Estrogen regulation of anti-apoptotic Bcl-2 family member Mcl-1 expression in breast

cancer

BY

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For my family

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ABSTRACT

INTRODUCTION

Estrogen is implicated as an important factor in stimulating breast cancer cell proliferation, and presence of estrogen receptor (ER) is an indication of a good prognosis in breast cancer patients. Mcl-1 is an anti-apoptotic Bcl-2 family member that is often overexpressed in breast tumors, correlating with poor survival. Estrogen has been previously shown to regulate Bcl-2 family members, leading to an evasion of apoptosis, however the role of estrogen in regulating Mcl-1 expression is unclear. I hypothesize that estrogen increases the expression of anti-apoptotic gene Mcl-1 through binding of ERα to a half estrogen response element (ERE) site within the promoter of Mcl-1 gene. This leads to increased Mcl-1 expression in breast cancer cells, ultimately contributing to an evasion of apoptosis.

METHODS

Four distinct breast cancer cell lines: MCF-7 and ZR-75, which both express ERα, and SKB-BR-3 and MDA-MB-231, which do not express ERα, to investigate the role of estrogen plays in regulating Mcl-1 expression. Cells were grown in serum-starved white media with charcoal-stripped FBS for five days prior to treatment with estrogen. Cells were treated with ERα antagonists Tamoxifen and Fulvestrant in combination with estrogen. Also, siRNA knockdown of ERα was performed and mRNA expression was evaluated. Chromatin immunoprecipitation (ChIP) was used to investigate if ERα binds to a specific ERE half-site within the Mcl-1 promoter. To further validate this data, a streptavidin pull-down assay was performed using a biotin-labeled probe specific to this region.

RESULTS

In ERα positive cell lines, estrogen treatment increased Mcl-1 expression at both the protein and mRNA level. In two ERα negative cell lines, SK-BR-3 and MDA-MB-231, estrogen failed to increase in Mcl-1 protein expression. ERα antagonists decreased estrogen mediated Mcl-1 expression at both the protein and message level. Upon knockdown of ERα, Mcl-1 mRNA expression after estrogen treatment was also decreased. ChIP showed an enrichment of ERα to the Mcl-1 promoter at a region 3683 bp upstream of the translation start site containing a half ERE site. Streptavidin-pull down assay showed both ERα and transcription factor Sp1 bind to this region and mutation of the half ERE site eliminated this binding.

CONCLUSIONS

These results suggest that estrogen is involved in regulating Mcl-1 expression specifically through a mechanism involving ERα. Ultimately, a better understanding of the role of estrogen in regulating Mcl-1 expression will determine whether Mcl-1 is a valid molecular target for breast cancer therapy.

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α	alpha
β	beta
μ	micro
μg	microgram
μL	microliter
μM	micromolar
%	percent
°C	degree Celsius
A	ampere
AF1	activating function 1
AF2	activating function 2
AI	aromatase inhibitors
Akt	Ak thymoma
AIF	apoptosis-inducing factor
AP-1	activator protein 1
Apaf-1	apoptotic protease activating factor 1
Asp	asparagine
Bad	Bcl-2 associated death promoter protein
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-B	B-cell lymphoma B
Bcl-w	B-cell lymphoma w
Bcl-$_{xl}$	B-cell lymphoma extra large
BCT	breast-conserving therapy
BH1-4	Bcl-2 homology domain 1-4
BCA	bicinchoninic acid
Bid	BH3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Blk	B lymphocyte kinase
Bmf	Bcl-2 modifying factor
Bnip3	Bcl-2 E1B 19 kDa protein-interacting protein 3
bp	base pair
BRCA1	breast cancer type 1 susceptibility protein
BRCA2	breast cancer type 2 susceptibility protein
Ca	calcium
cDNA	complimentary DNA
ChIP	chromatin immunoprecipitation
CK	cytokeratin
Cl	chloride
CO_2	carbon dioxide
CREB	cAMP response element-binding protein
DCIS	ductal carcinoma *in situ*
ddH$_2$O	double distilled water
DISC	death-initiating signaling complex

DMEM	Dulbecco's modified essential medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DR	death receptor
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
E2	estrogen
ECL	enhanced chemiluminescence reagent
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERBB2	V-Erb-B2 Erythroblastic Leukemia Viral Oncogene Homolog 2
ERE	estrogen response element
Erk1/2	Extracellular signal-regulated kinases 1/2
ETOH	ethanol
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
g	gram
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCl	hydrochloric acid
Hepes	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt
HER2	human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis proteins
ICI-182,780	Fulvestrant
IgG	immunoglobulin G
IP	immunoprecipitation
JAK	Janus kinase
K	potassium
KCl	potassium chloride
kDA	kilodalton
KH_2PO_4	Monopotassium phosphate
ki67	Antigen KI-67
L	litre
LCIS	lobular carcinoma *in situ*
LiCl	lithium chloride
M	molar
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia-1
Mcl-1$_s$	myeloid cell leukemia-1 short
$MgCl_2$	magnesium chloride

microRNA	micro ribonucleic acid
mL	milliliter
mm	milimeter
mM	millimolar
MMAC1	mutated in multiple advanced cancers 1
MOMP	mitochondrial outer membrane permeabilization
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
n	nano
Na	sodium
Na_2HPO_4	disodium phosphate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nM	nanomolar
N-P40	Nonidet-P40
NT	non-treated
OMM	outer mitochondrial membrane
P13K	Phosphoinositide 3-kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEST	proline, glutamic acid, serine, threonine rich domain
PgR	progesterone receptor
pGSK3	phospho Glycogen synthase kinase 3
pH	power of hydrogen
Poly dI-dC	Poly(deoxyinosinic-deoxycytidylic) acid sodium salt
PTEN	phosphatase and tensin homolog
qPCR	quantitative real-time polymerase chain reaction
Ras	rat sarcoma
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SERM	selective estrogen receptor modulator
SERD	selective estrogen receptor downregulator
SF-1	steroidogenic factor 1
SFRE	steroidogenic factor 1 response element
SH2	Src homology 2
SH3	Src homology 3
siRNA	small interfering ribonucleic acid
Smac	second mitochondrial-derived activator of caspases
SOS	son of sevenless

Sp1	specificity protein 1
Sp3	specificity protein 3
STAT	signal transducers and activators of transcription
Stat-3	signal transducers and activators of transcription 3
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20
TE	Tris-EDTA buffer
TEMED	N,N,N,N;-tetramethylethylenediamine
TM	transmembrane domain
TNF	tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
w/v	weight/volume
V	volts
X	times

1. INTRODUCTION

1.1 Hallmarks of Cancer

Cancer is a highly complex disease that results in uncontrolled cell proliferation, tissue invasion and metastasis (1). This occurs through a gradual accumulation of genetic changes involving both oncogenes and tumor suppressor genes (2-3).

In a healthy individual, proto-oncogenes are involved in regulating normal cell growth. In cancer, deleterious mutations can occur, allowing for the transformation of proto-oncogenes into oncogenes, resulting in uncontrolled cell proliferation and tumor growth. In addition, tumor suppressor genes, which normally act by inhibiting unnecessary cell division, may undergo a loss of function mutation. This results in unrestrained cell growth that allows for tumor formation (2).

As a cell becomes increasingly malignant, there are a number of changes that allow for uncontrolled cell division and growth. These changes can be understood as hallmarks of cancer progression and include: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (4).

A cancer cell can acquire the ability to produce its own growth signals, thereby allowing it to become self-sufficient and grow uncontrollably. A cancer cell can become insensitive to antigrowth signals, meaning that normal cell cycle controls are disrupted, allowing for unrestricted cell proliferation and growth. Evasion of apoptosis is another important hallmark, as apoptosis, or programmed cell death, is a tightly regulated process that

controls the cell population. In cancer, apoptotic pathways are often disrupted, allowing the cell to become resistant to apoptotic cell death. A cancer cell can also acquire the ability to replicate unrestrictedly without undergoing senescence. In addition, sustained angiogenesis allows for the growth of new blood vessels, resulting in the formation of large tumors. Finally, the combined effect of these cellular changes results in the cancer cell's acquired ability to undergo tissue invasion and metastasis, which allows for the cancer to spread into neighboring tissues and organs (4).

Recently, Hanahan and Weinberg (5) have added two new emerging hallmarks as well as two enabling characteristics. First, the acquired ability to reprogram energy metabolism provides the cancer cell with alternative mechanisms for attaining fuel for cell growth. Secondly, the ability for a cancer cell to evade immune destruction allows for the suppression of the immune system that may otherwise limit its growth. While not distinct hallmarks of cancer, genetic instability and inflammation are important enabling characteristics that promote tumor development and growth (5).

1.2 Breast Cancer

1.2.1 Incidence

According to the Canadian Cancer Society's statistics for 2013 (6), it is estimated that in Canada, approximately 23 800 women and 200 men will be diagnosed with breast cancer. Of these diagnoses, approximately 5 000 women and 60 men will die from their disease. This data demonstrates that in 2013, breast cancer cases make up 26% of all new cancer cases among

women. As well, 14% of all cancer related deaths among women result from breast cancer. Currently, breast cancer is the most commonly diagnosed cancer among women, excluding non-melanoma skin cancers. In addition, it is the 2nd leading cause of cancer related mortality among women (6). Breast cancer typically presents in older patients, however, younger women, especially those with a family history of breast cancer, may also develop the disease (7).

1.2.2 Clinical Features

1.2.2.1 Histology

According to the World Health Organization, there are approximately 18 diverse histological subtypes of breast cancer (8). Breast cancer typically occurs in the breast parenchyma or accessory breast tissue, with the bulk of cancer occurring in the upper outer region of the breast (7). The majority of malignant breast tumors are adenocarcinomas, meaning that they arise from glandular tissue within the epithelium (9). Typically, breast adenocarcinomas can be divided into two major types: ductal carcinoma, arising from the lining of the milk ducts, and lobular carcinoma, arising from the milk lobules (9). Furthermore, each subtype of adenocarcinoma can be further classified as invasive or non-invasive (7, 10).

Ductal carcinoma *in situ* (DCIS) is a non-invasive tumor that is often a precursor of invasive ductal carcinoma (9). Studies have shown that the presence of DCIS results in a 10-fold increased risk of developing invasive carcinoma (9). The location of DCIS within the breast typically indicates where invasive carcinoma may occur (9). While less than 10% of female patients have a palpable mass, the incidence of DCIS has increased with the advance of

screening technologies, such as mammography (9-10). The histologic patterns of DCIS include comedo, which is the most common, followed by micropapillary, cribriform, solid and papillary (9). The comedo type is associated with a high tumor grade and a poor prognosis (9). DCIS is heterogeneous and may present as low, intermediate or high-grade, each associated with different gene expression profiles (11). Low-grade DCIS is associated with monomorphic, evenly spaced cells, whereas high-grade DCIS is associated with pleomorphic, irregularly spaced cells (12). In addition, high-grade DCIS presents with large, irregular nuclei, coarse chromatin and a high occurrence of mitoses (12). As well, low-grade DCIS is associated with a loss of chromosome 16q, whereas high-grade DCIS is associated with a gain of chromosome 17q (12-13). A DCIS that presents with a high-grade tumor, palpable masses and a diameter larger than 5 cm is associated with a high risk of transforming into invasive carcinoma (10).

Multiple studies have suggested that the majority of DCIS eventually transforms into invasive carcinoma, as evidenced by the many common traits and chromosomal abnormalities shared between the two tumor types (11). In tumorigenesis, DCIS may represent an intermediate stage between normal ductal lumen and invasive carcinoma (11). Burstein et al. (11) suggest that DCIS represents a stage of cancer where the majority of genetic and molecular changes required for transformation into invasive carcinoma have already occurred. However, this idea remains controversial, as not all DCIS will transform into invasive carcinoma (10). Instead, DCIS and invasive carcinoma may arise from a common progenitor; yet evolve along separate pathways (10).

Unlike DCIS, lobular carcinoma *in situ* (LCIS) is not malignant, but instead acts as a marker for an increased risk of invasive carcinoma. This marker indicates that invasive carcinoma may be of either ductal or lobular type and may occur in either one breast, or both,

regardless of which breast LCIS first presents itself. LCIS does not require immediate treatment and should instead be closely followed over time (9).

Invasive carcinoma represents a heterogeneous group of malignant tumors with the majority arising from ductal or lobular tissues. The most common type of invasive carcinoma are invasive ductal carcinomas, followed by invasive lobular carcinoma, making up 70-75% and 5-15% of total invasive carcinomas, respectively. In addition, approximately 5% of invasive cancers may be classified as having both ductal and lobular components. As well, there are a large number of rare invasive cancer types, such as, but not limited to: invasive tubular carcinoma, invasive cribriform carcinoma, invasive mucinous carcinoma, invasive medullary carcinoma, invasive micropapillary carcinoma and invasive metaplastic carcinoma. In addition, there are many other less frequent malignant breast tumors, such as inflammatory breast cancer and Paget disease of the nipple (7).

1.2.2.2 Risk Factors

There are a number of risk factors associated with breast cancer, such as personal or familial history of cancer, presence of BRCA1 and BRCA2 mutations, Ashkenazi Jewish heritage, dense breasts, exposure to ionizing radiation, hormone replacement therapy, hormonal contraceptives, and lifestyle choices such as diet, smoking and alcohol consumption (14-20). In addition, the risk of breast cancer increases with increasing age (18). As well, females who experience an early menstruation, late menopause, or late or non-pregnancy, may be at higher risk of developing breast cancer (21-22).

1.2.2.3 Hereditary Features

While many cases of breast cancer arise sporadically, approximately 5-10% of breast tumors are associated with inherited genetic abnormalities (23). There are a number of high-penetrance mutations that are associated with a high risk of developing breast cancer, such as mutations within *BRCA1* and *BRCA2* (24-25). *BRCA1* and *BRCA2* are tumor suppressor genes that function in DNA damage repair (14, 25-26). The presence of *BRCA1* and *BRCA2* mutations are associated with a high lifetime risk of developing breast cancer. While the overall frequency of these genes are rare within the general population, approximately 50% and 30% of familial breast cancer cases are associated with mutations in *BRCA1* and *BRCA2*, respectively (20, 27).

The *BRCA1* mutation is frequently expressed in the Ashkenazi Jewish population, as approximately 1% carry the *BRCA1* 185delAG mutation, 0.1-0.2% carry the *BRCA1* 5382insC mutation and 1.3% carry the *BRCA2* 6174elT mutation (14, 27, 28-30). In addition, a 5 bp deletion occurring within exon 9 at nucleotide 999 within *BRCA2* (999del5 mutation) is associated with the Icelandic population, occurring with a frequency of 0.4% (14, 31-32).

As well, there are a number of other high penetrance mutations associated with hereditary breast cancer, such as mutations within the *TP53* tumor suppressor gene in Li-Fraumeni Syndrome and mutations in *PTEN/MMAC1* in Cowden Disease. While Li-Fraumeni Syndrome is rare, germline mutations within the TP53 gene are highly penetrant and occur in approximately 50% of Li-Fraumeni pedigrees. These mutations are associated with an increased risk of developing early-onset breast cancer. In Cowden Disease, mutations within

PTEN/MMAC1 are autosomal dominant and carriers are associated with a 30% increased risk in developing breast cancer (14).

1.2.2.4 Staging

Breast cancer is staged using the guidelines set by the American Joint Committee on Cancer (AJCC) TNM system. This system classifies breast cancer based on the tumor size ("T"), whether the cancer has spread to lymph nodes ("N") and if the cancer has metastasized ("M") (Table 1A). The combination of tumor size, lymph nodes and presence of metastasis are further categorized into breast cancer stages 0-IV (Table 1B) (33).

According to the National Cancer Data Base, the 5-year survival rate of a patient decreases with increasing breast cancer stage. For example, in 2001-2002, a Stage 0 breast cancer patient had a 93% 5-year survival rate, whereas a Stage IV breast cancer patient had a 15% 5-year survival rate. The 5-year survival rates are summarized in Table 1C (34).

All tables adapted from American Cancer Society
< http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-staging>

Table 1. Definitions of AJCC TNM System.

TUMOUR	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	≤ 2 cm

T2	≥ 2 cm - ≤ 5 cm
T3	≥ 5 cm
T4	Tumor of any size growing into the chest wall or skin.
NODE	
NX	Nearby lymph nodes cannot be assessed
N0	Cancer has not spread to nearby lymph nodes
N1	1-3 axillary lymph nodes affected and/or mammary lymph nodes affected
N2	4-9 axillary lymph nodes affected and/or enlarged mammary lymph nodes
N3	≥ 10 axillary lymph nodes affected; cancer has expanded to lymph nodes under clavicle; enlarged mammary lymph nodes
METASTASIS	
MX	Presence of metastasis cannot be assessed.
M0	No distant spread is found on x-rays (or other imaging procedures) or by physical exam
M1	Spread to distant organs is present

Table 2. AJCC breast cancer stages using TNM System.

STAGE	TUMOUR	NODE	METASTASIS
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0 - T1	N1mi	M0
IIA	T0 - T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0 - T2	N2	M0
	T3	N1 - N2	M0
IIIB	T4	N0 - N2	M0

| IIIC | Any T | N3 | M0 |
| IV | Any T | Any N | M1 |

Table 3. National Cancer Data Base 5-year survival rates for breast cancer stages 0-IV.

STAGE	5-YEAR SURVIVAL RATE
0	93%
I	88%
IIA	81%
IIB	74%
IIIA	67%
IIIB	41%
IIIC	49%
IV	15%

1.2.2.5 Molecular Subtypes

Breast cancer is a highly complex, heterogeneous disease that can be categorized into a number of intrinsic molecular subtypes (35-36). The most common molecular subtype is luminal A, which comprises approximately 50-60% of all breast cancer cases (35-36). Luminal A is characterized as expressing estrogen receptor (ER), progesterone receptor (PgR), B-cell lymphoma 2 (Bcl-2) and cytokeratin CK8/18 (35). Luminal A tumors do not express human epidermal growth factor receptor 2 (HER2) and have low cellular proliferation, as determined by Ki67 expression (35). In addition, luminal A tumors are associated with a good prognosis and a low tumor grade, with a relapse rate of 27.8% (35, 37). Patients with luminal A breast tumors are typically treated with hormonal therapy such as aromatase inhibitors (AIs) or selective estrogen receptor modulators (SERMs) like Tamoxifen (35).

Luminal B comprises approximately 10-20% of all breast cancer and is characterized as having a higher tumor grade and increased cellular proliferation compared to luminal A (35-36,

38). Patients with luminal B tumors have a more aggressive form of breast cancer with a poorer prognosis. The majority of luminal B tumors express ER, PgR and Ki67 but not HER2-. However, a small subset of luminal B tumors may express HER2. Treatment for luminal B tumors remains an issue, as they do not respond well to AIs or SERMs, however, some luminal B tumors do respond well to neo-adjuvant chemotherapy (35, 38).

