Aamodt, R.L., Rumble, W.F. & Henkin, R.I. (1986). Kinetic analysis of zinc metabolism and its regulation in normal humans. *American Journal of Physiology*, 251, R398-R408.
- Whittaker, P. (1998). Iron and zinc interactions in humans. *American Journal of Clinical Nutrition*, 68, 442S-46S.
- Wilson, D., Varigos, G. & Ackland, M. L. (2006). Research article-Apoptosis may underlie the pathology of zinc deficient skin. *Immunology and Cell Biology*, 84, 28-37.
- Wong, R. S. Y. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, 30, 87.
- Wu, B. W. (2003). *N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine-induced depletion of the labile intracellular pool of zinc suppressed the growth of MDA-MB-231 human breast cancer cells*. UBC (Thesis).
- Xu, Z. & Purtzki, M. (2010a). Zinc deprivation-induced apoptosis is associated with an altered expression of apoptotic regulatory genes in MDA-MB-231 breast cancer cells. *Canadian Nutrition Society Annual Meeting*. (Abstracts).
- Xu, Z. & Purtzki, M. (2010b). Zinc deprivation-induced apoptosis in human breast cancer MDA-MB-231 cells is calcium-dependent. *FASEB J*, 26, 519.13 (Abstract).
- Yanagisawa, N. & Wada, O. (2001). Increased apoptosis in a variety of tissues of zinc-deficient rats. *Life Sciences*, 69, 1639-1649.
- Zalewski, P. D., Forbes, I. J. & Betts, W. H. (1993). Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-p-toluene-sulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). *Biochemical Journal*, 296(Pt2), 403-408.
- Zhu, L., Ling, S., Yu, X. D., Venkatesh, L. K., Subramanian, T., Chinnadurai, G. & Kuo, T. H. (1999). Modulation of mitochondrial Ca²⁺ homeostasis by Bcl-2. *Journal of Biological Chemistry*, 274, 33267-33273.

Appendices

Appendix 1

(A)



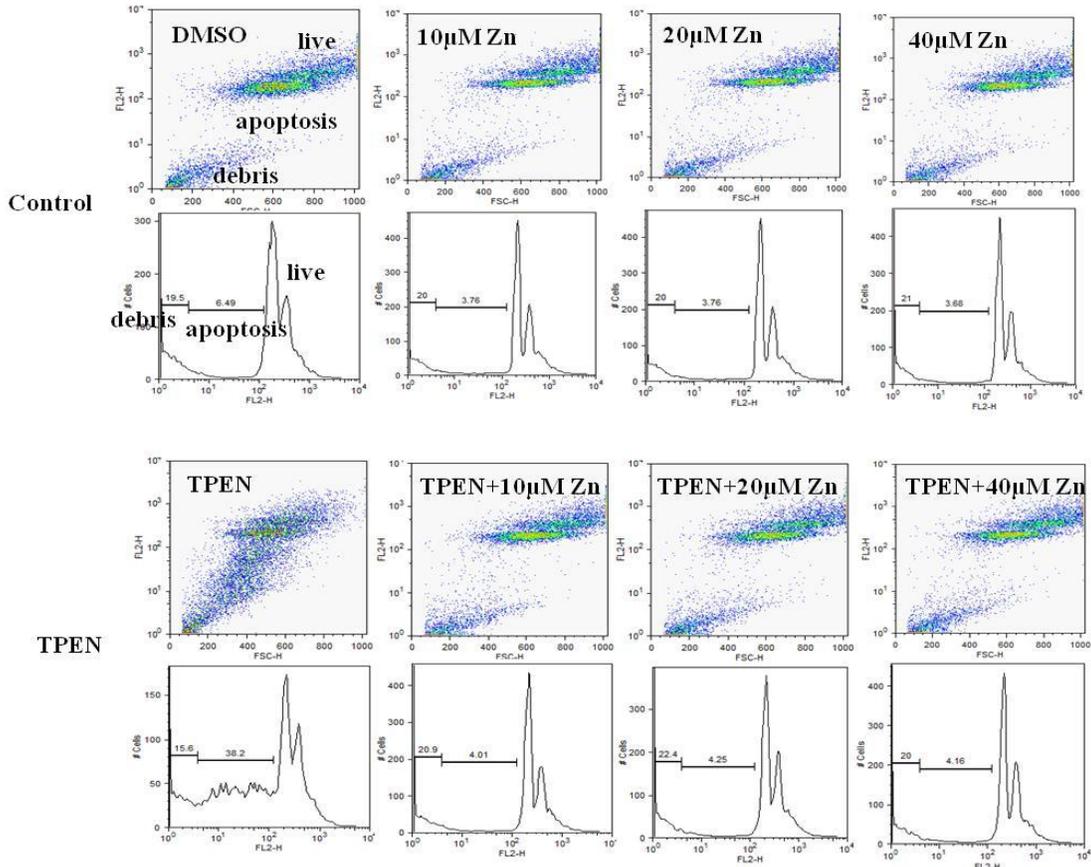
(B)

