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Chronic cadmium exposure alters ERα dependency and drug sensitivity of breast cancer cells

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Chronic cadmium exposure alters ERα dependency and drug sensitivity of breast cancer cells

By

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Abbreviations

Acronym	Word/Phrase
AF-1	activating function-1
AF-2	activation function-2
AP-1	activator protein-1
bax	bcl-2-like protein 4
bcl-2	B-cell lymphoma 2
CBZ	cabazitaxel
Cd	cadmium
$CdCl_2$	cadmium chloride
CRISPR	clustered regularly interspaced short palindromic repeats
CTSD	cathepsin D
DBD	DNA-binding domain
DE	differentially expressed
DMEM	Dulbecco's modified eagle medium
EC_{50}	half maximal effective concentration
EGR-1	early growth response 1
EMT	epithelial-to-mesenchymal transition
EPA	Environmental Protection Agency
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
ERR	estrogen receptor responsive
ESR1	estrogen receptor 1
FBS	fetal bovine serum
GI_{50}	growth inhibition of 50%
GPR30	G protein-coupled receptor 30
GR	glucocorticoid receptor

GREB1	growth regulating estrogen receptor binding 1
GTP	guanosine 5´-triphosphate
HER2	human epidermal growth factor receptor 2
IARC	International Agency for Research on Cancer
IF	immunofluorescence
IGF1R	insulin-like growth factor 1
LBD	ligand binding domain
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRIP1	nuclear receptor interacting protein 1
PARP1	poly(ADP-ribose) polymerase 1
PR	progesterone receptor
PRSS23	protease, serine 23
PS	phosphatidylserine
P/S	penicillin and streptomycin
qRT-PCR	reverse transcription polymerase chain reaction
RNAi	RNA interference
RNA-seq	RNA sequencing
SDF1	stromal cell-derived factor 1
SERDs	selective estrogen receptor down-regulators
SERMs	selective estrogen receptor modulators
siRNA	short interfering RNA
Sp-1	specificity protein 1
TNBC	triple negative breast cancer
TXT	docetaxel
5-FU	5-fluouracil

Abstract

The global prevalence of breast cancer in women illustrates the importance of identifying factors that contribute to disease onset and progression. Endogenous and environmental agents that interact with estrogen receptor alpha (ERα) have been shown to play a role in breast cancer etiology. Evidence from epidemiological studies and animal models has suggested that cadmium, a heavy metal that can activate ERα, contributes to the development and progression of breast cancer. Additionally, our lab previously showed that chronic cadmium exposure altered the expression of several ERα-responsive genes and increased the malignancy of MCF7 breast cancer cells. Although these studies support cadmium's function as a hormone disrupter, the role of ERα in cadmium-induced breast cancer progression remains unclear. In this study, we modulated the expression of ERα in MCF7 cells after chronic cadmium exposure (Cd7 and Cd12) in order to understand its role in cadmium-induced gene expression, cell growth, migration, and anchorage-independence. While all of the cancer phenotypes analyzed were altered in MCF7, Cd7, and Cd12 cells after the permanent loss of ERα, cell growth and migration ability were less affected in cadmium-adapted cells suggesting chronic cadmium exposure reduces the dependency of MCF7 cells on ERα for these characteristics. Furthermore, analysis showed the transcript levels of classical and non-classical ERα-regulated genes were reduced in MCF7 cells after transient and permanent modification of ERα expression, while the non-classical targets were not as affected in Cd7 and Cd12 cells after ERα knockout indicating cadmium exposure may have altered the regulation of these genes. Lastly, the effects of chronic cadmium exposure on sensitivity to chemotherapeutic drugs were also investigated. We found that the

cadmium-adapted cells were more resistant to taxane drugs than MCF7 cells, but showed a similar response to anthracycline and antimetabolite drugs. Collectively, our findings show that chronic cadmium exposure promotes breast cancer progression by increasing the ability of breast cancer cells to adapt to the loss of ERα as well as highlight a potential new role for chronic cadmium exposure in development of drug resistance.

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inspiration and motivation when things seem rough. No matter what happens, I know my parents and brother will be there for whatever I need and I love you all very much!

1. Chapter 1

1.1 Breast cancer

Breast cancer accounts for about 25% of all cancers diagnosed among women worldwide (1, 2). In the United States, it is projected breast cancer cases in 2018 will account for 30% of all cancer cases, thus making it the most prevalent female cancer (3). The estimated lifetime risk of developing breast cancer for women in the US is about 12%, and current estimates predict that this year over 40,000 women will die from breast cancer—about 14% of all cancer-related deaths among women in the US (3).

Breast cancers are classified into distinct subtypes based on the presence or absence of specific molecular biomarkers, including estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Over 70% of breast cancers are ERα-positive (ERα+), making it the most prevalent subtype (3); and 50-60% of breast cancers also express PR, while 5-10% express ERα only (4, 5). ERα+ tumors typically are less aggressive and have a favorable prognosis with a 5-year survival rate of over 90% (6). Additionally, a population-based study found that breast cancers expressing ERα and PR are more likely to be diagnosed at stage I or II, whereas tumors at more advanced stages are less likely to be ERα+/PR+ (7). The effects of estrogen, a hormone involved in mammary gland development, are mediated by the estrogen receptor and have been shown to stimulate breast cancer cell growth (8). Therapeutic agents targeting the receptor and/or the synthesis of estrogen have shown success in treating ERα+/PR+ and ERα+/PR- breast cancers (5, 9). Selective ER modulators (SERMs) and selective ER down-regulators (SERDs) are drugs that specifically target ERα and block estrogen binding (10), while aromatase inhibitors

reduce the overall levels of circulating estrogen by blocking its synthesis. Accordingly, patients with metastatic breast cancer expressing ERα have been shown to respond well to hormone therapy and have higher rates of survival than those lacking ERα (11). Clearly, ERα is a useful biomarker for the treatment as well as diagnosis of breast cancer.

Unlike ERα, HER2 is expressed in only 20-30% of all breast cancer cases (12). HER2+ tumors are generally more invasive, have a worse prognosis, and are more likely to recur and metastasize than ERα+/HER2- breast cancers (13-15). However, treatment of this subtype has improved with the introduction of therapies targeted to HER2, like trastuzamab—a monoclonal antibody that specifically binds to the extracellular domain of HER2 to (a) inhibit HER2 receptor dimerization and downstream signaling and (b) recruit immune cells to kill HER2+ tumor cells (16). A recent study carried out by Perez and colleagues showed that HER2+ breast cancer patients treated with chemotherapy alone had a 10 year survival rate of 75.2%, whereas patients treated with chemotherapy plus trastuzamab had a 10 year survival rate of 84% and experienced a 37% increase in overall survival (17).