HER2/ErbB2 positive tumors represent 15-20% of all breast cancer and are characterized by the expression of the HER2/ErbB2 gene (35, 36). HER2/ErbB2 positive tumors express high levels of proliferation and have a high tumor grade (35). Approximately 40% of HER2/ErbB2 positive tumors also express *TP53* mutations (35). In the past, HER2 positive patients had a poor prognosis, however, the advance of HER-2/ErbB2-targeted monoclonal antibody therapies, such as the drug trastuzumab (Herceptin), have positively impacted patient survival (35, 39).

Basal-like tumors comprise 10-20% of breast cancer and are characterized by the expression of cytokeratins CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and epidermal growth factor receptor (EGFR) (35-36). In addition, they do not express ER, PgR or HER2, and are often called Triple Negative. However, it should be noted that not all Triple Negative tumors are basal-like. Patients with basal-like tumors have a poor prognosis and a higher relapse rate compared to luminal tumors (35). As well, they are often associated with BRCA1 germ-line mutations and p53 mutations. While these tumors respond well to chemotherapy, there remains a high relapse rate, requiring superior targeted therapies (35).

Normal breast-like represents 5-10% of all breast tumors and unlike the other molecular subtypes, it is poorly characterized (35-36). They do not express ER, PgR or HER2 and do not respond well to neo-adjuvant chemotherapy (35). However, there is debate regarding whether

normal breast-like is a distinct molecular subtype or has been misclassified (35, 40).

Recently, microarray data has shown evidence of more molecular subtypes (35-36). For example, claudin-low is a relatively newer subtype that is categorized by a low expression of genes that are normally involved in cellular adhesion, such as ocludin and E-cadherin (35, 41). In addition, breast cancer tumors are also categorized as molecular apocrine and interferon-rich (36).

1.2.2.6 Diagnosis and Treatment

When a patient is diagnosed with breast cancer, they may be subject to a diagnostic mammogram, ultrasound and a needle biopsy. A diagnostic mammogram is a method of examining the breast using radiography. The mammogram produces an image of the breast that can be studied for the presence of calcifications and masses that would indicate if cancer were present. Ultrasonography may also be used as a diagnostic tool as it produces an image in real time using high frequency sound waves. While not as sensitive as mammography, ultrasonography may be preferred as it produces an image without using ionizing radiation (42). Recently, breast magnetic resonance imaging (MRI) may be used as part of the diagnostic process, as it is a more sensitive procedure than both mammography and ultrasonography. Currently, the use of breast MRI remains controversial, as it is expensive and may produce false positive results. In addition, there is not enough data to suggest that breast MRI is a superior diagnostic technique compared to mammography and ultrasonography (42-43).

Once a patient undergoes diagnostic imaging, a biopsy may be taken in order to confidently diagnose the cancer (42). Afterwards, the patient may be subject to surgery,

depending on the stage of their breast cancer (44). Since mammography has improved the

ability to detect cancer at earlier stages, many patients now have the option to undergo breast-

conserving therapy (BCT) (44-45). With BCT, the tumor, along with a small portion of healthy

tissue, is removed, followed by radiation therapy or chemotherapy (44-45). The success of BCT

ultimately depends on whether the surgeon is able to achieve tumor-free margins after surgery;

otherwise the risk of recurrence is greater (45). If the cancer is found in later stages, patients

often undergo a mastectomy, which is the complete removal of the breast (44).

In addition, a patient may undergo hormonal receptor and HER2 testing in order to

determine the molecular status of their cancer (42, 46). If a patient is diagnosed as ER positive,

they may be subject to neo-adjuvant endocrine therapy that specifically targets the ER.

Currently, the first line of response for ER positive patients is Tamoxifen, a selective estrogen

receptor modulator (SERM) that competitively inhibits estrogen from binding to its receptor

(Figure 1). While many patients respond well to Tamoxifen, resistance can still occur. This

resistance occurs as either *de novo* resistance, meaning that there is no initial response, or

acquired resistance, meaning that resistance develops over the course of treatment. As well,

Tamoxifen may engage in agonist activity depending on the differential expression of ER in

certain tissues. In order to counteract Tamoxifen resistance, another SERM, called Raloxifen,

has been developed, however studies have indicated that while it is less toxic, it does not have

superior activity compared to Tamoxifen (46).

As Tamoxifen displays agonist activity, selective estrogen receptor downregulators

(SERDs) have been developed in order to completely inhibit ER activity. One such SERD,

called Fulvestrant, binds to the ER and induces a conformational change that ultimately results

in ER degradation (Figure 1) (46-47). However, clinical trials have indicated that there is no

significant difference between Tamoxifen and Fulvestrant treatment (46). Fulvestrant is currently used as a second-line response (46).

A new class of estrogen inhibitors has emerged. Aromatase inhibitors (AIs) work by inhibiting aromatase activity, which prevents estrogen production (Figure 1). Currently, many third generation AIs have been developed, such as anastrozole, exemestane, and letrozole, and they have shown promising activity in postmenopausal ER positive patients. However, like Tamoxifen, drug resistance to AIs remains an issue (46).

Patients who express HER2 may be subject to monoclonal antibody therapy in the form of a drug called Herceptin (39). This monoclonal antibody targets ErbB2, which is overexpressed in HER2 positive patients (39). Studies have shown that Herceptin can improve the survival outcomes for patients expressing HER2 (48-50).

If a patient is Triple Negative, meaning that they do does not express ER, PgR or HER2, their current main treatment option is chemotherapy. In some situations, a patient may undergo neo-adjuvant chemotherapy in order to reduce the size of the tumor prior to surgery. In more advanced disease, a patient may undergo chemotherapy after surgery in order to prevent disease recurrence (51). In Triple Negative breast cancer, neo-adjuvant chemotherapy has been
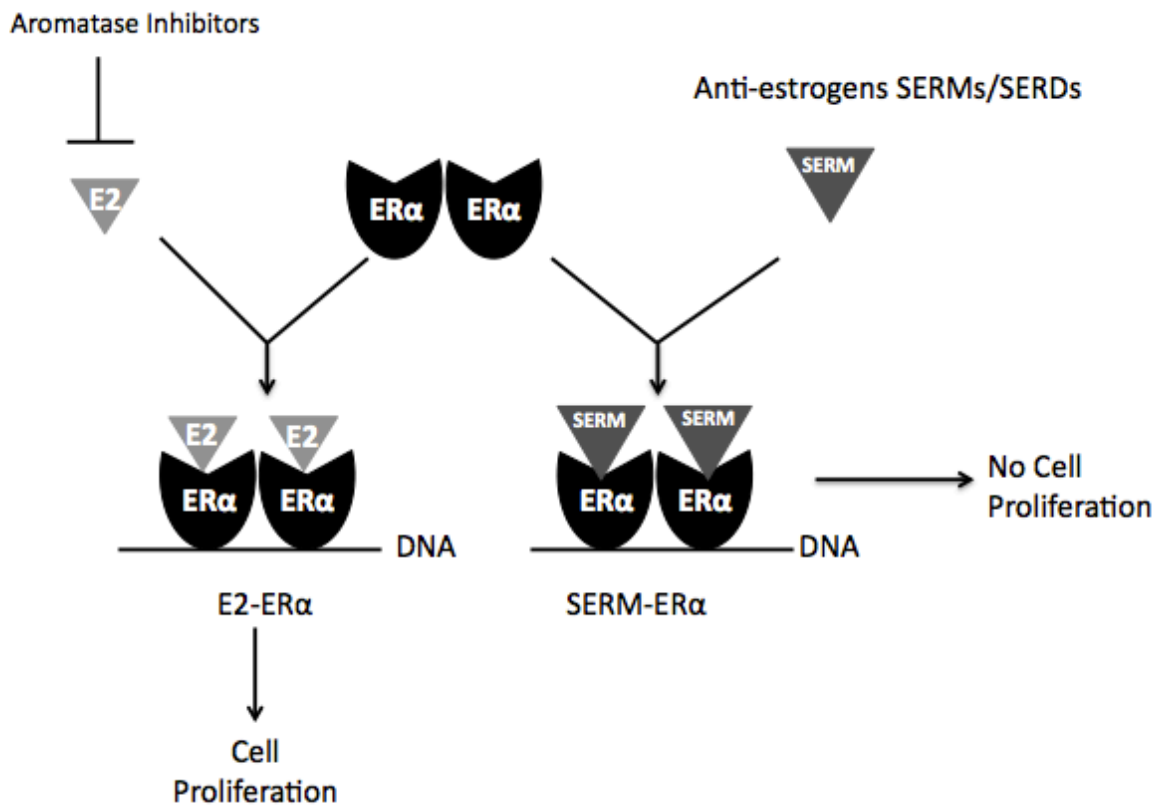
Figure 1. Mechanism of action on Estrogen Receptor alpha for the SERM Tamoxifen, SERD Fulvestrant and Aromatase Inhibitors. SERMs/SERDs inhibit estrogen from binding to ERα, preventing cell proliferation. Aromatase inhibitors block production of estrogen, also preventing cell proliferation.

Figure adapted from: <http://www.life.illinois.edu/shapiro/proliferation.html>

shown to increase the response rate to treatment, resulting in a better long-term outcome (52-53).

Finally, patients may undergo radiation therapy, which uses high-energy rays in order to remove malignant cells and tissues that may be left behind after surgery. Radiation therapy has been shown to increase survival and reduce recurrence rates in breast cancer patients (54).

1.3 Role of Estrogen

Estrogen is a steroid hormone that has been implicated as an important factor in stimulating cell proliferation in breast cancer (55). Working predominantly through the 17β-estradiol form, estrogen is involved in regulating cellular growth, development and differentiation within a variety of tissues (55-56). Estrogen regulates these important cellular activities through the estrogen receptor (ER) (55). The ER is part of a superfamily of nuclear receptors and is comprised of two subtypes, ERα and ERβ (55). Elwood Jensen first characterized ERα in the 1950's, whereas ERβ was not discovered until 1993 (56-58). The two receptor subtypes are located on different genes within different chromosomes; however, they share a high level of sequence homology within their DNA-binding domains (56, 59-61). ERα is located on chromosome 6q25.1 and ERβ is located on chromosome 14q23.2 (56, 59-61). ERα has been established as an important biomarker for breast cancer as its presence may indicate if hormonal therapy is a valid treatment option for patients (46). Approximately 40-70% of breast tumors express ERα and patients who present with ERα positive breast cancer are typically associated with a good prognosis (46, 59). The roles of ERα and ERβ differ, as ERα has a proliferative effect on cells, whereas the function of ERβ has yet to be fully

determined (46, 62). Recent literature suggests that ERβ may have two separate functions depending on the presence or absence of ERα (59, 62). When ERα and ERβ are expressed together, ERβ may act as a tumor suppressor, however, when ERβ is expressed alone, it may take on a proliferative role (59, 62).

The ER is regulated through two main mechanisms, a ligand-dependent pathway and a ligand-independent pathway (Figure 2) (55-56). The ligand-dependent pathway is the classical estrogen-signaling pathway, as it involves estrogen binding directly to its receptor (55). In turn, the ER can dimerize and bind to estrogen response element (ERE) sites within the promoter of a target gene (63).

 ERE sites are palindromic sequences that are found within many genes, either as full or half ERE sites (63). An ERE half-site is half of the palindromic ERE sequence and may involve other binding sites, such as SF-1 response elements (SFREs), or tethered transcription factors such as AP-1 (mediated through Fos and Jun) or Sp1 (59, 64). Once bound, ERE sites allow for the activation of a signaling cascade that ultimately leads to a response within target genes (55). ERα and ERβ share the same ERE sites, however, once bound they activate different transcription factors, co-activators and co-repressors (46).

The ligand-dependent pathway does not always involve direct binding of estrogen to its receptor, but instead estrogen may bind through other protein interactions or through rapid non-genomic effects (56). This stimulation of rapid non-genomic effects may lead to crosstalk between estrogen and growth factor signaling pathways, such as the mitogen activated protein kinase (MAPK) pathway, ultimately contributing to drug resistance (46, 65-66).
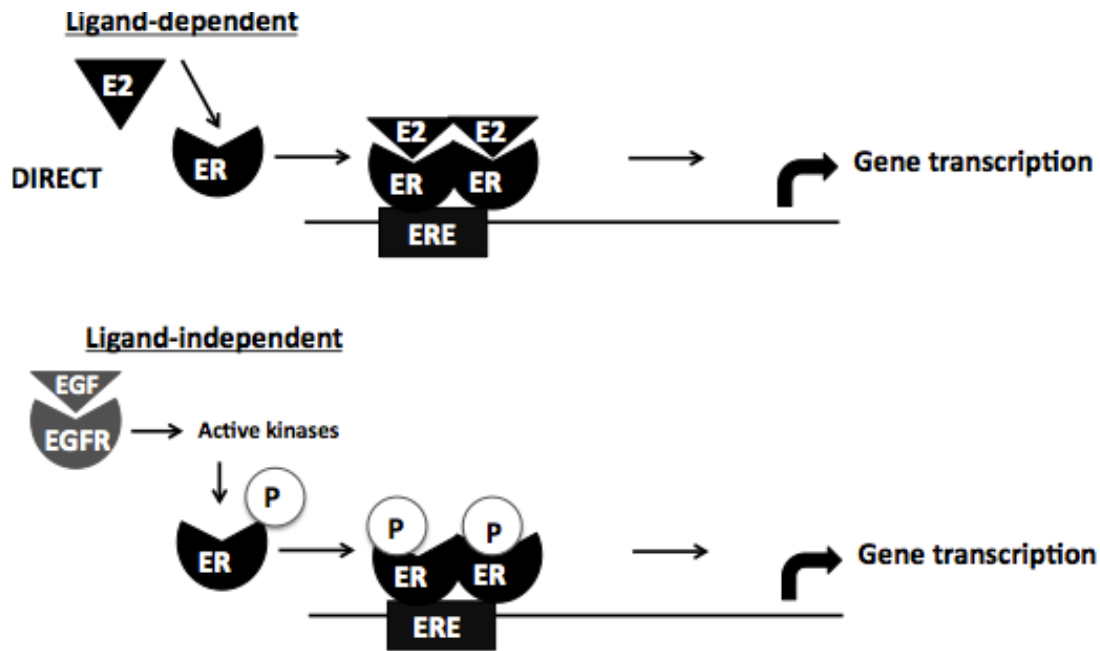
Figure 2. Regulation of Estrogen Receptor. Estrogen receptor is regulated through either a ligand-dependent pathway or a ligand-independent pathway. The ligand-dependent pathway involves either direct (shown) binding of estrogen to its receptor, binding through tethered interactions with transcription factors, or rapid non-genomic effects. The ligand-independent pathway involves phosphorylation of the estrogen receptor by growth factor signaling pathways. Figure adapted from Heldring et al. (56)

The ligand-independent pathway involves growth factor signaling that phosphorylates the ER, thus activating it without direct ligand binding (55-56). This pathway may contribute to drug resistance and hormone-independent growth in certain breast tumors (56, 59, 67). For example, the ER contains two activation function domains, AF1 and AF2 that are required for transcription (46, 59). Tamoxifen inhibits only AF2 sites within promoter of estrogen-regulated genes, leaving AF1 sites available for growth factor binding and activation (46, 59).

1.4 Role of Epidermal Growth Factor

Epidermal Growth Factor (EGF) plays an important role in regulating cellular proliferation and survival during mammalian development (68). It has been demonstrated to be upregulated in certain types of cancer, including breast cancer (69-70). EGF binds to four specific membrane receptors, called ErbB1, ErbB2, ErbB3, and ErbB4, where ErbB2 has an increased presence in some breast cancers, resulting in poor survival outcomes (68, 71). Once bound to its receptor, EGF can initiate multiple signaling cascades, such as the Ras/Erk (MAPK) pathway, JAK/STAT pathway and P13K/AKT pathway (Figure 3) (72).

Once the ErbB receptors are bound by ligands, they undergo either homo or heterodimerization that results in a signaling cascade (68, 73). While ErbB1, ErbB3 and ErbB4 may bind each other; their preferred binding partner is the ErbB2 receptor (68). Interestingly, ErbB2 is the only receptor that does not contain a ligand-binding domain (68, 74). In addition, ErbB3 does not contain a functional tyrosine kinase domain, therefore requiring it to act as a co-receptor with ErbB1, ErbB2 and ErbB4 (74). While ErbB2 and ErbB3 both lack specific components, they prefer to bind with each other and form the most active signaling dimer out
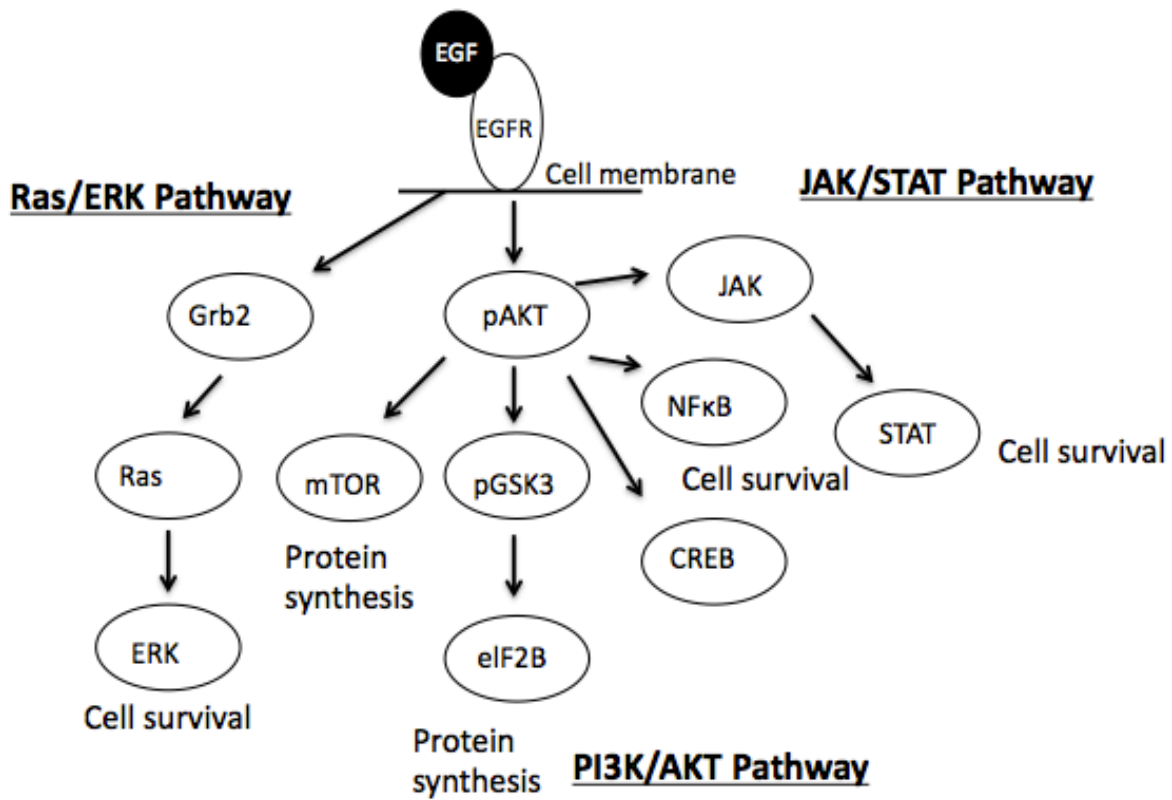
Figure 3. EGF initiates multiple signaling cascades such as the Ras/Erk (MAPK) pathway, JAK/STAT pathway and P13K/AKT pathway. These signaling cascades result in either cell survival or protein synthesis.