Control (h)	Debris (%)	Apoptosis (%)	TPEN (h)	Debris (%)	Apoptosis (%)
0	4.26	3.27	0	5.28	3.38
3	3.78	3.18	3	4.68	2.59
6	3.31	1.56	6	3.31	2.23
12	3.98	1.68	12	3.99	3.83
24	6.75	4.03	24	8.72	10
48	15.4	3.38	48	15.8	33.8
72	21.8	5.38	72	16.8	51.6

Figure A.1. TPEN induced DNA fragmentation in MDA-MB-231 cells. Cells were treated with DMSO (control) or TPEN (20 μM) for 0, 3, 6, 12, 24, 48 or 72 h. DNA content was analyzed by PI-staining flow cytometry. Apoptosis was indicated by cells with fragmented DNA. Distribution of cell debris, and apoptotic and live cells are located in the low-left, middle, and upper-right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B).

Appendix 2

(A)



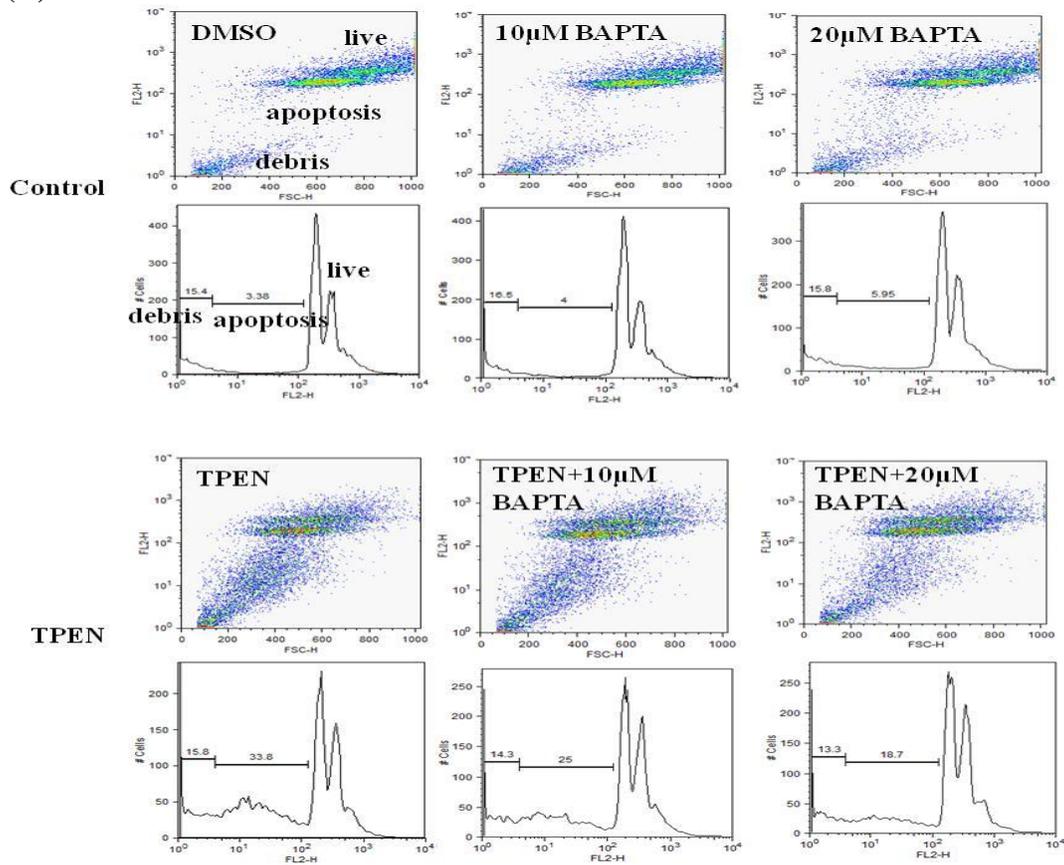
(B)