Triple-negative breast cancers (TNBCs) do not express ERα, PR, or HER2, and these tumors account for 10-20% of all breast cancer cases (12, 18, 19). Such tumors tend to be the most aggressive and have the worst prognosis of all the breast cancer subtypes (18-20). A study of TNBC patients found that 33% experienced distant recurrence within 5 years of diagnosis compared to 20% in other breast cancer patients, and the median time to death after recurrence was 9 months in TNBC patients and 20 months for non-TNBC patients (21). One-third of TNBCs are in either stage III or IV at the time of diagnosis, and this subtype accounts for 15% of all invasive breast cancer (22,

23). Chemotherapy is the most common form of therapy used in TNBC treatment, as targeted therapies are not currently available for these types of tumors.

1.2 Breast cancer risk factors

Environmental and biological risk factors are both thought to play important roles in the development of breast cancer. Biological risk factors that can contribute to the development of breast cancer include genetics, gender, race/ethnicity, and lifetime exposure to estrogen—the steroidal hormone responsible for the development of the mammary gland and other female characteristics (2, 24). As indicated earlier, given its role in mammary gland development and cell growth, the deregulation of estrogen signaling plays a central role in the development of hormone-dependent breast cancers. Lifetime estrogen exposure is influenced by the age at onset of menstruation, age at first pregnancy, age at which a woman enters menopause, and obesity (25, 26). Environmental risk factors include diet, cigarette consumption, physical activity, and environmental exposures to chemicals and heavy metals (2, 27). Furthermore, exposure to environmental agents that have estrogen-like activity may compound with lifetime exposure to estrogen and contribute to breast cancer (28). In addition to pharmaceutical and plant sources of estrogens, studies have suggested some heavy metals possess estrogenic activity and may contribute to breast cancer (29). These heavy metals are collectively referred to as metalloestrogens and include nickel, selenium, mercury, and cadmium, the latter of which is the best characterized of all the metalloestrogens (29-31).

1.3 Cadmium

Cadmium is found ubiquitously in the environment. It is present in the earth's crust at a concentration of 0.1-0.5 ppm and is a natural component of ocean water with

average levels as high as 110 ng/L in some coastal areas (32). Cadmium is primarily released into the environment through the mining and refining of metals, the burning of fossil fuels, and the disposal and incineration of waste. These activities can pollute the soil and water and then enter the food supply by accumulating in plants and animals (33). For non-smokers in the United States, dietary intake is the largest source of cadmium exposure, with estimated daily intakes of 0.35 and 0.30 μg Cd/kg/day for men and women, respectively (34-36). Shellfish, wheat, potatoes, and leafy vegetables are foods that tend to be higher in cadmium content. Each cigarette contains roughly 1.7 μg Cd and about 10% of this is inhaled when smoked. Accordingly, the cadmium burden of cigarette consumers is greater than that of non-smokers, with mean blood cadmium levels of 1.58 μg/L and 0.38 μg/L, respectively (37, 38). The body is only able to remove a fraction of our daily cadmium intake, and consequently cadmium tends to bioaccumulate in body tissues. As a result of such prolonged exposure, cadmium has a half-life in blood of about 20-30 years (36, 39, 40). Cadmium has been recognized as a carcinogen by the International Agency for Research on Cancer (IARC) and the U.S. Environmental Protection Agency (EPA) (41). While the primary target organs for cadmium are the kidney and lungs, other studies indicate that cadmium can also affect other tissues, like the liver, prostate, and breast (42-46).

1.4 Epidemiological evidence connecting breast cancer and cadmium exposure

Several epidemiological studies link cadmium exposure and breast cancer. A report by McElroy *et al.* measured urinary cadmium levels in 246 breast cancer patients and 254 age-matched controls using inductively coupled plasma mass spectrometry. They found that women with a higher cadmium burden had an increased risk of

developing breast cancer compared to women with lower cadmium levels (47). A similar study from Japan using flameless atomic absorption spectrometry showed that urinary cadmium levels positively correlated with breast cancer risk (48). Strumylaite *et al.* compared cadmium concentrations in the tissue, blood, and urine of 57 breast cancer and 51 benign tumor patients. The researchers revealed significantly higher cadmium levels in the tumor tissue and urine of breast cancer patients than those with benign tumors (49). Interestingly, this study also reported that ERα-positive breast cancers had significantly higher cadmium concentrations than ERα-negative cancers (49), which suggests that cadmium might be a more critical factor in tumors expressing ERα. A recent study showed that the cadmium content of breast cancers increased the risk of distant metastasis within the first 5 years (50). Conversely, two cohort-based studies could not conclude higher cadmium levels increased the risk of breast cancer mortality based on urinary cadmium measurements (51, 52), but this may be attributed to the small sample sizes of the studies ($n<45$) (53). There are also inconsistencies in epidemiological reports focusing on dietary cadmium exposure. While one report found that dietary cadmium exposure increased the breast cancer risk of post-menopausal women (54), another study based on self-reported surveys of food consumption in over 150,000 post-menopausal women showed no significant association between dietary cadmium exposure and ovarian, endometrial, and breast cancer risk (55). A study of dietary cadmium intake and the risk of hormone-defined breast cancer in 405 Japanese women found a significant association for ERα+ tumors in postmenopausal women (56). Finally, although a meta-analysis of previous studies on dietary cadmium exposure and breast cancer risk in post-menopausal women concluded there was no statistically significant association, the

authors suggested interpretation of the data should be made cautiously due to the difficulties in accurately determining cadmium intake from food consumption (57). All these reports highlight the need of additional studies to better understand the relationship between environmental cadmium exposure and breast cancer risk.

1.5 Animal and *in vitro* research on cadmium exposure and breast cancer

Research on the physiological and cellular effects of cadmium exposure have provided more insights into its potential role in breast cancer. Two *in vivo* studies demonstrated that ovariectomized rats subject to a single acute cadmium exposure displayed increased uterine weight and higher density of epithelial cells in the mammary gland, both of which are considered early events in breast cancer development (58, 59). Johnson *et al.* also found that the effects of cadmium were blocked when the rats were concurrently injected with a dose of the antiestrogen ICI-182,780, suggesting that ERα may play an important role in mediating cadmium's effects (58). Two *in vitro* studies found that acute cadmium exposure upregulated the expression of ERα-regulated genes and increased cell growth in ERα-positive MCF7 breast cancer cells (60, 61). Earlier work from our lab has shown that cadmium promotes expression of cyclin D and c-myc—two genes involved in cell proliferation—by potentiating the interaction between ERα and the transcription factors c-fos and c-jun (62). Although these studies support cadmium's role as a metalloestrogen in breast cancer, most humans are exposed to cadmium at low, chronic levels, not acute.