Figure adapted from:
< http://www.abcam.com/index.html?pageconfig=resource&rid=10723>

of all potential ErbB receptor combinations (39).

In cancer, the ErbB receptor family can be overexpressed and activate signaling cascades that result in uncontrolled cellular proliferation and an evasion of apoptosis (39). For example, HER2/ErbB2 is overexpressed in approximately 20% of breast cancer cases (75-77). Patients that show an overexpression of ErbB2 are often associated with a high tumor grade and a poor prognosis (75). Recently, targeted therapies towards EGF receptors ErbB1 and ErbB2, such as monoclonal antibodies Herceptin and Lapatinib have been developed (75). These treatments have shown an increase in disease-free survival and tumor regression for many patients (39, 70, 75). Unfortunately, drug resistance still remains an integral problem in treating patients, creating the need for superior targeted therapies (39).

1.4.1 Ras/Erk (MAPK) Pathway

EGF signaling cascades may result in the activation of the mitogen-activated protein kinase (MAPK) superfamily (Figure 4) (39). In order to activate the MAPK pathway, EGF first binds and phosphorylates an ErbB receptor. Once the tyrosine kinase receptor is phosphorylated, the adaptor proteins Grb2 or Shc bind to the ErbB receptor through their SH2 domains. This allows for the recruitment of guanylyl nucleotide-release protein son of sevenless (SOS), which binds Grb2 through its SH3 domain. After recruitment, SOS can exchange GDP for GTP, which activates the small G protein Ras. When Ras is activated, it can bind and phosphorylate mitogen-activated protein kinase kinase kinase Raf-1. This allows for active Raf-1 to phosphorylate mitogen activated protein kinase kinase Mek1/2. Active Mek1/2 phosphorylates mitogen activated protein kinase Erk1/2 (78). This allows for phosphorylated

Figure 4. Schematic of MAPK signaling pathway. EGF binds to its receptor (ErbB1 shown), Src homology 2 (SH2) domain recruits adaptor protein Grb2, activating signaling cascade. Guanyl-nucleotide-release protein SOS binds Grb2 via Src homology 3 (SH3) domain. SOS exchanges GDP for GTP, activating small G protein Ras. Activated Ras binds mitogen-activated protein kinase kinase kinase Raf-1. Activated Raf-1 phosphorylates mitogen activated protein kinase kinase Mek1/2. Activated Mek1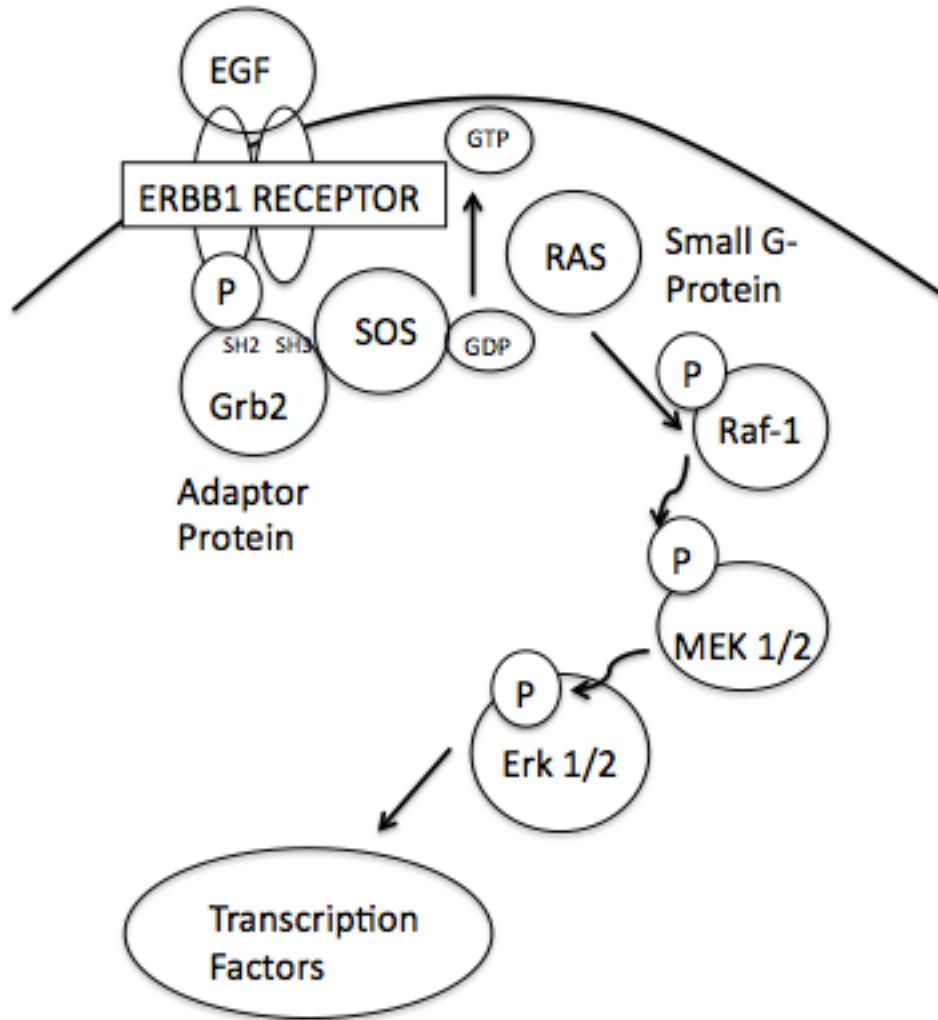/2 phosphorylates mitogen activated protein kinase Erk1/2. Once activated, Erk1/2 activates pathways that activate downstream transcription factors, leading to increased cell growth and survival.

Figure adapted from Henson and Gibson (39)

Erk to activate a number of downstream transcription factors, such as those affecting Bcl-2 family member Mcl-1, resulting in increased cell growth and survival (39, 78). In addition, the MAPK pathway may also be activated through crosstalk with other signaling pathways, such as either the PI3K/AKT pathway or estrogen signaling cascades (79-80).

1.5 Cell Death

In multicellular organisms, programmed cell death is an integral part of regulating the balance between life and death. Programmed cell death helps maintain cellular development and tissue homeostasis by carefully eliminating non-functional, damaged cells (81). Three major types of programmed cell death have been described, and include: type I, or apoptosis; type II, or autophagy; and type III, or programmed necrosis (81-82). Programmed cell death is a complex process where all three types may either function independently or work together in order to regulate cell death (83-85).

Type I programmed cell death, or apoptosis, is the best characterized type of cell death and is often deregulated in cancer (81-82, 86). Apoptosis is characterized by the formation of apoptotic bodies, chromatin condensation, nuclear fragmentation and membrane blebbing (81). Apoptosis is a tightly regulated process and will be discussed in more detail below.

Type II programmed cell death, or autophagy, is an important catabolic process that allows for the removal of damaged organelles (87-88). Autophagy is characterized by a double membrane vesicle called an autophagosome that engulfs cellular components and fuses with lysosomes in order to degrade the vesicle contents (87). This process is vital as it allows for the recycling of essential nutrients that may aid the cell during times of stress and starvation (89).

Autophagy is a caspase independent process that is subdivided based on how the lysosome receives cellular materials, and includes: macroautophagy, microautophagy and chaperone mediated autophagy (81). Autophagy as a method of programmed cell death remains controversial; it is involved in both cell survival and cell death processes and may protect cells from cell death depending on the environmental circumstances and stage of cancer development (85, 90).

Type III programmed cell death, or programmed necrosis, describes a series of carefully planned cellular events that ultimately result in necrotic cell death. It is characterized by a fragmented plasma membrane, swelling organelles and the appearance of large, hollow spaces within the cytoplasm that can merge together. Of all three types of programmed cell death, necrosis is the least well understood and may involve interplay with both apoptotic and autophagic cellular pathways (81). While necrosis is often considered a random and catastrophic form of cell death, programmed necrosis involves tightly regulated cellular events, such as those concerning the mitochondria, making it similar to apoptosis and autophagy (85, 91). In addition, programmed necrosis results in the release of cellular content that may result in an inflammatory response (85, 92). This may contribute to carcinogenesis, as an increase in inflammation can contribute to tumor development (85, 93-94).

1.5.1 Apoptosis

First described by Kerr et al. (95), apoptosis is a tightly regulated form of cell death that occurs in order to eliminate damaged and mutated cells without the initiation of inflammation (82, 96). Apoptosis is a highly conserved process that has been well documented in studies with

Caenorhabditis elegans (97-98). From these studies, three specific proteins, CED3, CED4 and CED9, were described as having a pivotal role in apoptosis (97-98). During survival conditions, CED3 is bound to CED9. However, under apoptotic conditions, CED9 is released from CED3 and is free to bind CED4, resulting in cell death. These proteins share a similar functional role to human proteins, for example, CED3 is homologous to caspases and CED9 is homologous to Bcl-2 family member proteins (99).

An apoptotic cell is typically characterized as one that has undergone cell shrinkage, chromatin condensation and nuclear fragmentation (Figure 5) (82). In addition, once a cell undergoes apoptosis, phosphatidylserine moves from the inner to the outer cell membrane, allowing for the recruitment of phagocytic proteins (82, 100). Apoptosis is a caspase-dependent process that involves either death receptor activation through the extrinsic pathway, or the Bcl-2 family and the mitochondria through the intrinsic pathway, which will be detailed below (99).
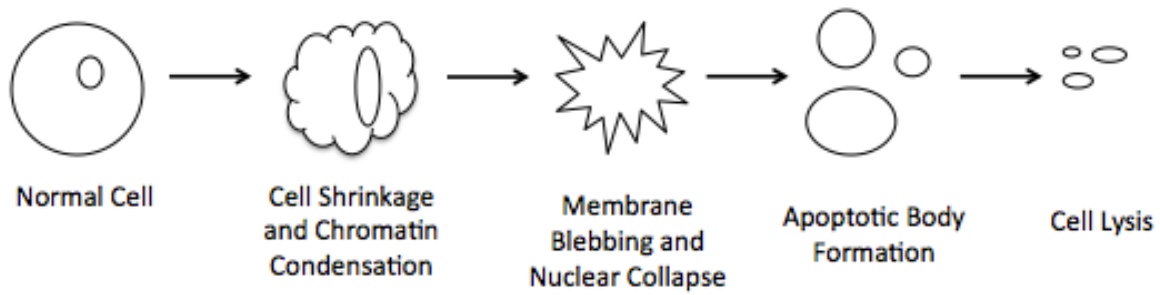
Figure 5. Generalized apoptotic pathway. In order to undergo apoptosis, a normal cell will first shrink and chromatin will condense. Next, the cell membrane will bleb and the cell will undergo nuclear collapse. This results in apoptotic body formation that will eventually be subject to cell lysis.

Figure adapted from:
< http://www.sameerkalghatgi.com/CancerTherapy.html>

1.5.1.1 Caspases

In order for apoptosis to occur, a family of cysteine proteases, called caspases, must be cleaved and activated, resulting in a caspase cascade that allows for the biochemical and morphological changes required for cell death (96, 99). Caspases are involved in both the extrinsic and intrinsic apoptotic pathway, functioning as both initiators and effectors (96). Apoptosis is initiated when an inactive form of caspases, called pro-caspases, are cleaved (101). This results in activating an upstream initiator caspase that can cleave downstream effector caspases, subsequently producing a caspase cascade that initiates apoptosis (96, 101). In humans, initiator caspases are comprised of caspase-8 and caspase-9, while effector caspases are comprised of caspase-3, caspase-6 and caspase-7 (101).

1.5.1.2 Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway involves the tumor necrosis factor (TNF) family of death receptors (Figure 6) (99). This pathway is initiated by the binding of a specific death ligand to a death receptor (DR), part of the TNF receptor gene super family, that is expressed on the cell surface (99, 102-103). There are a number of DRs that have been identified and play an important role in apoptosis, such as Fas (104). Once a death ligand binds to DR, an adaptor protein is required to allow for the recruitment and subsequent activation of caspases (99). For example, the death ligand FasL binds to Fas, allowing for the recruitment of an adaptor protein, called Fas-associated death domain (FADD) (105).
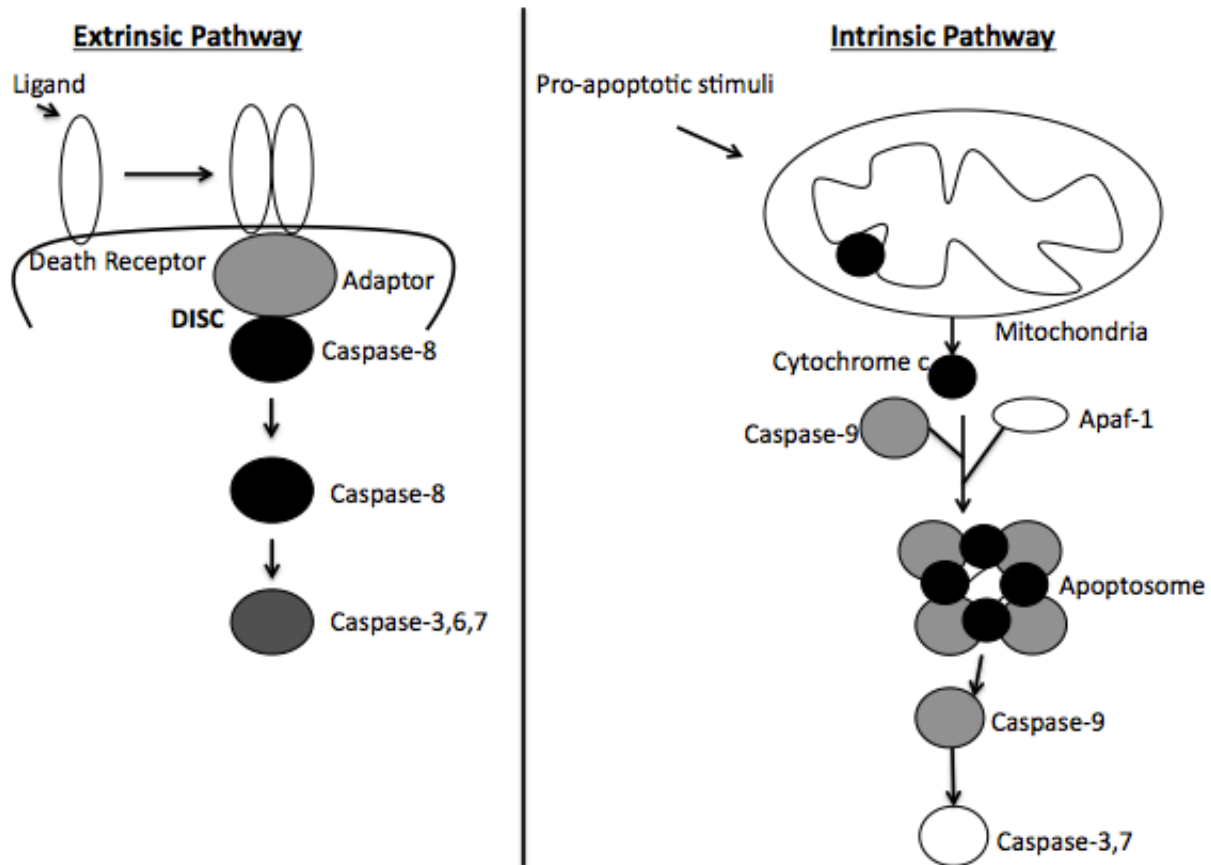
Figure 6. Schematic of extrinsic and intrinsic apoptotic pathways. In extrinsic pathway, death ligand binds to a death receptor, recruiting adaptor protein FADD, forming DISC complex that cleaves and activates caspase-8. Active caspase-8 cleaves and activates downstream effector caspases. In intrinsic pathway, pro-apoptotic stimuli initiate release of cytochrome c from mitochondria. This forms a complex with Apaf-1 and caspase-9 called the apoptosome. Caspase-9 is cleaved and activated. Once activated, caspase-9 can cleave and activate downstream effector caspases.

Figure adapted from:
< http://www.molecularbrain.com/content/5/1/15/figure/F1?highres=y>

Once FADD is recruited, a complex called death-inducing signaling complex (DISC) is formed, which then cleaves and activates pro-caspase-8 (82). Once activated, caspase-8 initiates a caspase cascade that results in the cleavage and activation of downstream effector caspases such as caspase-3 (99).

1.5.1.3 Intrinsic Apoptotic Pathway

The intrinsic pathway involves a caspase cascade mediated by Bcl-2 family members within the mitochondria (Figure 6) (82). This results in mitochondrial protein cytochrome c release, loss of membrane potential, and increased production of reactive oxygen species that eventually leads to cell death (82). In order to initiate the intrinsic apoptotic pathway, the mitochondria release cytochrome c, which then binds to apoptotic protease-activating factor-1 (Apaf-1) (106). Together, cytochrome c and Apaf-1 form a complex called an apoptosome that allows for the cleavage and activation of initiator pro-caspase-9, resulting in a caspase cascade (99, 106). In addition, the mitochondria can also release Smac/DIABLO, which activates caspases by interacting with the inhibitor of apoptosis (IAP) protein family (82, 107-108).

1.5.1.4 Bcl-2 Family

The intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins, which are comprised of both pro-apoptotic and anti-apoptotic members (Figure 7) (82). When a cell is not undergoing apoptosis, the pro-apoptotic proteins will form heterodimer associations with the anti-apoptotic proteins, therefore preventing cell death from occurring (82, 109). However,

under apoptotic conditions these heterodimers will disassociate, allowing the pro-apoptotic

proteins to form new associations that allow for apoptosis to occur (109). The majority of Bcl-2

family members associate with the mitochondria, however some members, such as Bax, are

primarily found in the cytosol and only move to the mitochondria when the cell is undergoing

apoptosis (109). In order to initiate apoptosis, Bcl-2 family members are regulated through

post-translational modifications such as phosphorylation and cleavage (85). In addition, Bcl-2

family members are also regulated by the expression levels of their members and their

localization within the cell (82). In many types of cancer, Bcl-2 family members are often

deregulated, resulting in an evasion of apoptosis by cancer cells (95, 110).

The Bcl-2 family mainly regulates the intrinsic pathway, however, there can be

crosstalk between both the intrinsic and extrinsic pathways (82). For example, the Bcl-2 family

member Bid is cleaved by caspase-8, which is part of the extrinsic apoptotic pathway (111).

The Bcl-2 family members are highly conserved and share regions of homology known

as Bcl-2 homology (BH) domains (82, 112). They are divided into three separate groups based

on this homology, which will be discussed further below (113).

Anti-Apoptotic

| BH4 | BH3 | BH1 | BH2 | TM | Eg. Bcl-2, Bcl-X$_L$, Mcl-1 |

Pro-Apoptotic

| BH3 | BH1 | BH2 | TM | Eg. Bax, Bak, Bok |

Multi-domain

| BH3 | TM | Eg. Bim, Bad |

| BH3 | Eg. Bid, Puma, Noxa |

BH3-only

Figure 7. Members of the Bcl-2 protein family. Bcl-2 family members are divided into 3 groups: anti-apoptotic, pro-apoptotic multi-domain and pro-apoptotic BH3-only proteins.