Control	Debris (%)	Apoptosis (%)	TPEN	Debris (%)	Apoptosis (%)
DMSO	19.5	6.49	TPEN	15.8	38.2
10 μM Zn	20.8	3.76	TPEN+10 μM Zn	20.9	4.01
20 μM Zn	20	3.76	TPEN+20 μM Zn	22.4	4.25
40 μM Zn	21	3.68	TPEN+40 μM Zn	20	4.16

Figure A.2. Zinc replenishment suppressed TPEN-induced DNA fragmentation in MDA-MB-231 cells. Cells were treated with TPEN (20 μM) plus zinc (0, 10, 20 or 40 μM) for 72 h. Cells treated with DMSO or zinc alone served as the corresponding controls. DNA content was analyzed by PI-staining flow cytometry. Apoptosis was indicated by cells with fragmented DNA. Distribution of cell debris, and apoptotic and live cells are located in the low-left, middle, and upper-right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B).

Appendix 3

(A)



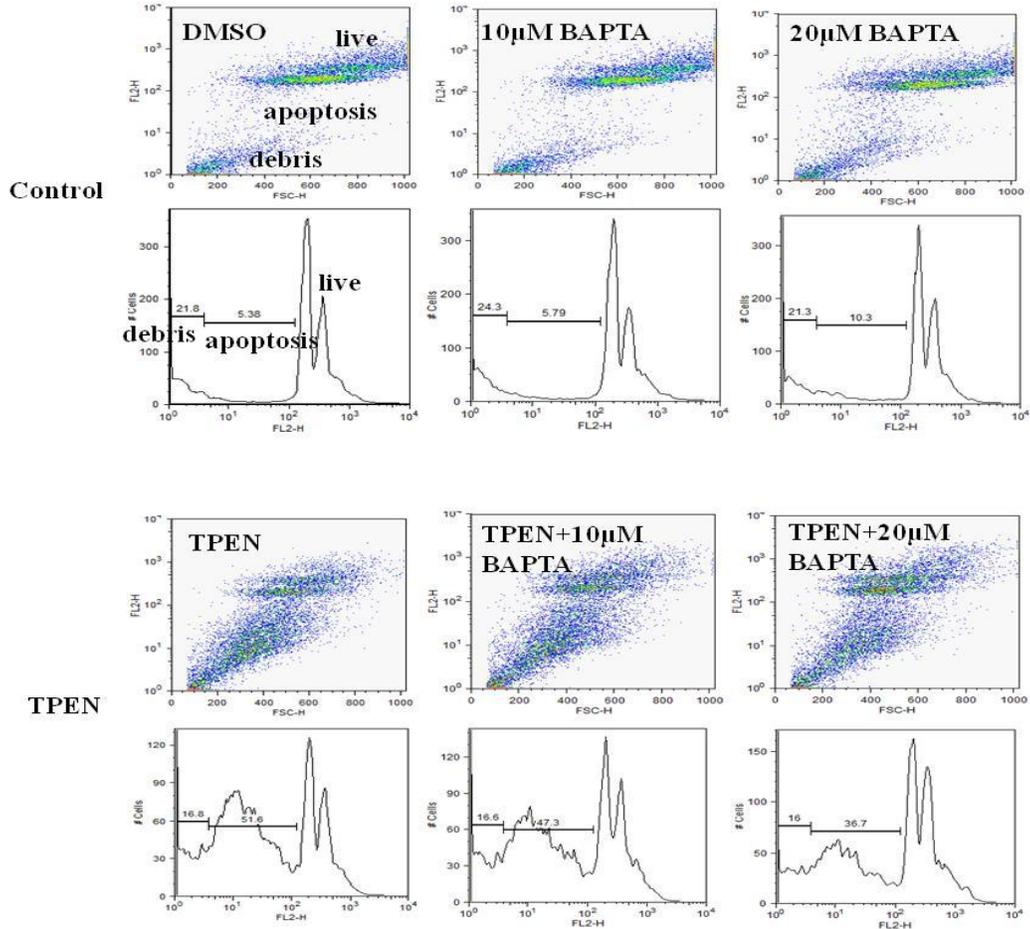
(B)

Control	Debris (%)	Apoptosis (%)	TPEN	Debris (%)	Apoptosis (%)
DMSO	15.4	3.38	TPEN	15.8	33.8
10 μM BAPTA	16.5	4	TPEN+10 μM BAPTA	14.3	25
20 μM BAPTA	15.8	5.95	TPEN+20 μM BAPTA	13.3	18.7

Figure A.3: Intracellular Ca²⁺ chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation (48 h). Cells were treated with TPEN (20 μM) plus BAPTA-AM (0, 10 or 20 μM) for 48 h. Cells treated with DMSO or BAPTA-AM alone served as the corresponding controls. DNA content was analyzed by PI-staining flow cytometry. Apoptosis was indicated by cells with fragmented DNA. Distribution of cell debris, and apoptotic and live cells are located in the low-left, middle, and upper-right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B).