Despite difficulties to accurately mimic environmental cadmium exposure at minute quantities over prolonged periods of time, some studies have managed to examine the effects of chronic cadmium exposure. For example, Alonso-Gonzalez *et al.* showed

an increase in the uterine weight, ductal branching, and lobuloalveolar development in ovariectomized mice after 7 weeks of low exposure to cadmium (63). Another report demonstrated that prolonged exposure to cadmium malignantly transformed breast epithelial cells *in vitro*, independent of ERα expression (64). Our lab has likewise mimicked chronic cadmium exposure by exposing ERα-positive MCF7 breast cancer cells to low levels of cadmium (100 nM $CdCl_2$) for six months. We have found that the cadmium-exposed MCF7 cells grow faster, have increased migration capabilities, and are more invasive, indicating that chronic cadmium exposure promotes breast cancer progression (65, 66). Our lab also demonstrated that low levels of cadmium promotes expression of SDF1, a chemokine that promotes tumor growth and metastasis (67, 68) by altering the interaction between ERα, c-jun, and c-fos, thus supporting cadmium's function as a metalloestrogen (65). Microarray analysis showed that the expression of genes that impact many pathways—both ERα-dependent and -independent—was altered in cadmium-exposed MCF7 cells (66). However, although chronic cadmium exposure has been shown to increase the malignancy of breast cancer cells, the underlying mechanisms that contribute to breast cancer development and progression are still not fully understood.

2. Chapter 2. The effects of chronic cadmium exposure on estrogen receptor gene regulation and cancer phenotypes

2.1 Introduction

ERα and ERβ, another estrogen target, belong to a superfamily of nuclear hormone receptors that have a highly conserved structure (69, 70). Estrogen binds to the ligand-binding domain (LBD) to initiate ligand-dependent transcriptional activation within the activation function-2 domain (AF-2). The binding of estrogen also induces a conformational change leading to dimerization and translocation into the nucleus. Once inside the nucleus, this conformational change allows for its interaction with other transcriptional cofactors (71, 72). The conserved DNA-binding domain (DBD) contains two zinc fingers which bind to a specific palindromic sequence—5`-GGTCAnnnTGACC-3`—called an estrogen response element (ERE) (73). Unlike the DBD and LBD, the NH_2-terminal is highly variable in sequence among the nuclear receptors and contains the activation function-1 domain (AF-1), which is responsible for ligand-independent activation of the receptor (74). While AF-1 and AF-2 have distinct functions, both are crucial for the transcriptional activity of ER and often work synergistically to induce a more robust transcriptional response (75).

ERs regulate gene expression and cellular processes through both genomic and non-genomic actions (Figure 1). The genomic effects of ER signaling occur through both classical and non-classical mechanisms. In the classical mechanism, ligand binding activates ER causing homodimerization and translocation into the nucleus where it binds directly to DNA to modulate expression of target genes (73). Classical ER-regulated genes contain an ERE site upstream of the transcription start site that facilitates DNA

binding and the recruitment of co-regulators by ER (76). Non-classical mechanisms are not dependent on a full-length ERE (77). In this form of gene regulation, ER forms heterodimers with other transcription factors to recognize half-ERE sites within promoter regions. Alternatively, ER can be recruited by other transcription factors as a co-regulator to alter gene expression independent of an ERE (78, 79). Transcription factors that associate with ERα, such as Sp-1, AP-1, and the NFkB families of transcription factors, are involved in regulating many cellular processes (80-84). ERs can also be activated through post-translational modifications in the AF-1 region independent of estrogen (74). Conversely, ERs can impact gene regulation without directly binding to nuclear DNA. Such non-genomic actions of estrogen can be mediated by cross-talking of cytosolic ER with other signaling pathways (85, 86) or through the membrane receptor known as GPR30, which activates signaling cascades to elicit a physiological response (87, 88).

Estrogen binds to the ER to regulate multiple physiological processes, including growth and development of the mammary gland (89). Lifetime exposure to endogenous estrogens is a key risk factor for breast cancer (25, 90), and exogenous agents with estrogenic activity have also been implicated in breast cancer. The heavy metal cadmium is an environmental pollutant that is known to have estrogenic activity (29). Epidemiological studies have found that cadmium levels positively correlate with breast cancer risk and tumor malignancy (47, 49). To better understand the estrogenic activity of cadmium on breast cancer, multiple studies have attempted to delineate the molecular interactions between cadmium and ERα. Stoica *et al.* measured the ability of cadmium to bind and activate both wild-type and mutant forms of ERα transfected into COS-1 cells.

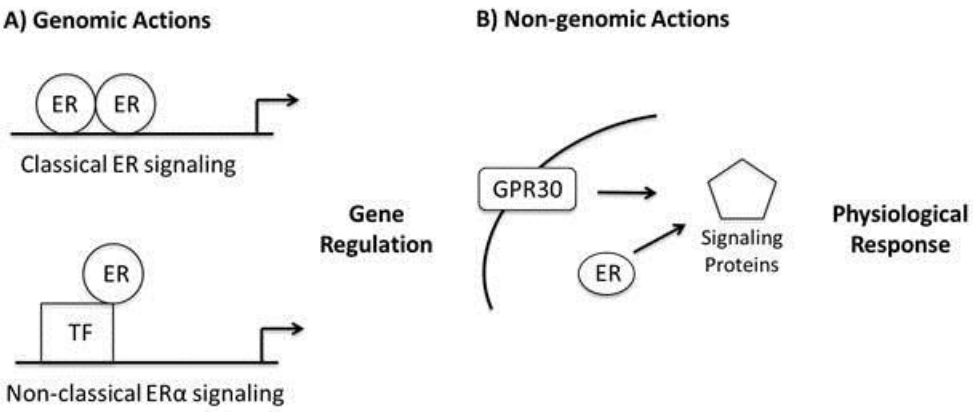


Figure 1. Genomic and non-genomic actions of estrogen signaling. A) Genomic actions of ERα occur in the nucleus through classic and non-classical mechanisms. In classical ERα signaling, the receptor homodimerizes and binds directly to DNA at an estrogen-specific site. In non-classical ERα signaling, the receptor interacts with other transcription factors to regulate gene expression. B) The non-genomics actions of estrogen can be mediated by the membrane receptor GPR30 and ER crosstalking with other proteins in the cytosol.