Figure adapted from:
<http://www.nature.com/nrm/journal/v9/n3/box/nrm2312_BX1.html>

1.5.1.4.1 Pro-apoptotic Bcl-2 Family Members

The pro-apoptotic Bcl-2 family members are divided into two groups, depending on their sequence homology and functional roles. The first group are homologous in their BH1-BH3 domains and consists of members: Bax, Bak, and Bok. The second group shares only the BH3 domain and consists of members: Bid, Bik, Bnip3, Bad, Bim, Blk, Bmf, Hrk, Noxa and Puma (113).

Currently, there are two models to explain how BH3-only proteins regulate apoptosis. In the Direct Activation model, activator BH3-only proteins, such as Bid, Bim and Puma, bind directly to pro-apoptotic proteins Bax and Bak, forming complexes that allow for initiation of apoptosis. As well, sensitizer BH3-only proteins, such as Bad, Noxa, Bik, Bmf, Hrk, and Bnip3, bind and sequester anti-apoptotic proteins, allowing for activator BH3-only proteins to initiate mitochondrial outer membrane permeabilization (MOMP). In the Displacement Model, pro-apoptotic proteins Bax and Bak are constitutively active. In order to initiate apoptosis, BH3-only proteins displace Bax and Bak from anti-apoptotic proteins, allowing for Bax and Bak oligomerization and MOMP initiation (114). For example, BH3-only protein Noxa sequesters anti-apoptotic Mcl-1 away from Bak, forming a Noxa-Mcl-1 complex that inhibits Mcl-1 activity, allowing apoptosis to occur (115). The role of BH3-only proteins in evading apoptosis will be discussed below.

1.5.1.4.2 Anti-apoptotic Bcl-2 Family Members

The anti-apoptotic proteins consist of one group that shares sequence homology in all

four BH domains (BH1-BH4). This group consists of members: Bcl-2, Bcl-$_{xl}$, Bcl-w, Mcl-1, Boo, and Bcl-B (113).

In order to evade apoptosis, the Direct Activation model suggests that anti-apoptotic proteins sequester pro-apoptotic proteins Bax and Bak by binding to activator BH3-only proteins. In the Displacement Model, pro-apoptotic proteins form heterodimers with anti-apoptotic proteins, preventing apoptosis from occurring. This allows for BH3-only proteins to regulate apoptosis by interacting with these heterodimer conformations (114). For example, under survival conditions, pro-apoptotic Bak can form a heterodimer with anti-apoptotic Mcl-1, allowing for inhibition of apoptosis (115).

1.5.1.4.3 Bcl-2 Family Members in Cancer

In cancer, anti-apoptotic Bcl-2 family members are often overexpressed, resulting in evasion of apoptosis and resistance to chemotherapy and radiation therapy (85, 116). Pro-apoptotic proteins are often downregulated by alterations in signaling pathways or mutations within pro-apoptotic genes (116). For example, anti-apoptotic protein Bcl-2 is frequently overexpressed in many types of cancer, including breast cancer, resulting in a poor prognosis (116). Studies have shown that overexpression of Bcl-2 results in resistance to cancer treatment (116). Also, studies in follicular B-cell lymphoma have demonstrated that chromosomal translocation of *BCL-2* next to an enhancer element results in gene amplification (116-117). This *BCL-2* gene amplification has been shown in other cancers, along with hypermethylation and chromosomal deletions, resulting in overexpression of Bcl-2 (116). In addition, overexpression of Bcl-2 is more detrimental to a cell when co-expressed with other gene

amplifications, such as c-myc (116, 118).

Anti-apoptotic protein Bcl-x_L has been shown to play an important role in cancer development by increasing drug resistance (116). For example, Bcl-x_L is often overexpressed in breast, prostate and pancreatic cancer, allowing for evasion of apoptosis (116, 119-120). Anti-apoptotic protein Mcl-1 is also often overexpressed in many types of cancer, including breast cancer, resulting in an inhibition of apoptosis and increased drug resistance (119). Mcl-1 will be discussed in more detail below.

Finally, downregulation of pro-apoptotic proteins has been associated with drug resistance in cancer (116). For example, pro-apoptotic protein Bax is often mutated in hematological malignancies resulting in a decrease in its expression and subsequent evasion of apoptosis (116). As well, loss of *TP53* results in a downregulation of its transcriptional targets, including pro-apoptotic BH3-only proteins Noxa and Puma, inhibiting apoptosis (116, 121-122). Overall, the Bcl-2 family has been shown to play an important role in allowing for the evasion of apoptosis in a cancer cell.

1.5.1.5 Mcl-1

Bcl-2 family member myeloid cell leukemia-1 (Mcl-1) was first discovered by Kozopas et al. (123) while examining the human myeloid leukemia cell line ML-1. Since its initial discovery, Mcl-1 has been shown to have an important functional role in preventing apoptosis (124).

Unlike the other anti-apoptotic proteins, Mcl-1 lacks the BH4 domain, and instead is composed of BH domains 1-3 (125). In addition, Mcl-1 also contains two proline, glutamic 1
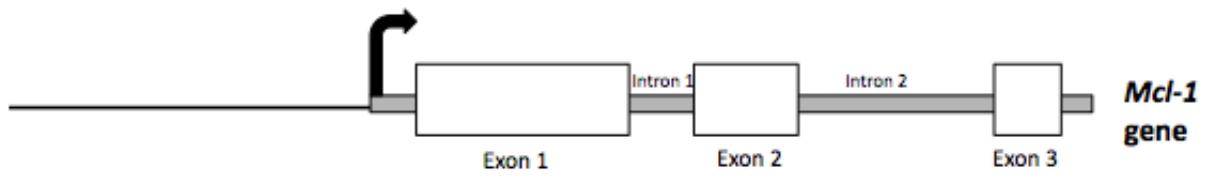
Figure 8. Schematic of *Mcl-1* gene. The *Mcl-1* full-length gene contains 3 exons and 2 introns.

Figure adapted from Bae et al. (127)

acid, serine and threonine (PEST) sequences as well as a transmembrane domain. As well, Mcl-1's full-length gene contains 3 exons and 2 introns (Figure 8) (125). Mcl-1's transmembrane domain allows it to bind to pro-apoptotic proteins Bax and Bak, thus preventing the release of cytochrome c and other mitochondrial proteins, blocking apoptosis (125-126). Mcl-1 is sequestered away from Bax or Bak by BH3-only members such as Noxa, allowing for activation of apoptosis (Figure 9). Full-length Mcl-1 (hereafter referred to as Mcl-1) is composed of 350 amino acid residues whereas short-length Mcl-1$_s$ is composed of 271 amino acid residue. In addition, Mcl-1$_s$ lacks all BH domains except for the BH3 domain. Mcl-1 is 40 kDa whereas Mcl-1$_s$ is 35 kDa (125).

Mcl-1 is regulated at both the transcriptional and translational level (126). At the transcriptional level, Mcl-1 expression is regulated by signaling pathways, such as MAPK and PI3K/AKT (125). In addition, Mcl-1 is regulated transcriptionally by alternative splicing (127). At the translational level, Mcl-1 expression is regulated by phosphorylation and ubiquitination (125). Mcl-1 possesses a short half-life, so post-translational modifications allow it to respond quickly to the needs of the cell, either by extension of its half-life or acceleration of degradation processes (125). For example, Mcl-1 is rapidly degraded by proteasome-dependent mechanism involving the E3 ligase MULE/LASU1 (128). This rapid degradation by MULE/LASU1 allows for rapid turnover in response to UV irradiation and other DNA damaging agents (128). Binding of BH3-only protein Noxa to Mcl-1 can counteract this degradation, preventing MULE/LASU1 from binding and ultimately stabilizing Mcl-1 (115, 128). Hyperphosphorylation of Mcl-1 allows for rapid proteasome-dependent degradation (125). Mcl-1 is also subject to caspase-degradation, where it is cleaved by caspase-3 at Asp$_{127}$ and Asp$_{157}$, resulting in the loss of an N-terminal domain, leaving the BH1-3 and C-terminal

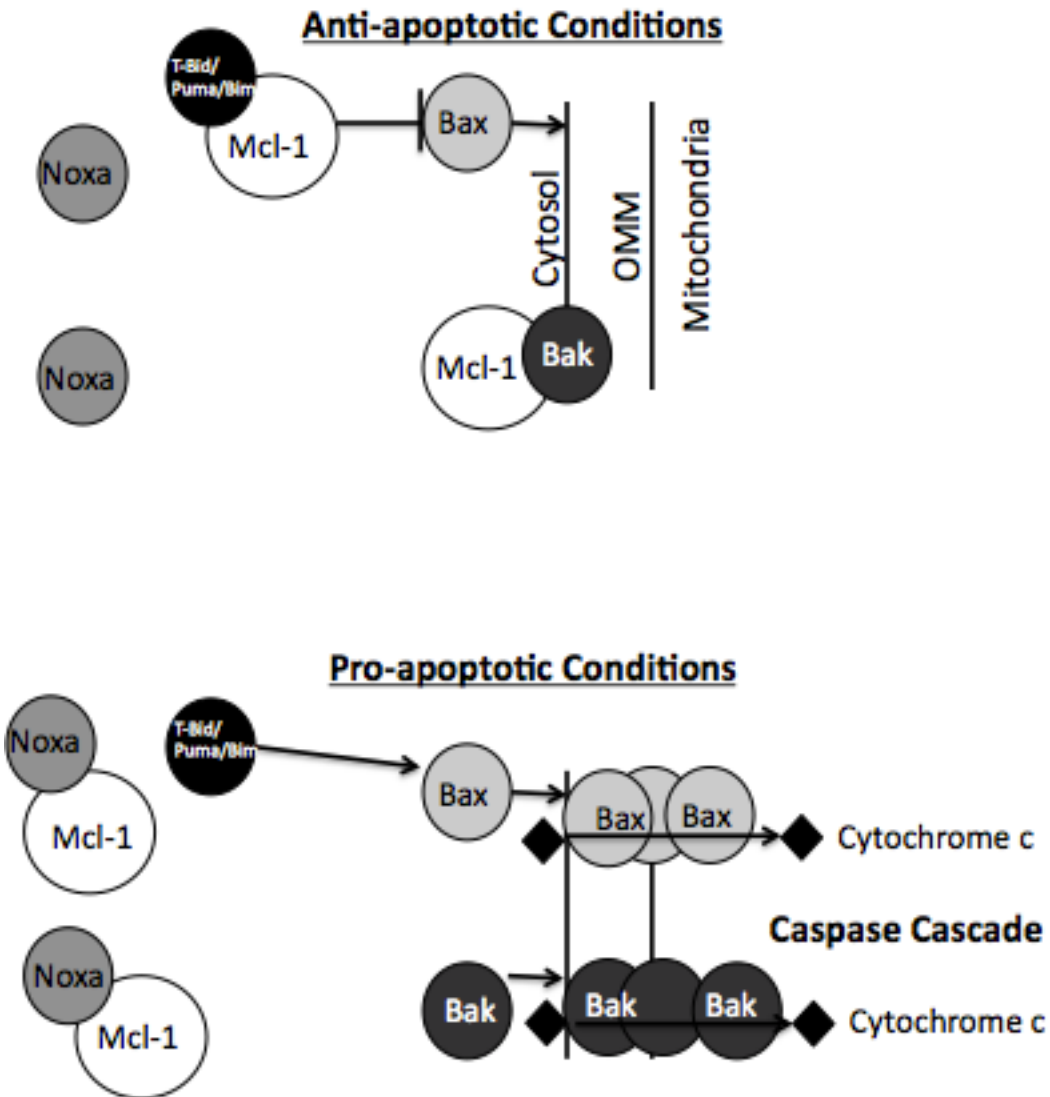Figure 9. Mechanism of Mcl-1 action under both survival and apoptotic conditions. Under survival (anti-apoptotic) conditions, pro-apoptotic proteins Bak and Bax form associations with Mcl-1, preventing release of cytochrome c. Under apoptotic conditions, BH3-only protein Noxa sequesters Mcl-1 away from Bak and Bax, allowing for cytochrome c release and caspase cascade, initiating apoptosis.

Figure adapted from Akgul (125)

domains intact, promoting cell death (125, 128). In addition, microRNAs, such as mir29b, have been implicated as having a role in regulating Mcl-1 at the translational level (129).

To overcome the anti-apoptotic function of Mcl-1, cells have devised three mechanisms to block Mcl-1 function. The first mechanism is to target Mcl-1 for degradation, which often occurs under stress-induced conditions. The second mechanism is to cleave Mcl-1 through activation of caspases, which occurs after activation of apoptosis (110, 125). The final mechanism is to express an alternative splicing mRNA lacking exon 2 that produces a shorter form, Mcl-1$_s$, which is pro-apoptotic (Figure 10) (110, 125, 127).

While these mechanisms may successfully promote apoptosis in healthy cells, Mcl-1 is often overexpressed in many types of cancer, including breast cancer, and has been associated with high tumor grade and poor prognosis (126, 130). Cancer cells frequently overexpress Mcl-1 overexpression, allowing them to develop resistance to traditional chemotherapy, ultimately avoiding cell death (126).

Previous literature has demonstrated that Mcl-1 is a downstream target of EGF in many different types of cancer, including breast cancer (69, 70). In addition, EGF mediated signaling cascades, such as the MAPK pathway, have been implicated in regulating Mcl-1 expression (69, 131-133). These signaling cascades result in the upregulation of transcription factors that may regulate Mcl-1 expression, such as Elk-1 and Stat-3 (69, 134-136). EGF-mediated activation of NFκB has also been shown to upregulate Mcl-1 expression (137). Overall, this suggests that targeting Mcl-1 may provide a mechanism for overcoming drug resistance in breast cancer patients (126).

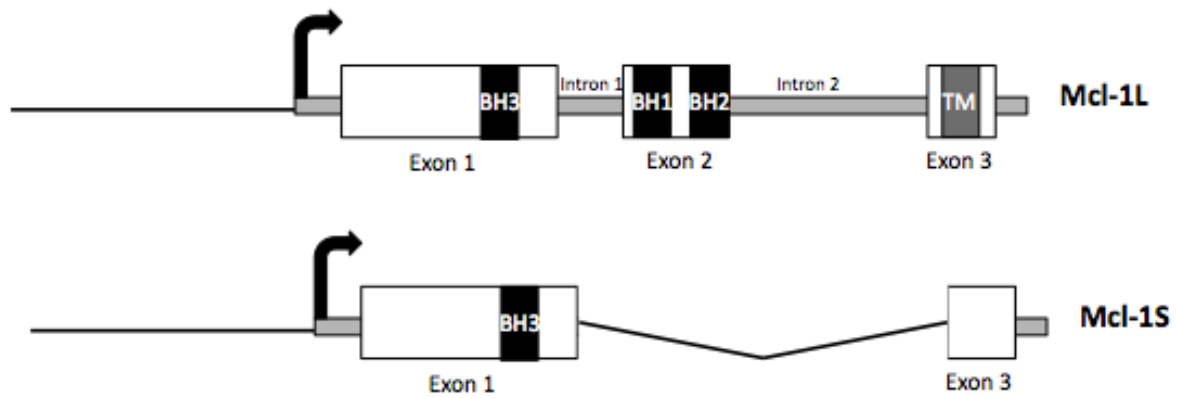Figure 10. Schematic comparing full-length Mcl-1 to splice variant Mcl-1$_s$. During post-transcriptional modification, the full-length Mcl-1 mRNA can undergo alternative splicing, producing the short form Mcl-1S. Mcl-1L contains 3 exons with 2 introns. Mcl-1$_s$ has exon 2 spliced out, removing BH-1, BH-2 and TM domain. Therefore, Mcl-1$_s$ behaves like a pro-apoptotic BH3-only protein.

Figure adapted from Bae et al. (127)

Currently, the role of Mcl-1 in breast cancer is unclear. Previous studies have shown that the Mcl-1 gene is located on chromosome 1q21 and is frequently amplified in many cancers including breast tumors (138). Beroukhim et al. (138) found that *Mcl-1* is amplified in approximately 11% of all cancers, with an amplification of approximately 4% in breast cancer. As well, previous literature has shown that estrogen may be involved in regulating the expression of other Bcl-2 family members such as anti-apoptotic protein Bcl-2 and therefore may have a role in regulating Mcl-1 expression (139). For example, a study by Wang et al. (140) found that estrogen treatment in the MCF-7 breast cancer cell line resulted in increased expression of Bcl-2 mRNA levels, which was decreased with Tamoxifen treatment. Zhang et al. (141) group found a similar result, as MCF-7 cells treated with Tamoxifen demonstrated a decreased Bcl-2 expression. In addition, a study of human endometrium found that Bcl-2 may bind to ERα, either directly or indirectly, at an ERE site within the c-Jun promoter (142).

Presently, there are few studies related to the association between Mcl-1 and estrogen. For example, Gauduchon et al. (143) demonstrated that 4-Hydroxytamoxifen treatment in multiple myeloma cells resulted in a decrease in Mcl-1 expression. Furthermore, many studies have shown that there is a link between EGF and estrogen signaling pathways that may contribute to drug resistance in patients (79-80, 144-145). For example, a study by Thrane et al. (146) found that ERα, in combination with EGF signaling pathways, might be an important component in mediating Tamoxifen resistance in breast cancer cells. Taken together, it is possible that estrogen may also be involved in upregulating Mcl-1, allowing for cell survival in breast cancer.

2. RATIONALE AND HYPOTHESIS

2.1 Rationale

Estrogen has been shown to be involved in regulating anti-apoptotic Bcl-2 in breast cancer. In addition, there is evidence to suggest that crosstalk between estrogen and EGF may result in increased drug resistance in breast cancer patients. It is known that EGF plays an important role in regulating Mcl-1 expression (69). Overall, a better understanding of the role of estrogen in mediating Mcl-1 expression may help validate Mcl-1 as a potential molecular target in combating drug resistance in breast cancer therapy.

2.2 Hypothesis

I hypothesize that estrogen increases the expression of anti-apoptotic gene Mcl-1 through binding of ERα to a half ERE site in the promoter of Mcl-1 gene. This leads to increased Mcl-1 expression in breast cancer cells.

3. MATERIALS AND METHODS

3.1 Tissue Culture

Human breast cancer cell lines MCF-7, SK-BR-3 and ZR-75 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) in 2010. Human breast cancer cell line MDA-MB-231 was obtained from Dr. Leigh Murphy (University of Manitoba) in 2012. MCF-7, SK-BR-3 and MDA-MB-231 cells were grown in Dulbecco's modified essential medium (DMEM) buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin. ZR-75 cells were grown in Roswell Park Memorial Institute medium (RPMI) buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were grown in a 37°C incubator with 5% CO_2. Cell lines were chosen based on ER status, as shown in Table 4.

Table 4. Estrogen Receptor status of breast cancer cell lines.