Appendix 4

(A)



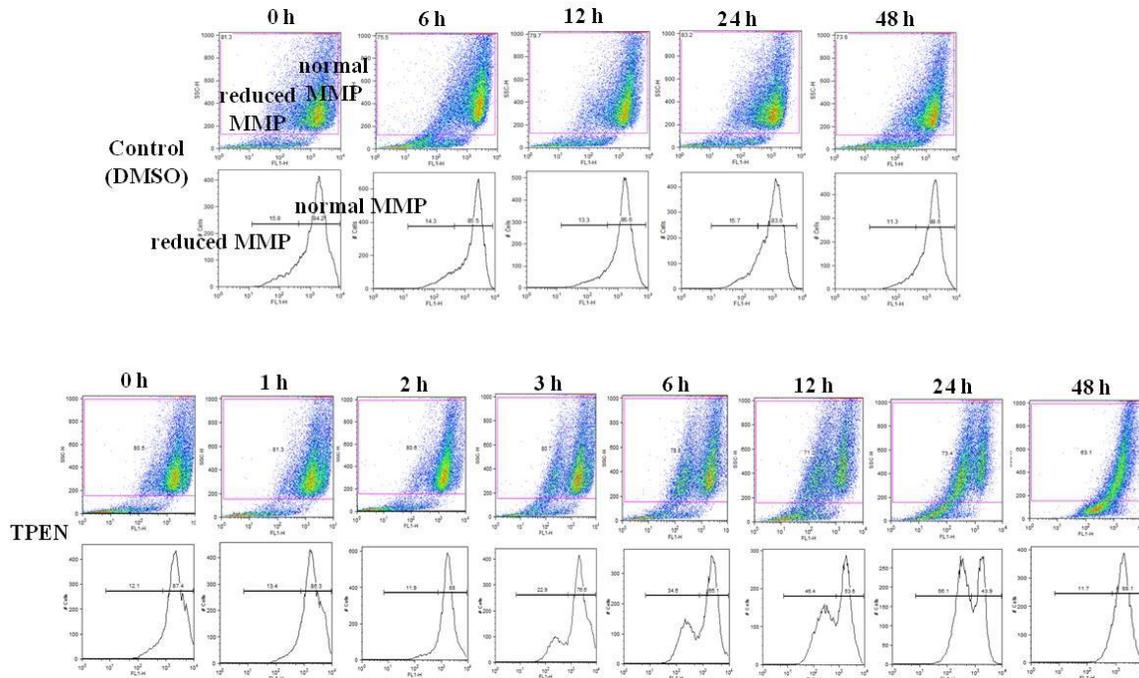
(B)

Control	Debris (%)	Apoptosis (%)	TPEN	Debris (%)	Apoptosis (%)
DMSO	21.8	5.38	TPEN	16.8	51.6
10 μ M BAPTA	24.3	5.79	TPEN+10 μ M BAPTA	16.6	47.3
20 μ M BAPTA	21.3	10.3	TPEN+20 μ M BAPTA	16	36.7

Figure A.4: Intracellular Ca^{2+} chelation with BAPTA-AM suppressed TPEN-induced DNA fragmentation (72 h). Cells were treated with TPEN (20 μ M) plus BAPTA-AM (0, 10 or 20 μ M) for 72 h. Cells treated with DMSO or BAPTA-AM alone served as the corresponding controls. DNA content was analyzed by PI-staining flow cytometry. Apoptosis was indicated by cells with fragmented DNA. Distribution of cell debris, and apoptotic and live cells are located in the low-left, middle, and upper-right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B).