This study revealed that cadmium activated ERα at concentrations as low as 10^{-11} M and also blocked estradiol binding in a noncompetitive manner indicating that cadmium interacts with ERα in the LBD (91). Another study carried out by Nesatyy and colleagues used chemical modification of ERα to identify potential cadmium interaction sites and found that cysteine residues within the LBD of the receptor have a strong affinity for cadmium (92). However, cys-381 and cys-447, two amino acids implicated as cadmium interaction sites previously (91), were not consistent with the cysteine residues identified by Nesatyy *et al.* (92). Due to chemical similarities between Zn^{2+} and Cd^{2+}, the zinc fingers of the DBD are another potential interaction site between cadmium and ERα. Accordingly, Predki and colleagues investigated the ability of several metals to replace zinc in the zinc fingers of hormone receptors and its effect on DNA binding *in vitro*. These studies demonstrated that even though ERα and glucocorticoid receptor (GR) were able to bind DNA even after cadmium displacement of zinc in the zinc fingers of the DBD, cadmium substitution slightly increased the DNA binding affinity of ERα (93, 94). While it is unclear how cadmium modulates the activity of ERα, these mechanisms may have implications relevant to breast cancer development.

Multiple studies, including those from our lab, have demonstrated that cadmium mimics estrogen and induces expression of ERα target genes under conditions of acute exposure to high concentrations (>1 μM) of cadmium (31, 60-62, 91, 95). In addition to acute studies, our lab has also evaluated the impacts of chronic, low-level exposure to cadmium on breast cancer and showed that prolonged exposure to cadmium even at low levels (10^{-7} M) increases the malignancy of MCF7 cells (65). Furthermore, after prolonged exposure to cadmium, the gene expression profile of these cells was altered

(66). Despite evidence that cadmium has estrogenic activity and may activate ERα upon acute exposure, less is known about the effects of chronic cadmium exposure on breast cancer and whether the estrogenic activity at low exposure levels is necessary for disease progression.

The goal of this project is to determine the role of ERα in chronic cadmium-induced gene expression and breast cancer progression. To achieve this, we modulated ERα expression in MCF7 breast cancer cells chronically exposed to cadmium and examined the effects on gene expression and phenotypic characteristics. Our results demonstrate that although ERα plays an important role in cadmium-mediated gene expression alterations and malignant phenotypes, chronic exposure to cadmium also increases the ability of MCF7 cells to adapt to the loss of ERα and aid in cell survival.

2.2 Materials and Methods

Materials

MCF7 cells were obtained from American Type Culture Collection (ATCC Manassas, VA). Cadmium chloride (Acros Organics, Geel, Belgium) was dissolved in autoclaved H_2O and sterile-filtered to make a 1M solution. Stock solution of ICI-182, 780 (Tocris Bioscience, Bristol, UK) was prepared at a concentration of 10^{-3} M in DMSO according to manufacturer's protocol.

Cell culture

MCF7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin and streptomycin (P/S) (Life Technologies).

Cadmium-adapted cell lines, MCF7-Cd7 and -Cd12, were generated as described previously (65, 66) and maintained in DMEM with 10% FBS, 1% P/S, and 10^{-7} M $CdCl_2$.

Identifying differentially expressed (DE) genes

Total RNA was collected from cells treated with 10^{-7} M of the antiestrogen ICI-182,780 or vehicle using Trizol Reagent (Life Technologies) and columns from the Direct-zol RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's protocol. Triplicate samples were sent to the bioinformatics core at the University of Minnesota Genomics Center. After passing quality check, mRNA was isolated from the total RNA via oligo-dT purification. This was followed by random-primed reverse-transcription, second-strand cDNA synthesis, and creation of a strand-specific library from the resulting dsDNA. The 50 bp paired-end reads were sequenced on a HiSeq 2500 (Illumina, Hayward, CA) at a depth of 22,000 reads. EdgeR (96) was used to determine DE genes and the resulting list of genes was ranked by false discovery rate (FDR) ranging from 10^{-3} to 10^{-6}. Different subsets of data were compared using Perl scripts (www.perl.org). Cluster 3.0 (97, 98) was used to organize data sets by DE genes, and heatmaps highlighting top 500 genes were created using http://jtreeview.sourceforge.net/.

RNA interference

Approximately $1x10^5$ cells were seeded into 6-well plates and transfected the following day with ERα siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using siRNA transfection reagents (Santa Cruz Biotechnology). A scrambled siRNA (Santa Cruz Biotechnology) was used as a control. The following day, the medium was replaced with DMEM containing 10% FBS and 1% P/S. Cells were harvested 24 and 48 hours later for

gene and protein expression analysis using reverse transcriptase PCR (qRT-PCR) and western blot, respectively.

Antiestrogen treatment

Approximately $2x10^5$ cells were seeded into 6-well plates and treated with a final concentration of 10^{-7} M ICI-182,780 or mock-treated with DMSO (Sigma Aldrich, St. Louis, MO). Cells were harvested 24 and 48 hours later for gene and protein expression analysis using qRT-PCR and western blot, respectively.

Western blot analysis

Cells were lysed in 1% sodium dodecyl sulfate (SDS)-HEPES buffer (0.05 M HEPES, 1% Triton, 0.002 M EDTA, 1% Deoxycholate, 0.002 M EGTA, 0.15M NaCl, and 0.01 M NaF) plus protease inhibitor cocktail (Thermo Fisher, Waltham, MA) for 15 minutes at 4°C. The cell lysate was then centrifuged at 20,000 x g for 15 minutes at 4°C. The total protein concentration was determined using the Bio-Rad Dc Protein Assay kit (Bio-Rad, Inc., Hercules, CA). Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Hayward, CA). The membranes were blocked with 5% milk-Tris-buffered saline with Tween (TBST) for one hour before protein expression was monitored using the following specific antibodies at dilutions ranging from 1:500 to 1:1000: ERα Ab-12 (6F11) (Neomarkers, Fremont, CA), Cathepsin D (C-5; Santa Cruz), SDF1 (Cell Signaling Technology), c-myc (D84C12; Cell Signaling Technology), Cyclin D (A-12; Santa Cruz), and Actin [AC-15] (Sigma). HRP-goat anti-mouse and -rabbit secondary antibodies (Invitrogen, Carlsbad, CA) were used at a concentration of 1:2000, and Clarity

Western ECL Substrate (Bio-Rad) was used for detection. Images were captured and analyzed using the iBright CL1000 imager (Invitrogen).

Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells using Trizol reagent (Life Technologies) and columns from the Direct-zol RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Inc., Foster City, CA). Gene expression was quantified using gene specific primers and Fast SYBR Green master mix (Applied Biosystems). The reaction was cycled 40 times with an annealing temperature of 60ºC. All gene-specific primers were synthesized by Integrated DNA Technologies, Inc. (IDT, San Diego, CA).

Table 1.	qRT-PCR Primer Sequences (5′ → 3′)
c-myc$_F$	CTCCACACATCAGCACAACT
c-myc$_R$	GTTTCCGCAACAAGTCCTCT
cyclin D1$_F$	AATGTGTGCAGAAGGAGGTC
cyclin D1$_R$	GAGGGCGGATTGGAAATGAA
CTSD$_F$	CTCTGTCCTACCTGAATGT
CTSD$_R$	GACAGCTTGTAGCCTTTG
SDF1$_F$	GTCAGCCTGAGCTACAGATGC
SDF1$_R$	CACTTTAGCTTCGGGTCAATG
pS2$_F$	GCGCCCTGGTCCTGGTGTCCA
pS2$_R$	GAAAACCACAATTCTGTCTTTC
Actin$_F$	GAGAAAATCTGGCACCACACC
Actin$_R$	ATACCCCTCGTCGATGGGCAC

Luciferase Reporter Assay

The ERE-Luc reporter plasmid contained an insert with three tandem ERE sequence repeats upstream of the TATA promoter and the sequence encoding a functional luciferase on a pGL-3 plasmid. An empty pGL-3 vector was used as a control. Approximately 2.5 x 10^4 cells were seeded in 24-well plates and allowed to grow

overnight. The following day, transfection with ERE-Luc or empty pGL3 plasmids along with the pRL-SV40 *Renilla* luciferase plasmid (Promega) was accomplished using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Five hours after transfection, the medium was changed to DMEM containing 10% FBS and 1% P/S, and cells were harvested 24 hours later. ICI-182,780 was added at a final concentration of 100 nM. Assays were performed in triplicate and analyzed using the DualGlo Dual Luciferase Assay system (Promega, Madison, WI).

Derivation of CRISPR/Cas-9-edited cell lines

Approximately $1x10^5$ cells were seeded into 6-well plates and transfected with ERα double nickase plasmids (Santa Cruz Biotechnology). To select for successfully transfected cells, 2.5 μg/mL puromycin (Santa Cruz Biotechnology) was added to the media for three days. Single cell clones were isolated by serial dilution in 96-well plate, and wells with only a single cell were expanded into clonal cell lines. Cell lines were initially screened for ERα protein expression by western blot analysis using an ERα-specific antibody (Ab-12, Neomarkers). Clones that did not express ERα at the protein level were candidates for DNA sequencing verification performed by Genewiz, Inc. (South Planfield, NJ). Sequence reads of ~800bp spanning the target region in the first exon of ESR1 were aligned using MacVector software (MacVector, Inc., Version 12.7.0 (214), Apex, NC) to identify frameshift mutations.

Cell Growth Assay

Approximately 50,000 cells were plated in 6-well plates. The next day, cells were counted in triplicate using a hemacytometer (Thermo Fisher), and total cell number was counted 2, 3, and 4 days later after the initial cell count. The doubling times were

determined using the exponential growth equation in Graphpad Prism v7.02 software (GraphPad Software, Inc., San Diego, CA).

Scratch Wound Assay

Cells were allowed to grow to approximately 80-90% confluence in 6-well plates before being scratched with a P200 pipette tip. The wound was imaged on the same day (day 0) and again after 4 days of growth (day 4). To quantify the migration capability of the cells, the surface area of the wound at day 0 and 4 was calculated using ImageJ software (NIH, Bethesda, MD). The percent of the wound that was healed was calculated using this equation:

% wound repaired = [1-(wound surface area day 4/wound surface area day 0)] x 100

Soft Agar Colony Formation Assay

Twenty-four well plates were coated with 1 mL 1% agar in supplemented DMEM with 20% FBS and 2% P/S, and this constituted the bottom later of the well. Once solidified, approximately 500 cells were mixed with 0.5 mL 0.6% agar DMEM containing 10% FBS and 1% P/S and poured on top of the bottom layer and incubated at 37°C and 5% CO_2. Fresh media was added to the top layer every 2-3 days. After two weeks, live colonies were stained using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; Invitrogen) and imaged using the ChemiDoc Imaging system (Bio-Rad). Colonies of 100 cells or greater were counted.

Statistical Analysis

To determine statistical significance, all data were analyzed using an unpaired, two-tailed T test in Graphpad Prism.

2.3 Results

While our lab previously demonstrated that breast cancer cells chronically exposed to cadmium had altered gene expression profiles in comparison to parental cells—including estrogen-responsive and breast cancer-associated genes (66)—the role of ERα in mediating these changes is unclear. To determine if chronic cadmium exposure alters gene expression through ERα-dependent mechanisms, MCF7 cells and cadmium-adapted cells (Cd7 and Cd12) were treated with the antiestrogen ICI-182,780 (ICI) to downregulate ERα, and a non-biased global gene expression analysis was conducted using RNA sequencing (RNA-seq) (University of Minnesota Genomics Center, Minneapolis, MN). Figure 2A shows the hierarchical clustering of the top 500 differentially expressed (DE) genes (FDR $\leq 10^{-6}$), demonstrating that the reduction of ERα alters expression of many genes in both MCF7 and the cadmium-adapted cell lines, including many of the ERα-regulated genes, such as GREB1, PR, SDF1, MYC, IGF1R, CTSD, NRIP1, and PRSS23 (65, 66, 91, 99). Moreover, under normal conditions (in the absence of ICI) these genes appear to be upregulated in the cadmium-adapted cells compared to the MCF7 cells, suggesting that chronic cadmium exposure alters expression of these ERα-regulated genes (Figure 1A). In total, the RNA-seq analysis identified 3,706, 4,721, and 4,628 DE genes in MCF7, Cd7, and Cd12, respectively, when treated with ICI compared to mock treated cells. Of the DE genes, 2,477 are shared by all three cell lines, while 251, 981, and 1,314 were unique to MCF7, Cd7, and Cd12, respectively (Figure 2B). In all, 67.3% and 59.5% of the DE genes in Cd7 and Cd12 cells, respectively, were shared with MCF7 cells suggesting that ERα continues to play an important role in gene expression after prolonged cadmium exposure.