Breast Cancer Cell Line	Estrogen Receptor Status
MCF-7	ERα positive; ERβ positive
ZR-75	ERα positive; ERβ positive
SKBR3	ERα negative; ERβ positive
MDA-MB-231	ERα negative; ERβ negative

3.2 Treatment of Cell Lines

All cells were grown in serum-starved media for 5 days prior to treatment with estrogen. MCF-7, SK-BR-3 and MDA-MB-231 cells were grown in serum-starved DMEM buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% charcoal-stripped fetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. ZR-75 cells were grown in serum-starved RPMI buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% charcoal-stripped FBS and 100 units/mL penicillin and 100 µg/mL streptomycin. In transfection experiments, cells were plated to ~60-70% confluency. For chromatin immunoprecipitation and streptavidin pull-down assay, cells were plated to ~80-90% confluency. For all experiments, β-Estradiol (Sigma-Aldrich, Oakville, ON, Canada) was made up in ethanol to a stock concentration of 10 mM and stored at -20°C. Tamoxifen (Sigma-Aldrich, Oakville, ON, Canada) was made up in ethanol to a stock concentration of 2 mM and stored at -20°C. Fulvestrant (ICI-182,780) was obtained from Sigma-Aldrich (Oakville, ON, Canada) and was made up in dimethyl sulfoxide to a stock concentration of 500 mM and stored at -20°C.

2.3 Protein Lysate Preparation

Protein lysates were prepared from cell lines using Nonidet-P40 (NP-40) buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). Cells were plated in 6-well tissue culture dishes at a concentration of 1 X 10^5 and were grown in

serum-starved conditions for 5 days prior to treatment. Cells were harvested using a cell scraper (Corning Incorporated, Corning NY, USA) and were collected in 1.5 mL tubes (Axygen, Inc. Union City, CA, USA). Cells were centrifuged at 5000 rpm for 5 minutes at 4°C and the supernatant was removed. Cells were resuspended in 1 mL phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and centrifuged at 5000 rpm for 2 minutes at 4°C. The supernatant was removed and cells were resuspended in NP-40 buffer, vortexed for 2 seconds and left on ice for 10 minutes. The cells were centrifuged at 13 000 rpm for 10 minutes at 4°C and protein lysates were collected in new 1.5 mL tubes. Protein lysate concentration was quantified by bicinchoninic acid (BCA) assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

3.4 Immunoblotting

To perform western blot, protein lysates were diluted to equal amounts using ddH_2O to a total volume of 30 μL. Loading dye was composed of 200 μL 6X SDS loading dye (diluted 1:6 in ddH_2O) and 10 μL β-mercaptoethanol and 5 μL of this 6X loading dye was added to each sample. The samples were vortexed for 2 seconds, boiled at 98°C for 5 minutes and spun down at 13 000 rpm for 1 minute prior to be loading in gel. SDS-polyacrylamide gels were made with a 10% resolving gel layer (1.5 M Tris pH 8.8, 10% SDS, 30% acrylamide, 10% ammonium persulphate and TEMED) and a 5% stacking gel layer (1 M Tris pH 6.8, 10% SDS, 30% acrylamide, 10% ammonium persulphate, and TEMED). Proteins were loaded in equal amounts into polyacrylamide gel along with 5 μL of Pageruler prestained protein ladder (Thermo Fisher Scientific, Waltham, MA, USA). Samples were run through stacking gel at 100 V for 15

minutes and then through resolving gel at 150 V for 45 minutes. Afterwards, proteins were

transferred using a wet transfer apparatus onto a polyvinyl difluoride membrane (GE

Healthcare Limited, Amersham Place, Little Chalfont, Buckinghamshire) for 1 hour at 100 V

and 350 A at 4°C. After transfer was complete, membranes were blocked with 5% skim milk

(Safeway, Calgary, AB, Canada) for 1 hour at room temperature. Membranes were then

washed 3X 5 minutes with Tris-buffered saline-Tween 20 (TBS-T) (50 mM Tris-Cl pH 7.5,

150 mM NaCl, 0.1% Tween-20). Membranes were incubated with primary antibody overnight

at 4°C. The following day, membranes were washed in TBS-T for 3X 5 minutes and incubated

with secondary antibody conjugated with horseradish peroxidase for 1-2 hours at room

temperature. Membranes were washed in TBS-T for 3X 10 minutes. Afterwards, membranes

were incubated in 2 mL enhanced chemiluminescence (ECL) (Thermo Fisher Scientific,

Waltham, MA, USA) made up according to manufacturer's instructions. Membranes were

exposed in dark room onto autoradiography film (Thermo Fisher Scientific, Waltham, MA,

USA) for 1-10 minutes and were developed using an automatic developer (AGFA Model:

#CP1000). Afterwards, membranes were stripped using 1X Western Reprobe reagent

(Calbiochem, La Jolla, CA, USA) and incubated with a loading control antibody.

3.5 Antibodies

The primary antibodies that were used were: mouse anti-Mcl-1 (22) (sc-12756, Santa

Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-β-actin (A2066, Sigma-Aldrich,

Oakville, ON, Canada), mouse anti-progesterone receptor (clone 16) (NCL-PGR-312,

Novocastra), rabbit anti-estrogen receptor alpha (D8H8) (86445, Cell Signaling Technology,

Inc., Danvers, MA, USA), chicken anti-estrogen receptor beta (15-288-22678, GenWay

Biotech Inc., San Diego, CA, USA), rabbit anti-SP1 (07-645, Millipore, Billerica, MA, USA)

and rabbit anti-SP3 (D-20) (sc-644, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The

secondary antibodies that were used were: goat anti-chicken HRP conjugate (103-035-155,

Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), goat anti-mouse HRP

conjugate (172-1011, Bio Rad, Mississauga, Ontario, Canada), and goat anti-rabbit HRP

conjugate (170-6515, Bio Rad, Mississauga, Ontario, Canada).

3.6 RNA Isolation

To perform RNA isolation, cells were plated in 6-well tissue culture dishes at a

concentration of 1×10^5 per mL and were grown in serum-starved conditions for 5 days prior

to treatment. Total RNA was isolated using the Qiagen RNeasy Plus mini kit (Qiagen, Toronto,

Ontario, Canada) according to the manufacturer's protocol. RNA was quantified using a

NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Afterwards, 100 ng of RNA for each sample was diluted to an equal volume of 10 μL with

RNA/DNAse free ddH$_2$O. cDNA was made by adding 2 μL qScript cDNA SuperMix (Quanta

Biosciences, Gaithersburg, MD, USA) to each sample and incubating at 25°C for 5 minutes,

42°C for 30 minutes and 85°C for 5 minutes. Samples were stored in -20°C freezer until

quantitative real-time polymerase chain reaction (qPCR) was performed.

3.7 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

One-step RT–PCR was performed using a reaction mix containing iTaq Universal

SYBR Green Supermix (Bio Rad, Mississauga, Ontario, Canada), Mcl-1 full-length primers

(Table 2A) and double distilled water. This reaction mix was added to each cDNA sample. The

cycling and data collection were performed on a Bio Rad CFX Real-Time Detection System

(Bio Rad, Mississauga, Ontario, Canada) using the supplied software. The housekeeping gene

cyclophilin was used to standardize the results (Table 2A). The qPCR reaction was run as

follows: 50°C for 10 minutes, 95°C for 5 minutes and then 40 cycles of 95°C for 10 seconds

and 55°C for 30 seconds. In order to validate primer specificity, samples were run out on an

agarose gel following qPCR reaction.

Table 5. Real-Time PCR Primers.

Real-Time PCR Primers	
Mcl-1 Forward	5`-GCCAAGGACACAAAGCCAAT-3`
Mcl-1 Reverse	5`-AACTCCACAAACCCATCCCA-3`
Cyclophilin Forward	5`-GCTGCGTTCATTCCTTTG-3`
Cyclophilin Reverse	5`-CTCCTGGGTCTCTGCTTTG-5`

3.8 siRNA Transfection

In order to perform siRNA transfection, GenePORTER-2 Transfection Reagent

(Genlantis, San Diego, CA, USA) was used according to the manufacturer's protocol. A total of

2 µg of 3-pooled siRNA was transfected per sample using prevalidated siRNA duplexes that

were obtained from Origene (ESR1 (ID 2099) Trilencer-27 Human siRNA). The following

ERα siRNAs were used: 5`-ACCUUGCAGAUAUGUUUAACC AAGC-3` (SR301461A), 5`-

ACACCAUAGUAAUGUCUAAUAUUCA-3` (SR301461B), and 5`-

GGCAAAUAGAGUCAUACAGUAGCTC (SR301461C). Cells were transfected with either

siRNA against ERα or control siRNA. Cells were treated with 10 nM estrogen overnight and

total RNA was isolated 48 hours post transfection. Total RNA was isolated and quantitative

real-time PCR was performed as previously described. In order to validate knockdown, western

blot analysis was performed using antibodies against ERα and β-actin as a loading control.

3.9 Chromatin Immunoprecipitation

To perform chromatin immunoprecipitation (ChIP), a total of 20×10^6 cells were plated

in 15 cm dishes per time point. These cells were serum-starved for 5 days prior to the addition

of estrogen. Cells were treated with estrogen (10 nM) and ChIP was performed at both 6 and 24

hours post-treatment. ChIP was performed using the EZ-ChIP Chromatin Immunoprecipitation

kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. After

estrogen stimulation, cross-linking was performed by adding 19 mL of PBS containing 1%

formaldehyde directly to each plate. Plates were incubated for 10 minutes with shaking at room

temperature. The cross-linking reaction was stopped by the addition of glycine to a final

concentration of 2.5M. The plates were incubated with glycine for 5 minutes with shaking at

room temperature. Next, the plates were rinsed with 2X ice-cold PBS and cells were scraped

and transferred to a 50 mL centrifuge tube. Cells were spun in the centrifuge at 1000 g for 5

minutes. Afterwards, cells were resuspended in 750 μL Lysis Buffer A (1% SDS, 10 mM

EDTA, 50 mM Tris-HCL pH 8 and protease inhibitor cocktail). Cells were transferred into a

microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16 mm sonication tube (Covaris, Woburn, MA,

USA). Samples were sonicated on a Covaris sonicator (Transition Technologies, Model S220,

Serial #001736) for 8 minutes at 200 cycles/burst with a peak power of 120 V and a duty factor of 5. Desired fragment size of 500 bp was determined by agarose gel electrophoresis. Samples were centrifuged for 10 minutes at 4°C in order to pellet cell debris. Next, samples were diluted 10-fold with ChIP Dilution Buffer (Millipore, Billerica, MA, USA) and protease inhibitor cocktail II (Millipore, Billerica, MA, USA) in order to reduce SDS concentration. Cell lysates were pre-cleared for 1 hour with 50 μL Protein G agarose beads (Millipore, Billerica, MA, USA) at 4°C with rotation. Samples were briefly centrifuged at 3000 rpm in order to pellet beads and transferred to a new tube. In addition, 10 μL of sample was saved as input at 4°C. The samples were incubated with 5 μg of anti-ERα antibody overnight at 4°C with rotation. As well, positive and negative controls were incubated with 1μg of anti-RNA Polymerase II and Normal Mouse IgG, respectively, overnight at 4°C with rotation. The following day, 60 μL of Dynabeads protein G (Invitrogen, Burlington, ON, Canada) were added to each immunoprecipitation and were incubated for 1 hour at 4°C with rotation. Samples were centrifuged briefly at 3000 rpm to pellet beads and supernatant was carefully removed. Beads were washed for 5 minutes each with the following buffers: Low Salt Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris-HCL pH 8.1, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris-HCL pH 8.1, 500 mM NaCl), and LiCl Wash Buffer (250mM LiCl, 1% N-P40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCL pH 8.1). Beads were washed for 2X 5 minutes with TE Buffer (10 mM Tris-HCL pH 7.5, 1mM EDTA). Afterwards, 200 μL of Elution Buffer (1% SDS, 100mM NaHCO$_3$) was prepared for each sample, including input. Samples were incubated with 100 μL Elution Buffer for 15 minutes at room temperature, centrifuged briefly at 3000 rpm and the eluate was collected in a new tube. This process was repeated and eluates for each condition were combined. The

samples were incubated at 65°C overnight with 5 M NaCl in order to reverse the DNA-protein crosslinks. The following day, samples were incubated with 1μg RNase A (Millipore, Billerica, MA, USA) at 37°C for 30 minutes. After, samples were incubated with 0.5 M EDTA (Millipore, Billerica, MA, USA), 1 M Tris-HCl (Millipore, Billerica, MA, USA) and 100 μg proteinase K (Millipore, Billerica, MA, USA) at 45°C for 1-2 hours. DNA was isolated using EZ-ChIP Chromatin Immunoprecipitation kit Spin Columns and Bind Reagents A, B and C (Millipore, Billerica, MA, USA). DNA concentration was determined using PicoGreen dsDNA quantification assay (Invitrogen, Burlington, ON, Canada). RT-PCR and qPCR were performed with 0.1 ng ChIP DNA using primers specific to the Mcl-1 promoter (Table 2B). The fold enrichment values were calculated using the cT value of each ChIP sample compared to the cT value of 0.1 ng Input DNA.

Table 6. ChIP primers.

ChIP Primers	
Mcl-1 Promoter Site 1 Forward	5`-GCACCCGCCACCATCCCCAGCTAATTTTTCGTATTTTTTTT-3`
Mcl-1 Promoter Site 1 Reverse	5`-GCCATTGCAACTGGCCCTGTTTGTTAGGAAACAAGTCTTGG-3`
Mcl-1 Promoter Site 2 (control primer) Forward	5`-CTTTACCACCTGATAAAATTTTACTTTATAAAGCATGAGAG-3`
Mcl-1 Promoter Site 2 (control primer) Reverse	5`-GTTTCTCTATTTAAGGAATGTCTAATCTTGTACAGCAACTA-3`

3.10 Biotin-Labeled Probe Preparation

HPLC purified biotin-labeled probes were ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA, USA). The probes were designed to specific 50

bp regions located at 3704, 2723 and 2588 bp upstream of the translation start site. Single-stranded probes were annealed by boiling at 98°C for 5 minutes and cooled at room temperature. After annealing, probe concentration was 10 μM and probes were diluted 100-fold for binding reaction.

3.11 Streptavidin Pull-Down Assay

In order to perform streptavidin pull-down assay, 20×10^6 cells were plated per time point. These cells were serum-starved for 5 days prior to treatment with estrogen. Cells were treated with 10 nM estrogen for 24 hours. After treatment, cells were rinsed twice with ice-cold PBS and scraped into a 15 mL centrifuge tube. Cells were spun in centrifuge at 1200 rpm for 5 minutes. The cell pellets were resuspended in Nuclear Extract Buffer 1 (25 mM Hepes pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$ with protease and phosphatase inhibitors). Next, 200 μL of Nuclear Extract Buffer 2 (25 mM Hepes pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, 1% N-P40 with protease and phosphatase inhibitors) was added to cell lysates. The tubes were rotated at 4°C for 15 minutes and then centrifuged at 2500 rpm for 1 minute in order to pellet the nuclei. After, the supernatant was discarded and the cell pellet was washed in a 1:1 mixture of Nuclear Extract Buffer 1 and Nuclear Extract Buffer 2. The lysates were centrifuged at 2500 rpm for 1 minute to pellet the nuclei and resuspended in Nuclear Extract Buffer 3 (25 mM Hepes pH 7.9, 10% w/v sucrose, 350 mM NaCl, 0.01% N-P40 with protease and phosphatase inhibitors). After resuspension, the tubes were vortexed for 30 seconds and rotated for 1 hour at 4°C. The cells were then spun at 13 000 rpm for 10 minutes in order to pellet the cell debris. The supernatant was transferred to a new tube and protein concentration was quantified using BCA assay

reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. At this point 10 μL of nuclear extract was saved as Input for each sample. The lysates were pre-cleared with 50 μL streptavidin agarose beads (Life Technologies, Eugene, OR, USA) for 30 minutes at 4°C with rotation. Next, a binding reaction was prepared, including: 500 μg nuclear extract, 50 ng/μL Poly dI-dC (Sigma-Aldrich, Oakville, ON, Canada), 1/5 volume 5X Binding Buffer (50 mM Tris pH 7.5, 250 mM KCl, 5 mM DTT), and 100 nM biotin labeled probe. A total of 3 site-specific biotin-labeled probes, as well as a scrambled probe and 3 unlabelled probes were used (Table 2C). The binding reaction was incubated for 30 minutes at room temperature. Afterwards, 50 μL of streptavidin-agarose beads were added and the samples were incubated for 30 minutes. Next, the beads were spun at 3000 rpm for 1 minute and washed in PBS for 3X 5 minutes. The beads were resuspended in 50 μL of 2X SDS Loading dye and the samples were boiled for 5 minutes. Finally, SDS/polyacrylamide gel electrophoresis and western blotting were performed. Antibodies specific to ERα, ERβ, Sp1 and Sp3 were used. Cold-competition was performed was adding an excess of unlabeled probe 15 minutes before the addition of the biotin labeled Mcl-1 probe.

Table 7. Mcl-1 site-specific biotin-labeled probes.

Mcl-1 Site-Specific Biotin Labeled Probes	
Site 1 Forward	5`-CCAGGATGGTCTTGATCTCCTGACCTCGTGATCTGCCCGCCTCAGCCTCC-3`
Site 1 Reverse	5`-GGAGGCTGAGGCGGGCAGATCACGAGGTCAGGAGATCAAGACCATCCTGG-3`
Site 1 Scrambled Forward	5`-CCAGGATGGTCTTGATCTCCGCTCATCGTGATCTGCCCGCCTCAGCCTCC-3`
Site 1 Scrambled Reverse	5`-GGAGGCTGAGGCGGGCAGATCACGATGAGCGGAGATCAAGACCATCCTGG-3`
Site 2 Forward	5`-GGCGCACTCTCAGCTCACCGCAACCTCCGCCTCCCAGGTTCAAGCGATTC-3`
Site 2 Reverse	5`-GAATCGCTTGAACCTGGGAGGCGGAGGTTGCGGTGAGCTGAGAGTGCGCC-3`
Site 3 Forward	5`-TTTCTCCATGTTGGTTGGGCTGGTCTCAAACTCCTGACCTCAGATGATTC-3`
Site 3 Reverse	5`-GAATCATCTGAGGTCAGGAGTTTGAGACCAGCCCAACCAACATGGAGAAA-3`

3.12 Statistical Analysis

All studies compared treated breast cancer cell samples to a non-treated control sample. Statistical analysis was performed using XLSTAT software in Microsoft Excel. A Two-Way ANOVA variance statistical test was performed, comparing Mcl-1 mRNA expression in treated samples to Mcl-1 mRNA expression in untreated samples. Afterwards, a post-hoc test was performed in order to determine any significant differences. Statistical significance is noted in figures as *. Densitometry was performed using ImageJ software.