Appendix 5

(A)



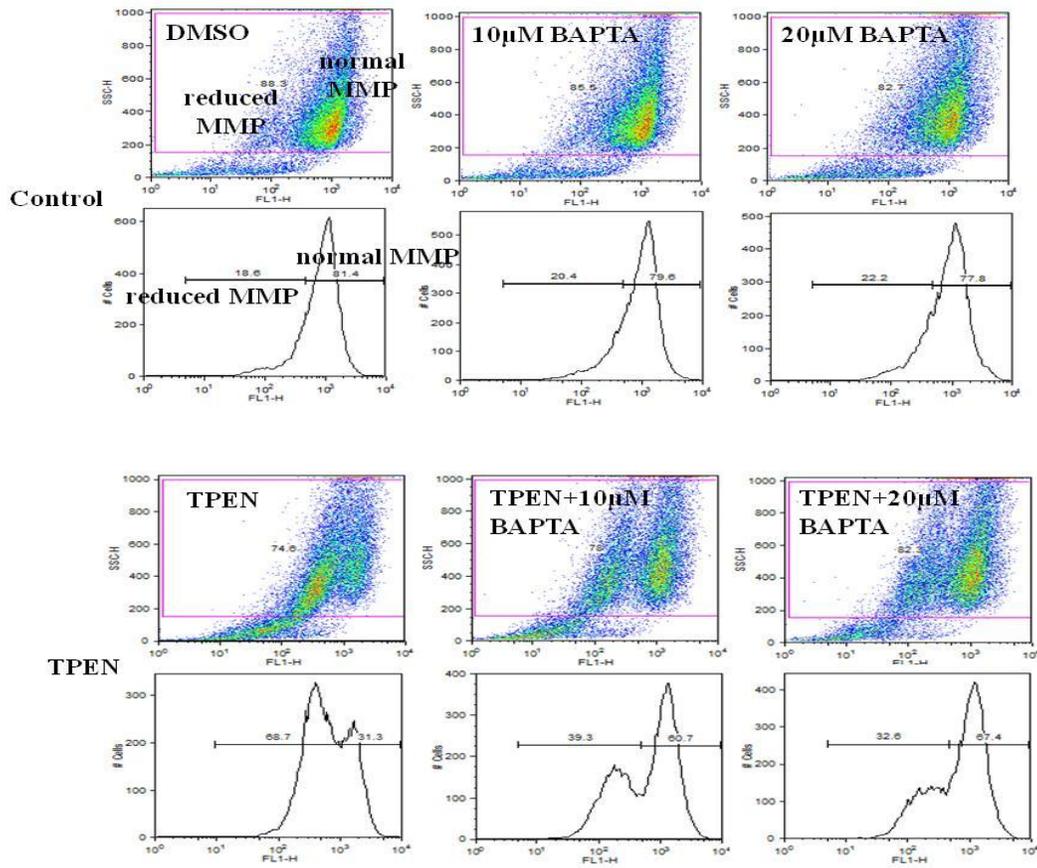
(B)

Control (h)	Reduced MMP (%)	Normal MMP (%)	TPEN (h)	Reduced MMP (%)	Normal MMP (%)
0	15.8	84.2	0	12.1	87.4
6	14.3	85.5	1	13.4	86.3
12	13.3	86.6	2	11.9	88
24	15.7	83.6	3	22.9	76.6
48	11.3	88.6	6	34.6	65.1
			12	46.4	53.6
			24	56.1	43.9
			48	11.7	88.1

Figure A.5: TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells. Cells were treated with TPEN (20 μ M) for 0, 1, 2, 3, 6, 12, 24 or 48 h. Cells treated with DMSO only served as the control. MMP was determined using DiOC₆ dye-staining flow cytometry. Distribution of cells with reduced MMP, and normal MMP are located in the left and right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B)

Appendix 6

(A)



(B)

Control	Reduced MMP (%)	Normal MMP (%)	TPEN	Reduced MMP (%)	Normal MMP (%)
DMSO	15.8	84.2	TPEN	12.1	87.4
10µM BAPTA	14.3	85.5	TPEN+10µM BAPTA	13.4	86.3
20µM BAPTA	13.3	86.6	TPEN+20µM BAPTA	11.9	88

Figure A.6: Intracellular Ca^{2+} chelation with BAPTA-AM prevented TPEN-induced loss of mitochondrial membrane potential in MDA-MB-231 cells. Cells were treated with DMSO only (Control), BAPTA-AM alone (10 or 20 µM), TPEN alone (20 µM), or TPEN plus BAPTA-AM (10 or 20 µM) for 24 h. MMP was determined by DiOC₆ dye-staining flow cytometry. Distribution of cells with reduced MMP, and normal MMP are located in the left and right quadrants, respectively (upper panel of each treatment in A) and histogram (lower panel of each treatment in A), and tabled in B).