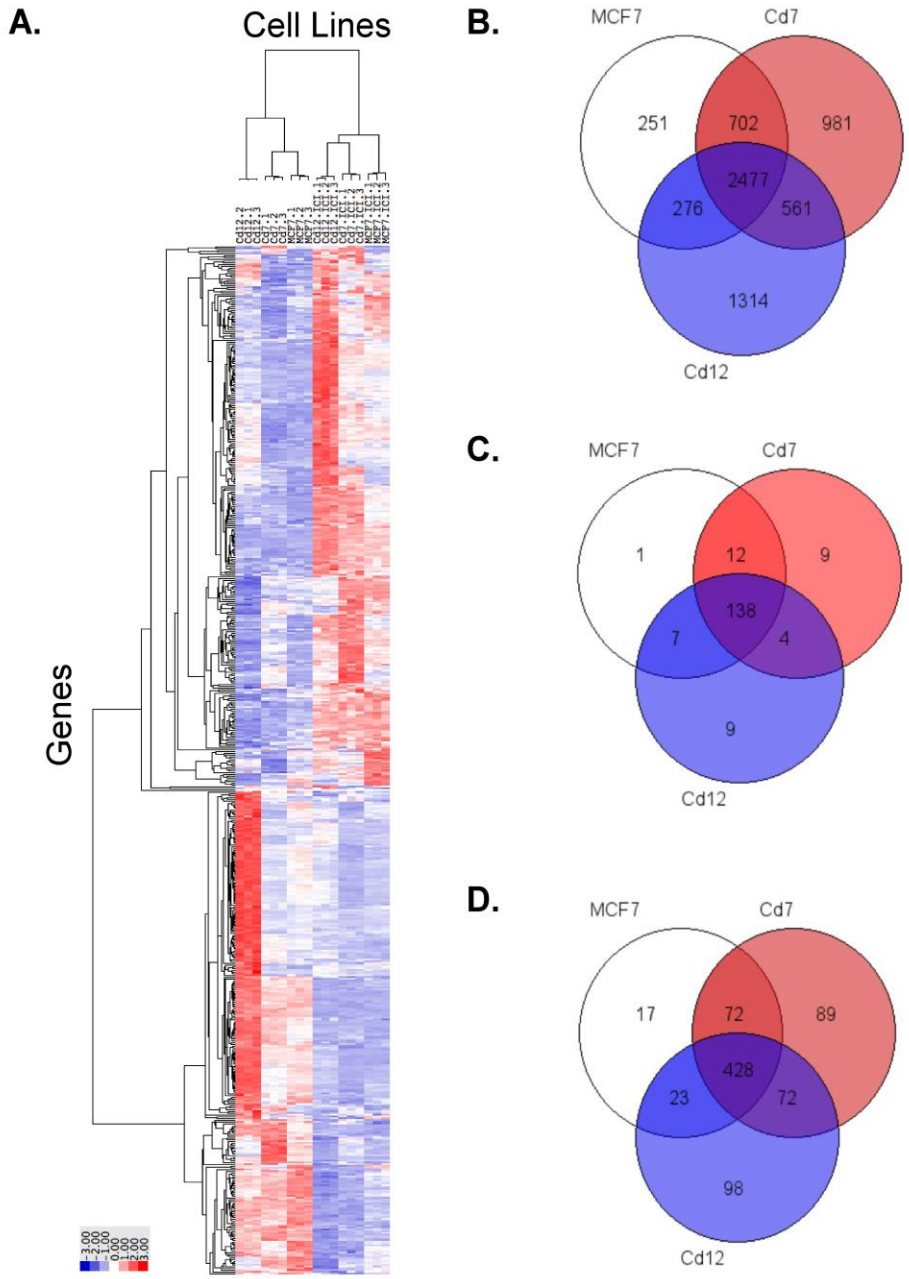


Figure 2. The effects of chronic cadmium exposure on ERα-responsive gene expression. (A) MCF7 , Cd7, and Cd12 cells were treated with either the antiestrogen ICI-182,780 or vehicle in triplicate for 24 hours. Total RNA was collected and RNA-seq was performed by the University of Minnesota Genomics Center. The top 500 differentially expressed genes (FDR≤10^{-6}) after ERα knockdown were hierarchically clustered. The Venn diagrams represent the (B) total, (C) ERE, and (D) ERR genes that were differentially altered to the same extent in MCF7, Cd7, and Cd12 cells after antiestrogen treatment.

To better understand how chronic cadmium exposure affects ERα-regulated genes, we compared how the classical ERα (ERE) genes and ERα-responsive (ERR) genes (100, 101) were altered after antiestrogen treatment in the MCF7 and cadmium-adapted cells. The results in figure 2C show that there were 180 total ERE genes that were altered by ERα modulation. Of those, 138 ERE genes (76.7%) were changed in the same direction (either up- or down-regulated) in MCF7, Cd7, and Cd12 cells (Figure 2C). As for the ERR genes, 428 (53.6%) of the 799 genes were altered in the same direction for all three cell lines (Figure 1D). There were only 17 ERR genes that were only differentially expressed in MCF7 after ICI treatment, while 89 and 98 ERR DE genes were unique to Cd7 and Cd12, respectively (Figure 2D). These findings show that while a majority of ERE genes responded in the same manner to decrease levels of ERα, more variability existed within the ERR genes, suggesting that chronic cadmium exposure may have expanded the function of ERα.

To confirm the RNA-seq data, ERα expression was down-regulated by either RNA interference (RNAi) or ICI for 24 and 48 hours, and cells were collected for protein and gene expression analyses. Consistent with the RNA-seq analysis, the expression of the ERα target genes SDF1, CTSD, c-myc, and cyclin D1 were decreased in all three cell lines at both the protein (Figure 3A) and transcription levels when ERα function was impaired ($p<0.05$; Figure 3B). To assess the ability of ERα function to mediate the transactivation of an ERE promoter, we transfected an ERE-luciferase reporter plasmid into MCF7, Cd7, and Cd12 cells and measured the transcriptional output. Once again, ERα expression was modulated by treating cells with and without ICI. Results in figure 3C show that transactivation decreased 58-, 47-, and 112-fold in MCF7, Cd7, and Cd12