4. RESULTS

4.1 Sequence analysis of Mcl-1 promoter reveals presence of 5 ERE half-sites.

Previous studies suggest that EGF is involved in increased Mcl-1 expression through activation of the MAPK signaling pathway (132-133). Recently, we have shown that EGF signaling can activate the transcription factor Elk-1, which mediates increased Mcl-1 transcription (69). Several studies have suggested that crosstalk between EGF signaling pathways and the estrogen receptor (ER) can result in increased drug resistance in breast cancer patients (79, 145). However, the role of estrogen in regulating Mcl-1 expression is currently unknown. To evaluate whether Mcl-1 expression is regulated by ER activity in breast cancer cells, we performed a sequence analysis of the Mcl-1 promoter region. As shown in Figure 11, the Mcl-1 promoter region includes 5 ERE half-sites. These half-sites are located at regions 3683 bp, 3376 bp, 2713 bp, 2554 bp and 1068 bp upstream of the translation start site (Figure 11). In addition, we identified multiple Sp-1 transcription factor sites within the promoter (Figure 11). Three of the ERE half-sites, located 3683 bp, 2713 bp and 2554 bp upstream of the translation start site, are in close proximity to Sp1 binding sites (Figure 11). Half ERE sites located beside an Sp1 site are considered to be potential ER binding sites in complex with Sp1, leading to gene expression. This sequence analysis suggests that estrogen may regulate Mcl-1 expression through a mechanism involving ER and Sp1 binding within the Mcl-1 promoter.
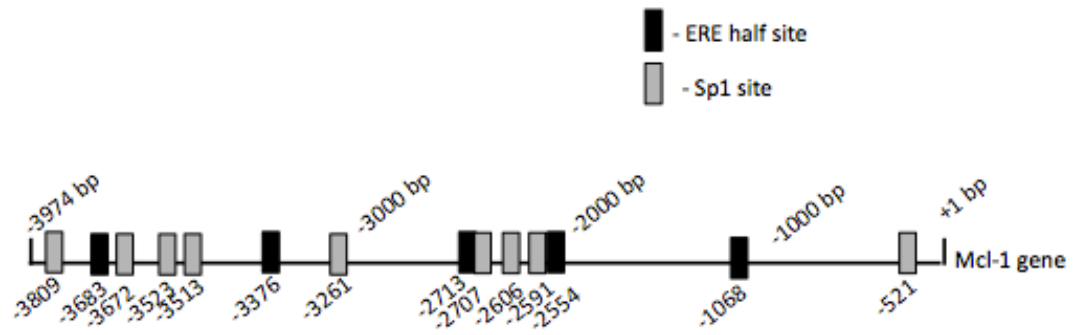
Figure 11. Schematic representation of *Mcl-1* gene showing approximate locations of ERE half-sites and Sp1 binding sites.

4.2 Estrogen treatment increases Mcl-1 expression in ERα+ breast cancer cell lines.

Given the presence of 3 ERE half-sites with adjacent Sp1 sites within the Mcl-1 promoter region, I evaluated whether estrogen is involved in regulating Mcl-1 transcription. Two serum-starved ERα expressing breast cancer cell lines, MCF-7 and ZR-75, were used as models of ERα+ breast cancer and treated them with estrogen (10 nM) overnight. Total RNA was isolated total RNA at both 6 hours and 24 hours post estrogen treatment. Mcl-1 mRNA levels were detected by quantitative real-time PCR. Mcl-1 RNA expression increased approximately 1.5-fold in both MCF-7 and ZR-75 after 6 hours of estrogen treatment, (Figure 12) and increased 2-fold in both MCF-7 and ZR-75 after 24-hours post-estrogen treatment (Figure 12). Control cells (untreated and vehicle control) failed to show a similar increase in fold-change (Figure 12). This suggests that in ERα+ breast cancer cells, estrogen signaling is involved in upregulating Mcl-1 transcription.

In order to determine the role of estrogen in regulating Mcl-1 protein expression, MCF-7 and ZR-75 were treated with increasing concentrations of estrogen and evaluated total protein levels. Cell lines were treated with a range of estrogen concentrations (10^{-2} nM – 10 nM) for 24 hours and isolated whole cell lysates. In both MCF-7 and ZR-75, the highest increase in Mcl-1 protein expression was found at 10 nM of estrogen treatment (Figure 13). These values were normalized using densitometry and found at 10 nM of estrogen, there was a 5-fold and 2.5-fold increase in MCF-7 and ZR-75 protein expression, respectively (Figure 13). Control cells (untreated and vehicle control) failed to show a similar increase in fold-change (Figure 13). This data suggests that estrogen signaling is involved in upregulating Mcl-1 protein expression in ERα+ breast cancer cell lines.
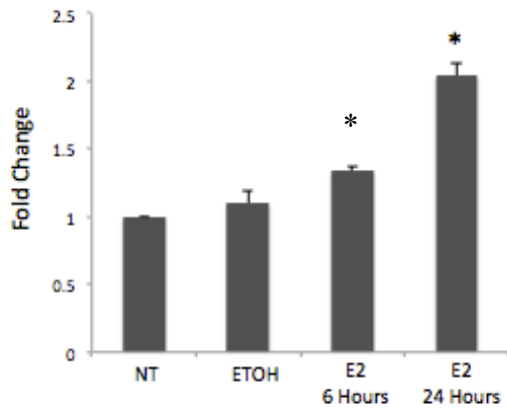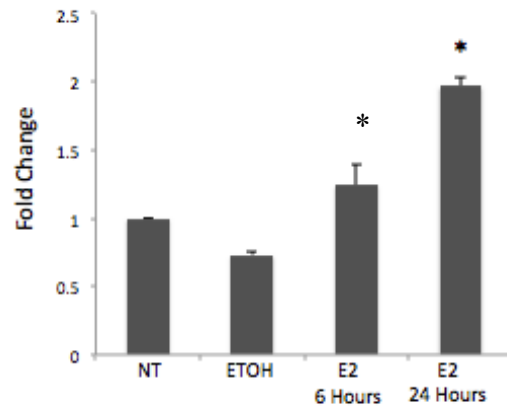
A **B**

MCF-7   • p ≤ 0.05

ZR-75   • p ≤ 0.05

Figure 12. Estrogen increases Mcl-1 expression at the message level. (A) Real-time PCR analysis of Mcl-1 transcript levels in MCF-7 shows increase following 24-hour stimulation with estrogen (10 nM). (B) Real-time PCR analysis of Mcl-1 transcript levels in ZR-75 shows increase following 24-hour stimulation with estrogen (10 nM). In both experiments 100 ng template RNA was amplified using primers specific to Mcl-1. qPCR results were standardized using primers for housekeeping gene cyclophilin. Fold change represents the results relative to changes in basal levels observed in untreated sample. Data represents the mean of three independent experiments ± standard error. (* indicates $p \leq 0.05$ compared to untreated control cells).
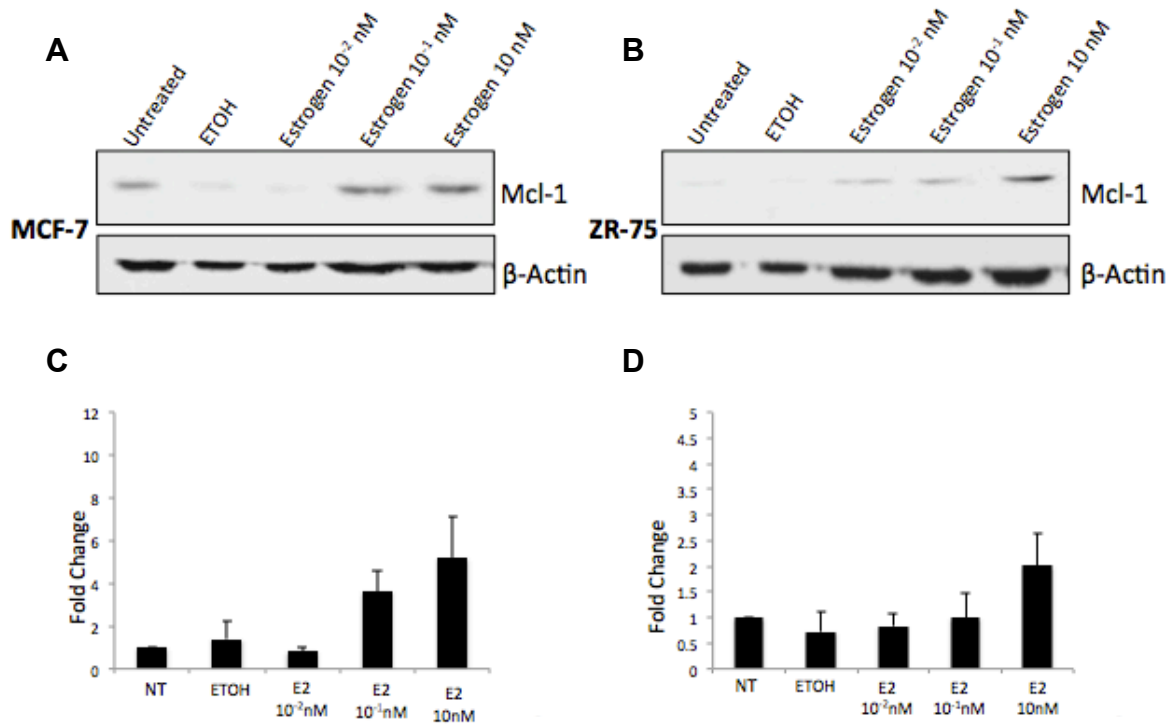
Figure 13. Stimulation by estrogen increases Mcl-1 protein expression in ERα+ breast cancer cell lines. (A) Western blot analysis of MCF-7 following 24-hour stimulation with increasing concentrations of estrogen (10^{-2} nM – 10 nM). (B) Western blot analysis of ZR-75 following 24-hour stimulation with increasing concentrations of estrogen (10^{-2} nM – 10 nM). In both experiments, cells were serum-starved for 5 days prior to treatment with estrogen. Blots were reprobed with anti-β-actin as a loading control. (C) Relative accumulation of Mcl-1 protein expression in MCF-7 cells, confirmed by densitometry. Data represents mean of three independent experiments ± standard error. (D) Relative accumulation of Mcl-1 protein expression in ZR-75 cells, confirmed by densitometry. Data represents mean of three independent experiments ± standard error.

4.3 Estrogen treatment does not increase Mcl-1 protein expression in ERα- cell lines.

Estrogen appears to regulate Mcl-1 expression at both the protein and mRNA level in ERα+ breast cancer cell lines, suggesting that ER mediates this expression. To determine the role of ER, the effect of estrogen on total protein expression was evaluated in two ERα- breast cancer cell lines, SK-BR-3 and MDA-MB-231. SK-BR-3 do not express ERα, however, they do express ERβ alone. MDA-MB-231 does not express ERα or ERβ (24, 62). Cell lines were treated with estrogen (10 nM) and whole cell lysates were obtained 24-hours post-estrogen treatment. After 24 hours, an increase in Mcl-1 protein expression was not detected in either SK-BR-3 or MDA-MB-231 (Figure 14). Using densitometry, there was a negligible fold-change in Mcl-1 protein expression after estrogen treatment when compared to both an untreated and vehicle control (Figure 14). Overall, this data suggests that estrogen signaling requires the presence of ERα to mediate changes in Mcl-1 mRNA and protein expression.
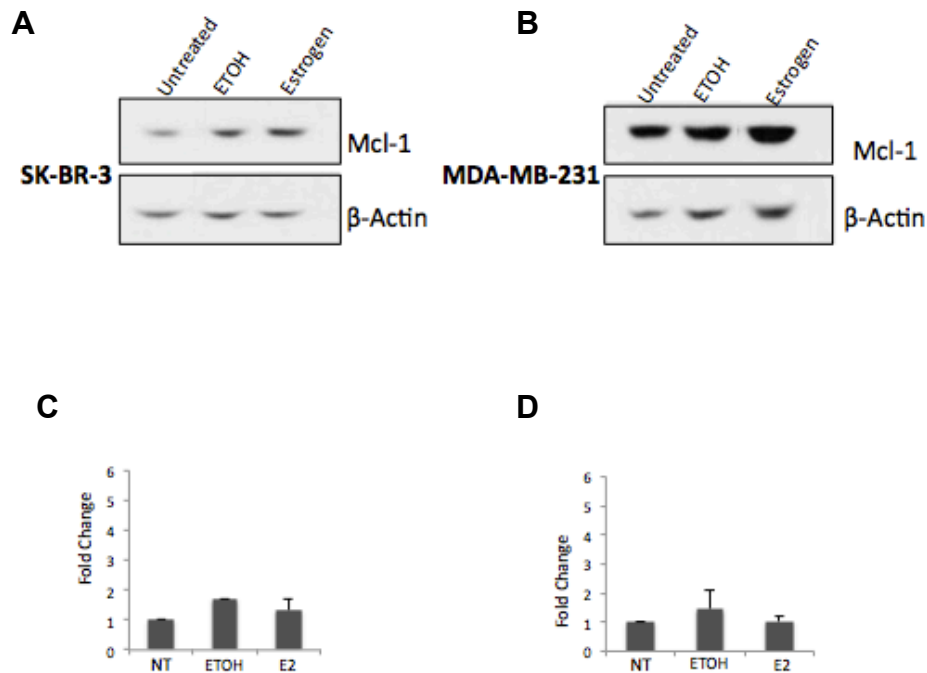
Figure 14. Stimulation by estrogen has no effect on Mcl-1 protein expression in ERα- breast cancer cell lines. (A) Western blot analysis of SK-BR-3 following 24-hour stimulation with estrogen (10 nM). (B) Western blot analysis of MDA-MB-231 following 24-hour stimulation with estrogen (10 nM). In both experiments, cells were serum-starved for 5 days prior to treatment with estrogen. Blots were reprobed with anti-β-actin as a loading control. (C) Relative accumulation of Mcl-1 protein expression in SK-BR-3 cells, confirmed by densitometry. Data represents mean of two independent experiments ± standard error. (D) Relative accumulation of Mcl-1 protein expression in MDA-MB-231 cells, confirmed by densitometry. Data represents mean of two independent experiments ± standard error.

4.4 Estrogen antagonists Tamoxifen and Fulvestrant decrease Mcl-1 expression at both the protein and mRNA level.

Estrogen antagonists are used in breast cancer therapy to block estrogen receptor activation. I determined whether estrogen regulates Mcl-1 expression through a ligand-dependent mechanism involving ERα activation. Two anti-estrogens, Tamoxifen and Fulvestrant, were used, which antagonize the ER by inhibiting estrogen binding to ERα (46-47). Serum-starved MCF-7 and ZR-75 cells were treated with either Tamoxifen (200 nM) or Fulvestrant (500 nM) in combination with estrogen (10 nM) for 24 hours and extracted total RNA. These findings were compared to cells treated with only estrogen, Tamoxifen or Fulvestrant. In MCF-7, estrogen treatment resulted in a 3.5-fold increase in RNA expression, whereas both Tamoxifen and Fulvestrant treatment in combination with estrogen resulted in a negligible fold-change (Figure 15A). In ZR-75, estrogen treatment resulted in a 2-fold increase in Mcl-1 mRNA expression whereas both Tamoxifen and Fulvestrant treatment in combination with estrogen also resulted in a negligible fold-change (Figure 15B). These results were compared to both an untreated and vehicle control, which showed a no significant fold increase (Figure 15). In addition, the effects of Tamoxifen and Fulvestrant on Mcl-1 expression at the protein level were studied. In both MCF-7 and ZR-75 cell lines, Tamoxifen and Fulvestrant treatment in combination with estrogen resulted in a decrease in Mcl-1 protein expression (Figure 16). This data was normalized using densitometry, which showed a reduction of approximately 1.8-fold in MCF-7 cells after both Tamoxifen and Fulvestrant treatment (Figure 16C). In ZR-75, a reduction of 3-fold and 6.5-fold was seen after Tamoxifen and Fulvestrant treatment, respectively (Figure 16D). Taken together, it appears that Tamoxifen and Fulvestrant antagonize ERα, resulting in a decrease in Mcl-1 expression at both the protein and mRNA

level. Therefore, estrogen may be involved in regulating Mcl-1 expression specifically through a ligand-dependent mechanism involving ERα.
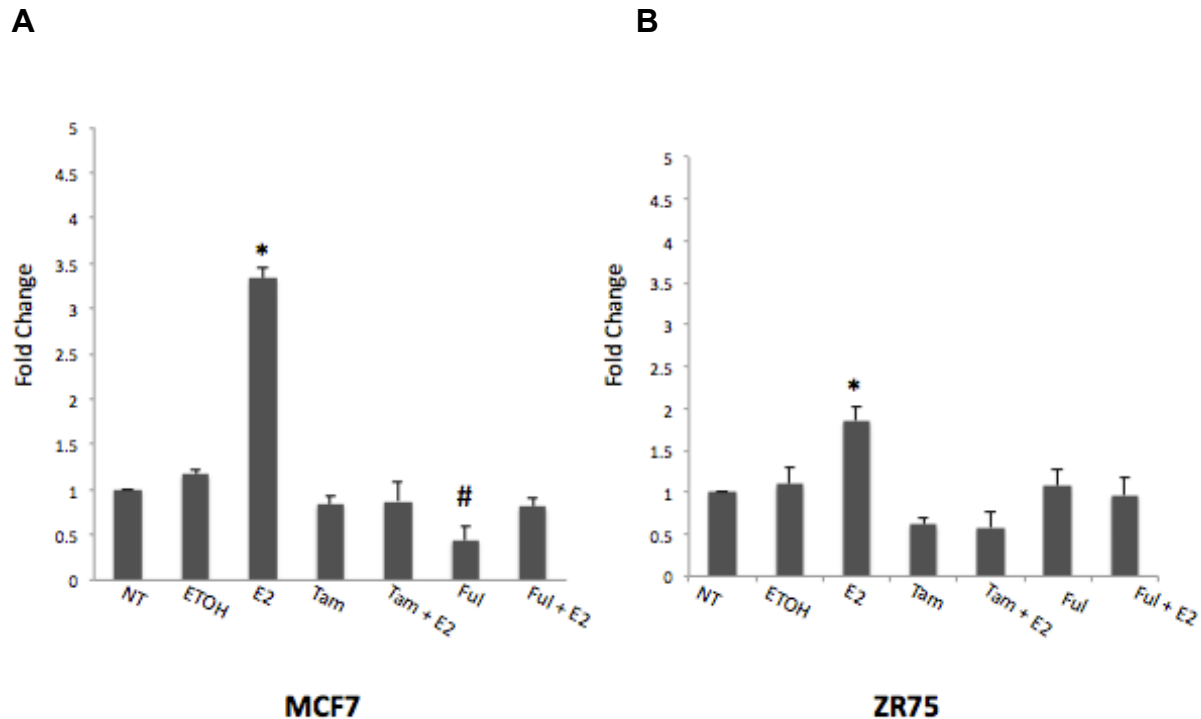
A   **B**