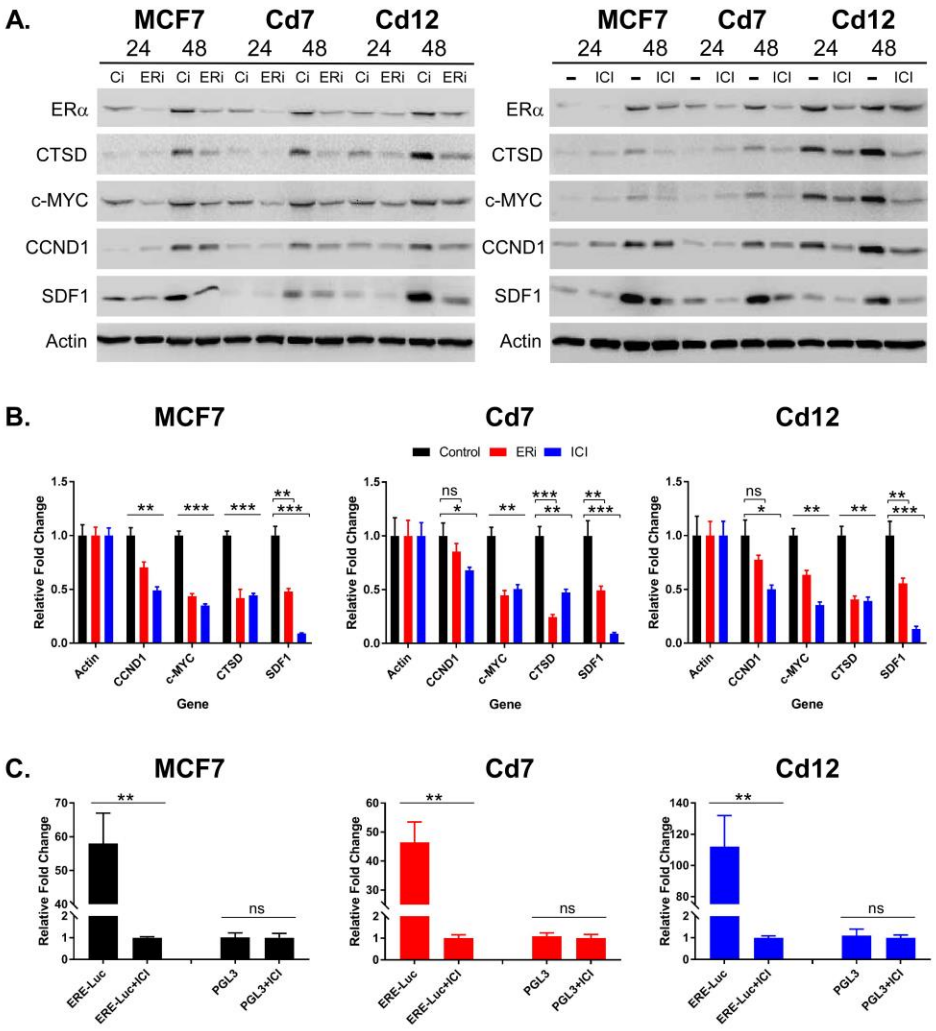


Figure 3. ERα function remains intact after prolonged exposure to cadmium. (A) MCF7, Cd7, and Cd12 cells were transfected with si-ERα (ERi) or si-control (Ci) or treated with 100 nM ICI-182,780 (ICI) or vehicle (-) and collected after 24 and 48 hours for protein expression analysis using western blot with actin as the loading control. Results represent three independent experiments. (B) RNA was collected after 24 hours for gene expression analysis using qRT-PCR (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). Results represent three independent experiments with quadruplicate samples. (C) The ability of ERα to mediate ERE transactivation in MCF7, Cd7, and Cd12 cells was measured using a luciferase reporter assay. Cells were transfected with either ERE-Luc plasmids or empty vector (pGL-3) and treated with ICI or vehicle control for 24 hours. Results represent three independent experiments with triplicate samples (** = $p<0.01$; ns = not significant).

cells, respectively, after a decrease in ERα levels ($p<0.01$) suggesting that the loss of ERα activity had a similar effect on MCF7 and Cd7 cells, but appears to have a slightly greater effect on Cd12 cells. As expected, cells transfected with a control, empty pGL-3 plasmid exhibited little to no activity in the absence or presence of ICI. Collectively, these findings demonstrate that the loss of ERα results in significant reduction of ERE transcriptional activity and alteration of estrogen responsive gene expression in the cadmium-adapted cells.

Since ERα function remains intact after chronic cadmium exposure, we questioned whether ERα is responsible for the cadmium-induced malignant phenotypes (65). To test this, we developed a stable system of ERα loss using the CRISPR/Cas9 gene editing system to permanently knockout ERα expression. In brief, two short-guide CRISPR RNA oligonucleotides were used to specifically target adjacent sequences in exon 1 of the ESR1 gene (Figure 4A). Upon recognition of these target sequences, the mutant Cas9 enzyme nicks both strands of DNA to induce a double strand break with minimal off-target effects (102). Correction of this break by non-homologous end joining often introduces insertions or deletions of nucleotides, which may lead to permanent loss of gene function. DNA sequencing and protein expression analysis of the MCF7, Cd7, and Cd12 CRISPR-edited clones revealed that 8 contained mutations that resulted in a lack of ERα protein expression (Figure 4B-C), and these clones were selected for further characterization. Clones that still expressed ERα of either the same or different molecular weight were not used for further experimentation.

To confirm that ERα function is absent in the CRISPR-edited clones, ERα transactivation was measured in clones transfected with ERE-Luc reporter plasmids and