Figure 15. Anti-estrogens Tamoxifen and Fulvestrant decrease Mcl-1 mRNA expression in ERα+ breast cancer cell lines (A) Real-time PCR analysis of Mcl-1 transcript levels in MCF-7 shows decrease following 24-hour treatment with Tamoxifen (200 nM) or Fulvestrant (500 nM) in combination with estrogen (10 nM). (B) Real-time PCR analysis of Mcl-1 transcript levels in ZR-75 shows increase following 24-hour treatment with Tamoxifen (200 nM) or Fulvestrant (500 nM) in combination with estrogen (10 nM). In both experiments 100 ng template RNA was amplified using primers specific to Mcl-1. qPCR results were standardized using primers for housekeeping gene cyclophilin. Results are expressed as fold change relative to changes in basal levels observed in untreated sample. Data represents the mean of three independent experiments ± standard error. (* indicates $p \leq 0.05$ compared to untreated control cells; # indicates $p \leq 0.1$ compared to untreated control cells).
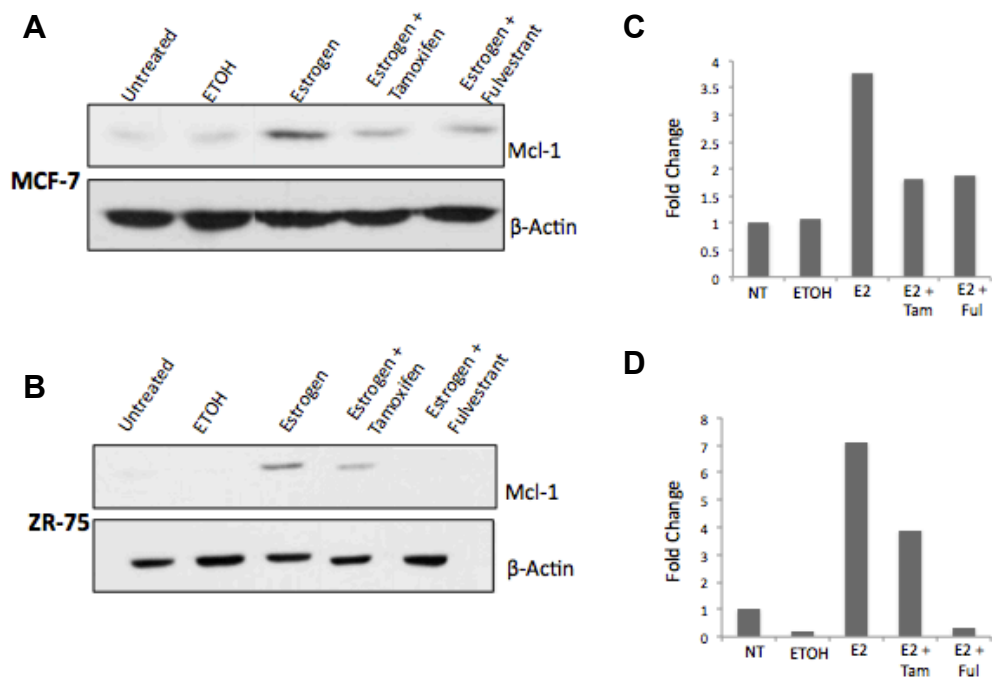
Figure 16. Treatment with anti-estrogen Tamoxifen and Fulvestrant decrease Mcl-1 protein expression in ERα+ breast cancer cell lines. (A) Western blot analysis of MCF-7 following 24-hour treatment with either Tamoxifen (200 nM) or Fulvestrant (500 nM) in combination with estrogen (10 nM). (B) Western blot analysis of ZR-75 following 24-hour hour treatment with either Tamoxifen (200 nM) or Fulvestrant (500 nM) in combination with estrogen (10 nM). In both experiments, cells were serum-starved for 5 days prior to treatment with estrogen. Blots were reprobed with anti-β-actin as a loading control. (C) Relative accumulation of Mcl-1 protein expression in MCF-7 cells, confirmed by densitometry. (D) Relative accumulation of Mcl-1 protein expression in ZR-75 cells, confirmed by densitometry.

4.5 Knockdown of ERα decreases Mcl-1 RNA expression in ERα+ breast cancer cell line.

   To investigate the role of ERα in regulating Mcl-1 expression, a knockdown of ERα in MCF-7 cells was performed using siRNA. Serum-starved MCF-7 cells were transfected with either a pool of 3 small interfering (si)RNAs against ERα or control siRNA. Silencing of ERα was confirmed by western blot analysis (Figure 17A). After transfection, cells were treated with estrogen (10 nM) over a 24-hour period. Total RNA was extracted and mRNA expression levels were evaluated using quantitative real-time PCR. After estrogen treatment, knockdown of ERα resulted in a 1-fold decrease in Mcl-1 mRNA expression, both alone and in combination with estrogen (Figure 17B). This was compared to control siRNA, which resulted in a 2-fold increase in Mcl-1 mRNA expression when treated with estrogen (Figure 17B). Overall, this suggests that ERα plays an important role in regulating Mcl-1 mRNA expression, as when it is silenced, Mcl-1 expression is negligible.
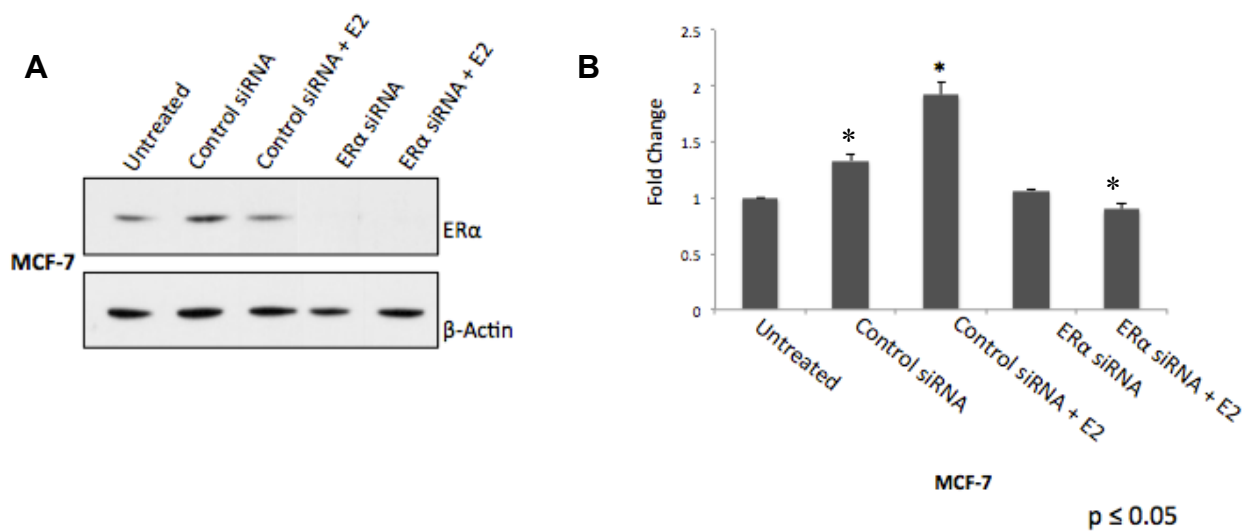
Figure 17. Knockdown of ERα results in decrease in Mcl-1 mRNA expression in ERα+ breast cancer cell line. (A) Western blot analysis confirms ERα silencing in MCF-7 after siRNA transfection. Blot was reprobed with anti-β-actin as a loading control. (B) Real-time PCR analysis of Mcl-1 transcript levels in MCF-7 shows decrease following transfection with siRNA against ERα. Cells were serum starved for 5 days prior to transfection. After transfection, cells were stimulated with estrogen (10 nM) for 24-hours. For qPCR,100 ng template RNA was amplified using primers specific to Mcl-1. qPCR results were standardized using primers for housekeeping gene cyclophilin. Results are expressed as fold change relative to changes in basal levels observed in untreated sample. Data represents the mean of three independent experiments ± standard error. (* indicates p ≤ 0.05 compared to untreated control cells).

4.6 ERα binds to the Mcl-1 promoter.

To evaluate whether ERα binds to the Mcl-1 promoter, a chromatin immunoprecipitation (ChIP) experiment was conducted with ERα+ MCF-7 cells. To assess ERα binding, primers directed to a 200 bp region containing an ERE half site and Sp1 transcription factor site were designed (Figure 18D). Serum-starved MCF-7 cells were treated with estrogen (10 nM) and ChIP was performed 6 hours and 24 hours post-treatment. ChIP was performed using antibodies directed against ERα as well as a positive anti-RNA Polymerase II antibody, negative antibody and a no antibody (beads alone) control. After stimulation with estrogen, ERα was detected on the Mcl-1 promoter at both 6 and 24 hours post-treatment (Figure 18). After 6 hours, estrogen treatment resulted in a 5-fold enrichment of ERα at the Mcl-1 promoter (Figure 18A). After 24 hours, estrogen treatment resulted in a 3-fold enrichment of ERα at the Mcl-1 promoter (Figure 18B). Both the negative control (non-specific control Normal Mouse IgG) and no antibody (beads alone) control had negligible fold-enrichment values, suggesting that they failed to immunoprecipitate Mcl-1 promoter fragments (Figure 18). In addition, immunoprecipitation of ERα was confirmed by resolving the samples on an agarose gel (Figure 18C). Overall, this suggests that ERα is involved in regulating Mcl-1 expression by binding to an ERE site within the Mcl-1 promoter.
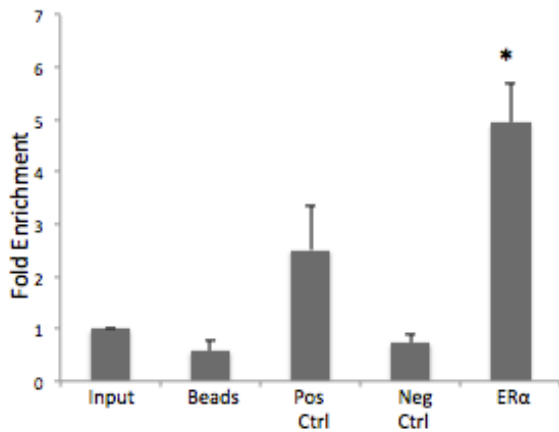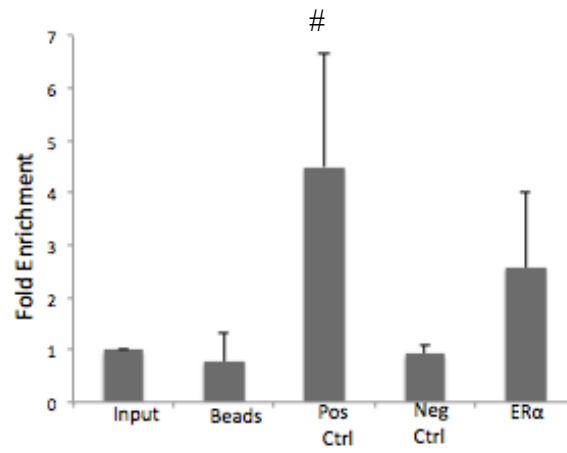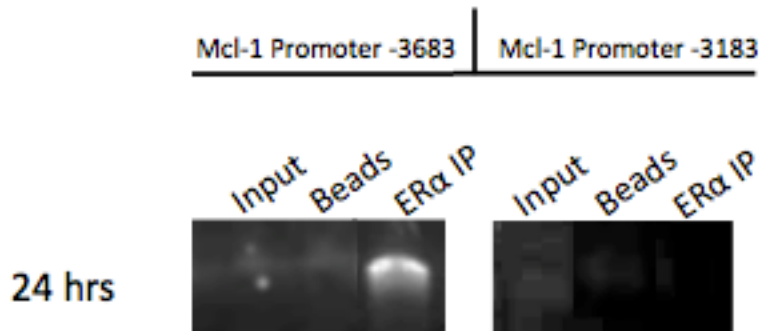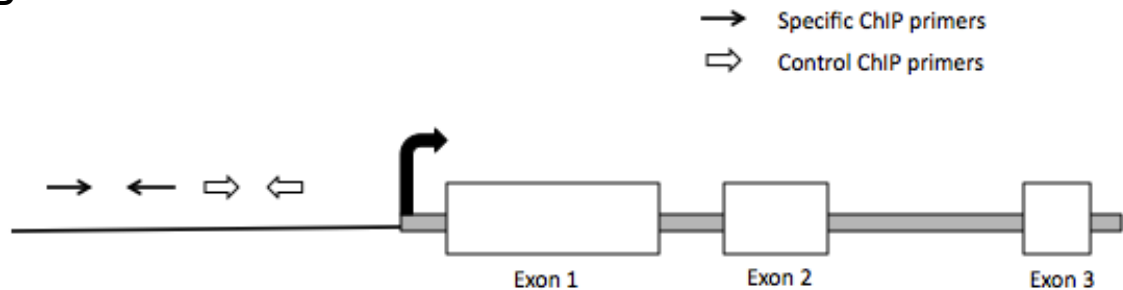
Figure 18. ERα binds to specific region within Mcl-1 promoter in MCF-7 cells. (A) Chromatin immunoprecipitation (ChIP) was performed using antibody specific to ERα. ChIP was performed 6 hours after estrogen (10 nM) treatment. (B) ChIP was performed using antibody specific to ERα 24-hours post-estrogen (10 nM) treatment. In both experiments, positive control (RNA Polymerase II), negative control (Normal Mouse IgG) and no antibody control (beads alone) are shown. Results represent fold enrichment values obtained by comparing cT values of ChIP samples to cT values of input. Data represents the mean of 3 independent experiments ± standard error. (C) PCR products were run on agarose gel to evaluate ChIP specificity. Primers specific to ERE half-site located 3683 bp upstream of translation start site were compared to control primers representing region lacking ERE half-site. (D) Schematic representation of *Mcl-1* gene showing approximate locations of ChIP primers and control primers. (* indicates $p \leq 0.05$ compared to input; # indicates $p \leq 0.3$ compared to input).

4.7 Estrogen increases ERα and Sp1 binding to a half ERE and Sp1 site of the Mcl-1 promoter.

After demonstrating that ERα binds to the Mcl-1 promoter using ChIP, this was further validated by performing a streptavidin pull-down assay. 50 bp biotin-labeled probe was designed to be complementary to the region of interest, a half ERE site that is 3683 bp (Site 1) upstream of the translation start site (Figure 19G). ERα+ MCF-7 cells were treated with estrogen and performed nuclear extraction at both 6 hours and 24 hours post-treatment. The Mcl-1 promoter specific probe was able to pull down ERα at 6 hours post-estrogen treatment (Figure 19A). This data was quantified using densitometry and showed that there is a 2.5-fold increase in ERα expression at 6-hours post-estrogen treatment (Figure 19E). This was further validated this data using a scrambled probe, which was unable to pull down ERα (Figure 19A). In addition, cold competition was performed using an excess of unlabeled probe. Cold competition was able to successfully compete away ERα (Figure 19A). ERβ binding was also evaluated, however, the Mcl-1 promoter specific probe was unable to pull down ERβ after both 6 hours and 24 hours of estrogen treatment (Figure 19B). Since the ERE site under study is a half-site, I was interested in determining whether transcription factors Sp1 or Sp3 are also involved in regulating Mcl-1 expression. Upon evaluation with antibodies against Sp1 and Sp3, the Mcl-1 promoter specific probe was able to pull down Sp1 but not Sp3 at 6-hours post-estrogen treatment (Figures 19C and 19D). Densitometry showed a 5-fold increase in Sp1 expression at 6 hours post-estrogen treatment (Figure 19F). Two other regions within the Mcl-1 promoter that contained ERE half sites were studied. These regions are located 2713 bp (Site 2) and 2554 bp (Site 3) upstream of the translation start site. Using 50 bp biotin-labeled probes

directed to these sites, the probes were unable to pulldown ERα, ERβ, Sp1 or Sp3 to these regions (Figure 20 and Figure 21). Overall, these results suggest that estrogen is involved in regulating Mcl-1 expression through a mechanism involving ERα and Sp1 binding to a specific ERE half-site and Sp1 site that is approximately 3683 bp upstream of the translation start site within the Mcl-1 promoter.
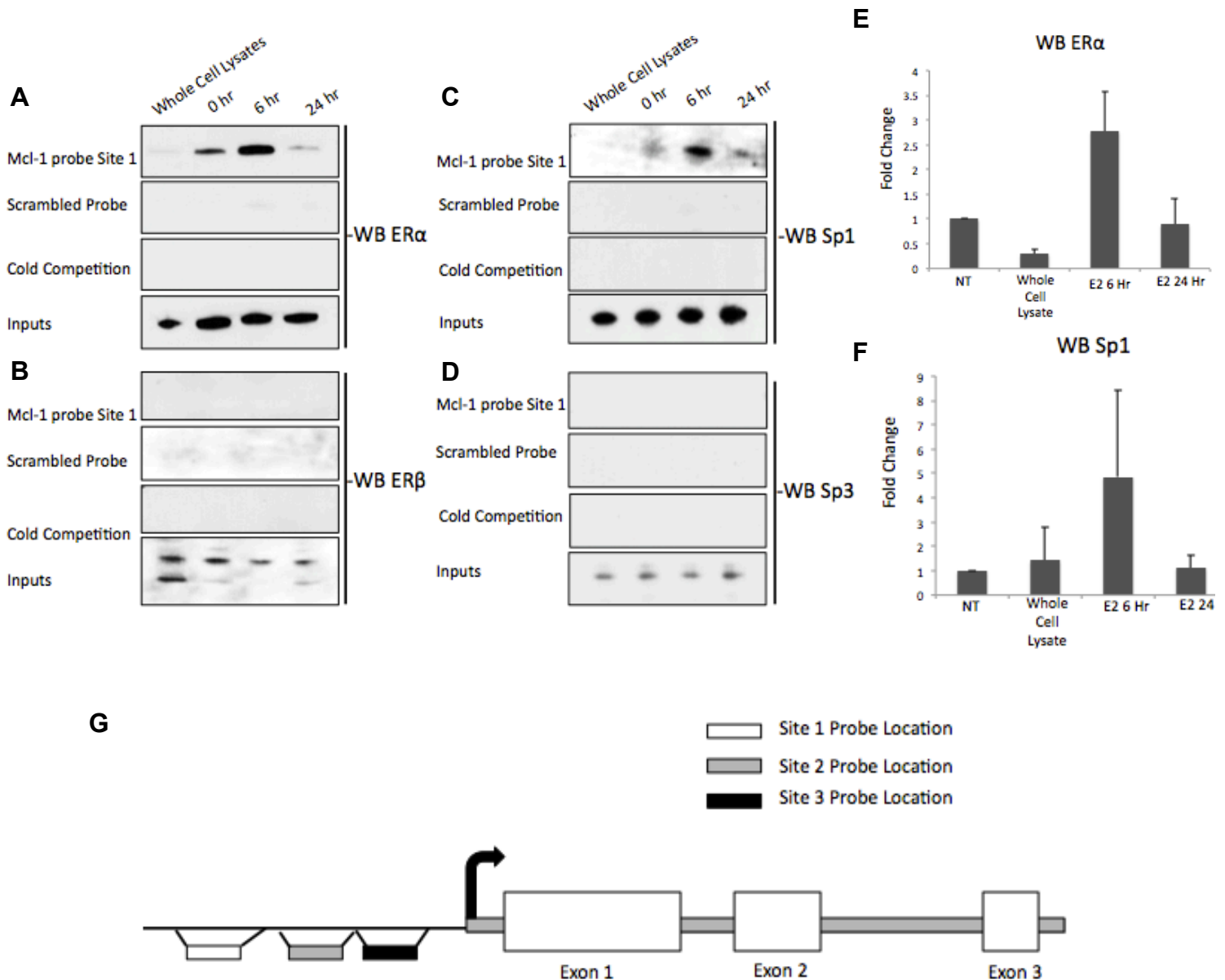
Figure 19. Estrogen increases ERα binding to specific region on Mcl-1 promoter. (A) Streptavidin pull-down assay to detect ER and transcription factor binding to a 50 bp double-stranded biotin labeled probe specific to Mcl-1 promoter region of interest (Site 1). Cells were stimulated with estrogen (10 nM) and nuclear extracts were taken 6 and 24-hours post-estrogen treatment. Pull-down products were analyzed using SDS/polyacrylamide gel electrophoresis and western blotting. Both a scrambled probe and an excess of unlabeled probe were used as a control. Blot was probed with antibody specific for ERα. (B) Blot was probed with antibody specific for ERβ. (C) Blot was probed with antibody specific for Sp1. (D) Blot was probed with antibody specific for Sp3. (E) Relative accumulation of ERα protein expression, confirmed by densitometry. (F) Relative accumulation of Sp1 protein expression, confirmed by densitometry. (G) Schematic representation of *Mcl-1* gene showing approximate locations of biotin labeled probes used for Streptavidin pull-down.
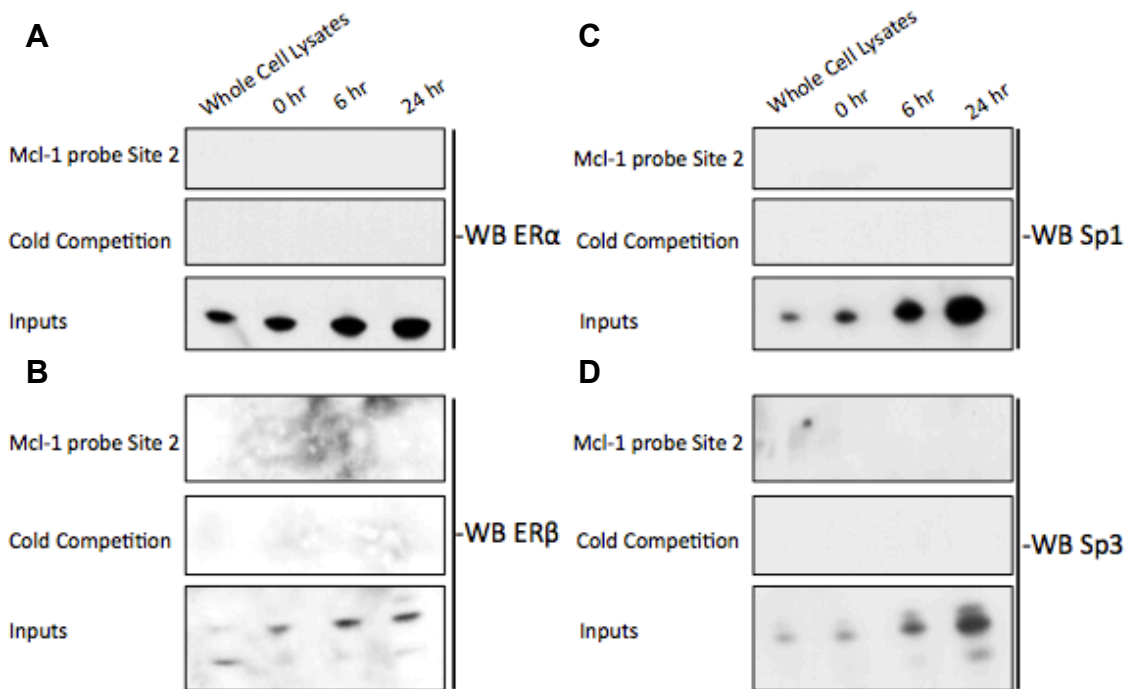
Figure 20. Estrogen does not increase ERα binding to specific region 2713 bp upstream of translation start site on Mcl-1 promoter. (A) Streptavidin pull-down assay to detect ER and transcription factor binding to a 50 bp double-stranded biotin labeled probe specific to Mcl-1 promoter region of interest (Site 2). Cells were stimulated with estrogen (10 nM) and nuclear extracts were taken 6 and 24-hours post-estrogen treatment. Pull-down products were analyzed using SDS/polyacrylamide gel electrophoresis and western blotting. Both a scrambled probe and an excess of unlabeled probe were used as a control. Blot was probed with antibody specific for ERα. (B) Blot was probed with antibody specific for ERβ. (C) Blot was probed with antibody specific for Sp1. (D) Blot was probed with antibody specific for Sp3.

Figure 21. Estrogen does not increase ERα binding to specific region 2554 bp upstream of translation start site on Mcl-1 promoter. (A) Streptavidin pull-down assay to detect ER and transcription factor binding to a 50 bp double-stranded biotin labeled probe specific to Mcl-1 promoter region of interest (Site 2). Cells were stimulated with estrogen (10 nM) and nuclear extracts were taken 6 and 24-hours post-estrogen treatment. Pull-down products were analyzed using SDS/polyacrylamide gel electrophoresis and western blotting. Both a scrambled probe and an excess of unlabeled probe were used as a control. Blot was probed with antibody specific for ERα. (B) Blot was probed with antibody specific for ERβ. (C) Blot was probed with antibody specific for Sp1. (D) Blot was probed with antibody specific for Sp3.

5. DISCUSSION

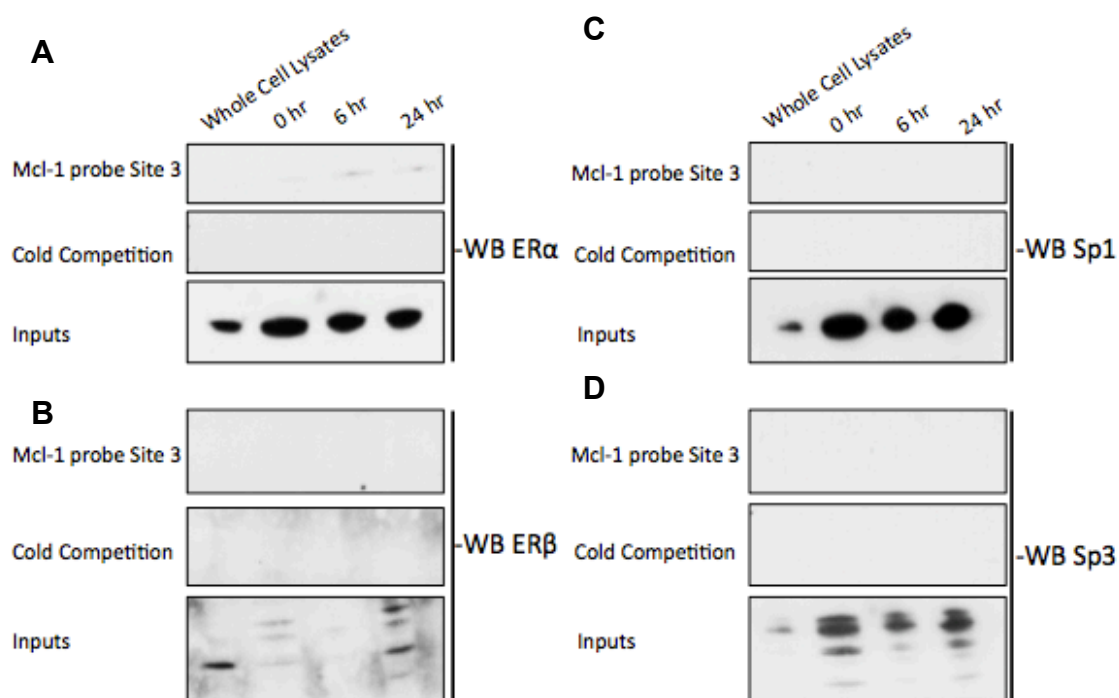Breast cancer is a heterogeneous disease that currently affects approximately 24 000 Canadian women and men every year (6). Estrogen has been implicated as an important factor in stimulating DNA synthesis and cell proliferation in breast cancer, specifically through ERα (55, 147). While many patients respond to hormonal therapy, drug resistance remains a critical issue in the treatment of breast cancer (80). Additionally, overexpression of anti-apoptotic protein Mcl-1 has been shown to contribute to drug resistance in breast cancer patients (125, 138). My research suggests that estrogen upregulated Mcl-1 expression through a mechanism involving ERα and ultimately could contribute to drug resistance in ER positive breast cancer patients.

Mcl-1 is an anti-apoptotic member of the Bcl-2 protein family, which are involved in the mitochondrial-mediated intrinsic pathway of apoptosis (125). Mcl-1 has a very short half-life, indicating that tight regulation at both the transcriptional and translational level is required (125). At the transcriptional level, Mcl-1 is regulated by several transcription factors, such as members of the STAT family and Elk-1 (69, 125). Additionally, Mcl-1 is modified post-transcriptionally by alternative splicing that results in two Mcl-1 isoforms, Mcl-1 and Mcl-1$_s$, which have opposing roles in regulating cell death (127). Mcl-1 is also regulated at the translational level by several mechanisms controlling both caspase and proteasomal degradation (125). As well, post-translational modifications, such as phosphorylation and ubiquitination, allow for tight regulation of Mcl-1 stability and degradation (125).

I have shown that estrogen is involved in regulating Mcl-1 transcription through a mechanism involving ERα. Estrogen upregulates Mcl-1 mRNA and protein expression

specifically in ERα positive breast cancer cell lines. In breast cancer, the *Mcl-1* gene is frequently amplified, allowing for its overexpression despite its short half-life (138). However, Mcl-1 expression is tightly controlled by both proteasomal and caspase-dependent degradation pathways (128). Therefore, malignant cells must develop alternative mechanisms in order to overcome Mcl-1's rapid degradation. My results indicate that ERα is an important regulator of Mcl-1 expression. I have shown that estrogen treatment increases Mcl-1 mRNA and protein expression 2-fold, and silencing of ERα inhibits Mcl-1 mRNA expression in ERα positive breast cancer cell lines. This suggests that ERα-mediated upregulation of Mcl-1 may counteract post-translational Mcl-1 degradation, allowing for evasion of apoptosis in ERα positive breast cancer.

Currently, ERα positive breast cancer patients are treated with anti-estrogen hormonal therapies such as the drugs Tamoxifen and Fulvestrant (80). These drugs antagonize ERα by binding and inhibiting estrogen-induced activation of transcription (80). My results indicate that both Tamoxifen and Fulvestrant treatment in combination with estrogen can decrease Mcl-1 mRNA and protein expression. This suggests that Mcl-1 expression is mediated through a mechanism involving ERα.

Many studies have indicated that crosstalk between estrogen and growth factor signaling pathways may promote drug resistance to hormonal therapy (80). Previous literature has demonstrated that Mcl-1 is a downstream target of EGF in many different types of cancer, including breast cancer (69, 148). Further studies have shown that estrogen may be involved in upregulating signaling pathways that are associated with EGF, such as the MAPK or PI3K/AKT pathways (149-150). EGF may initiate signaling cascades that phosphorylate and activate AF1 sites within ERα, contributing to Tamoxifen resistance (79, 144). Estrogen may

upregulate important components of EGF-mediated signaling cascades, such as activating

MAPK protein Erk in a mechanism involving ERα in MCF-7 cells (80).

My results indicate that estrogen increases Mcl-1 mRNA and protein expression.

Similarly, estrogen has been implicated in regulating several members of the Bcl-2 family of

proteins. Like Mcl-1, Bcl-2 is an anti-apoptotic protein that is frequently overexpressed in

cancer (138). Several studies have indicated that estrogen may upregulate Bcl-2, allowing for

evasion of apoptosis (147, 151-153). Estrogen-mediated overexpression of Bcl-2 is regulated

by ERα, as both Tamoxifen and Fulvestrant can decrease Bcl-2 expression (154). In addition,

estrogen may be involved in regulating pro-apoptotic BH3-only protein Noxa, which is

associated with regulating Mcl-1 expression (155). While the role of estrogen in regulating Bcl-

x_L expression in breast cancer remains unknown, a study with cultured hippocampal neurons

demonstrated that estrogen increases Bcl-x_L expression through a mechanism involving an ERE

site within the *Bcl-x_L* gene (156).

While the role of estrogen in regulating Mcl-1 expression is unclear, my findings

suggest that ERα plays an important role in upregulating Mcl-1 expression at both the mRNA

and protein level. Previously, we have performed a luciferase assay showing that estrogen

treatment results in a 2-fold increase in Mcl-1 promoter activity (Elizabeth Henson,

unpublished data). Our current findings suggest that ERα is involved in upregulating Mcl-1

expression by binding to a specific half ERE site in complex with Sp1 sites within the Mcl-1

promoter. This is in agreement with previous literature, as estrogen has been shown to mediate

gene transcription through a complex involving ERα and Sp1 (157). For example, Wu-Peng et

al. (158) found that in order for estrogen to activate creatine kinase B, a complex between Sp1

and an ER DNA-binding domain was required. Additionally, Dubik and Shiu (159) identified

an interaction between Sp1 and an ERE half-site required for the activation of c-myc. Subsequently, many studies have found numerous interactions between GC-rich Sp1 binding domains and ERE half-sites required for estrogen-mediated activity (157, 160-163).

ERα-mediated overexpression of Mcl-1 may contribute to drug resistance by providing a mechanism by which breast cancer cells can evade apoptosis. Under survival conditions, Mcl-1 sequesters BH3-only protein Noxa, preventing it from binding to pro-apoptotic proteins Bax and Bak (125). This action prevents the loss of membrane potential, production of reactive oxygen species, and release of mitochondrial protein cytochrome c, which are required for initiation of apoptosis (125). My results suggest that estrogen upregulates Mcl-1 expression, allowing for an increase in cell survival which would ultimately prevent apoptosis. Recently, small molecular inhibitors against Bcl-2 family members, such as the drugs ABT-737 and obatoclax, have shown promise in combatting drug resistance in breast cancer (164-168). Furthermore, a small molecular inhibitor specifically targeting Mcl-1, called maritoclax, has been developed and has shown effectiveness in combating resistance to ABT-737 treatment (169). Therefore, a better understanding of estrogen-mediated Mcl-1 upregulation may allow for superior targeted therapies for breast cancer patients.

My results indicate that estrogen is involved in upregulating Mcl-1 expression specifically through ERα. I propose that ERα binds to a specific ERE half-site 3683 bp upstream of the translation start site in complex with a GC-rich Sp1 binding domain within the promoter of Mcl-1. Once bound to the promoter, this ERα-Sp1 complex is capable of mediating gene transcription and upregulates Mcl-1 in the presence of estrogen. I suggest that ERα in complex with Sp1 binds to the Mcl-1 promoter in a ligand-dependent mechanism involving estrogen. However, it is possible that estrogen also regulates Mcl-1 expression through a

ligand-independent mechanism involving crosstalk with EGF and the MAPK signaling

pathway (Figure 22).

New insight into the role of ER in breast cancer has revealed two distinct ER subtypes,

ERα and ERβ, which may have differing roles when co-expressed in ER positive tumors (62).

The role of ERβ in regulating Mcl-1 expression remains unclear and the mechanism is currently

unknown.

Overall, Mcl-1 expression appears to be regulated through a mechanism involving ERα,

possibly through a complex with Sp1. Estrogen treatment upregulates Mcl-1 expression,

conceivably providing a mechanism of drug resistance in hormonal therapy. Future studies

regarding the role of estrogen in mediating apoptosis will help determine whether Mcl-1 is a

valid molecular target for breast cancer therapy.
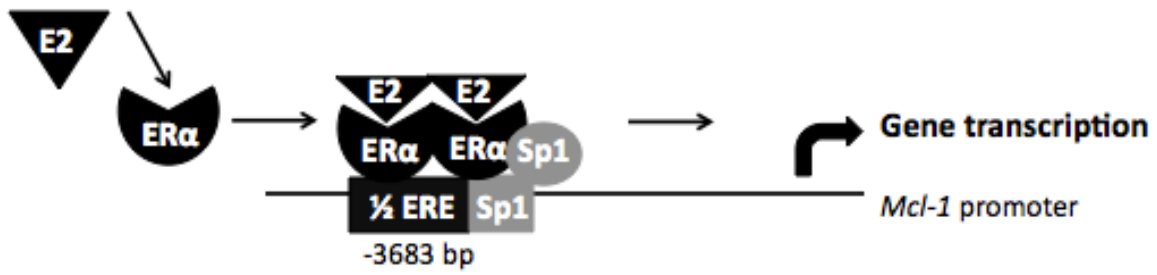
Figure 22. Proposed mechanism of ERα-mediated Mcl-1 regulation. Estrogen binds to ERα, which forms a complex with Sp1 in order to initiate Mcl-1 gene transcription.

6. CONCLUSION

       Overall, drug resistance remains a critical issue when treating breast cancer patients. Mcl-1 is an important Bcl-2 family member that is capable of promoting cell survival (125). My findings demonstrate that estrogen is involved in regulating Mcl-1 expression. This regulation occurs at both the message and protein level and is specifically regulated through ERα. Estrogen may be involved in regulating Mcl-1 expression through a mechanism involving a complex between ERα and Sp1. It is expected that estrogen treatment will cause increased expression of Mcl-1, promoting cell survival. In the future, our research will assist in resolving the role of ERα in mediating Mcl-1 expression and determine whether Mcl-1 is a valid molecular target for breast cancer therapy.

7. FUTURE DIRECTIONS

In the future, further studies are required to understand the association between EGF and ERα in mediating Mcl-1 expression. Future experiments may include treating ER positive breast cancer cells with a combination of estrogen and EGF in order to analyze Mcl-1 mRNA and protein expression. I expect that a combination of estrogen and EGF treatment will have a synergistic effect on Mcl-1 expression. In addition, it will be important to determine whether EGF is capable of phosphorylating the ERα-Sp1 complex in the Mcl-1 promoter in the absence of estrogen. ChIP and pull-down assays will be performed using antibodies for phospho-ERα. This would suggest that growth factor signaling initiates Mcl-1 transcription through a ligand-independent mechanism involving ERα.

In addition, previous studies have indicated that ERβ upregulates pro-apoptotic protein Bak, suggesting that ERβ has an opposing role to ERα-induced cell proliferation (147). An in-depth analysis of the role of ERβ in regulating Mcl-1 expression is required in order to gain a better understanding of estrogen-mediated regulation of apoptosis. In preliminary data, prolonged exposure of estrogen in ERβ expressing MD MBA 231 cells resulted in reduced Mcl-1 protein expression (data not shown). Therefore, I expect that ERβ may have a role in reducing Mcl-1 expression when co-expressed with ERα in ER positive breast cancer. In the future, Mcl-1 protein and mRNA levels will be determined in breast cancer cells either over-expressing or knock-down of ERβ in the presence or absence of ERα. In addition, ChIP analysis and pull-down assays similar to ERα experiments described above will clarify the role of ERβ in regulating Mcl-1 expression by determining whether ERβ binds to an ERE half-site within the Mcl-1 promoter. These experiments will determine the roles of EGF and ERβ in estrogen regulation of Mcl-1.

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