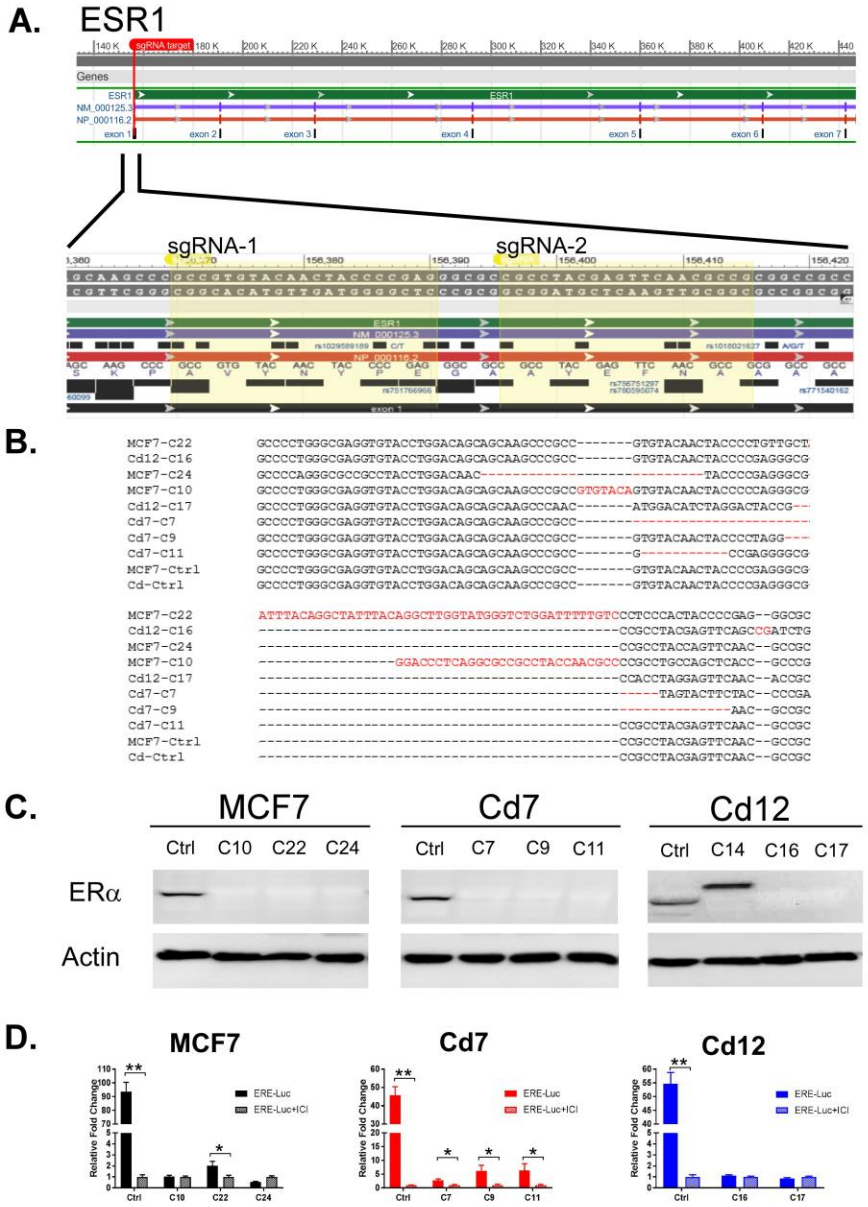
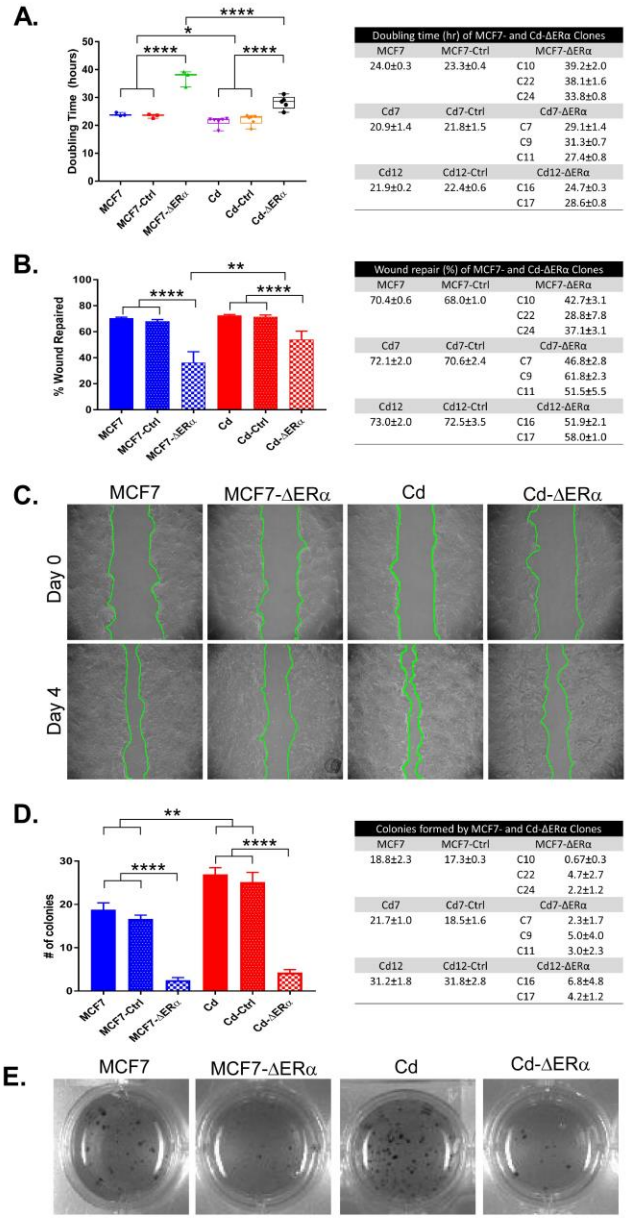


Figure 4. Generation of ERα-KO clones using the CRISPR/Cas9 gene-editing system. (A) Two CRISPR short guide RNA (sgRNA) oligos directed Cas9-mediated DNA cleavage at a specific site in exon 1 of the ESR1 gene. (B) DNA sequence alignment of a region that flanks the CRISPR target sequence showing the presence of insertion or deletion mutations (shown in red) in single cell-derived clones after transfection with plasmids containing sgRNA oligos and Cas9 enzyme. (C) The presence or absence of ERα in control or CRISPR-transfected clones was verified using western blot analysis with actin used as the loading control. (D) Presence or absence of ERα function in CRISPR-edited clones was confirmed using ERE-Luc reporter assay to monitor ERα transactivation. Cells were transfected with ERE-Luc plasmids and then treated with ICI (ERE-Luc + ICI or the vehicle control (ERE-Luc) for 24 hours (* = $p<0.05$; ** = $p<0.01$).

then treated with ICI for 24 hours. Figure 4D shows the MCF7, Cd7, and Cd12 control cell lines that express ERα exhibited 94-, 46-, and 55-fold increase in ERE activity, respectively, compared to cells treated with ICI ($p<0.01$), confirming ERα activity. On the other hand, the transcriptional activity of the ERE-Luc promoter was significantly reduced in all ΔERα clones relative to the unedited parental controls ($p<0.01$). Although several clones exhibited above background levels of ERE transactivation activity in comparison to their ICI-treated counterparts (2- to 6-fold difference; $p<0.05$), this activity was 40- to 92-fold lower than the wildtype ERα activity in unedited parental cells (Figure 4D). To verify ERα was not significantly expressed in these clones, protein expression analysis was repeated and again showed no ERα expression (data not shown), suggesting that other nuclear hormone receptors and/or estrogen-related receptors (ERβ) that are also sensitive to ICI may be activating the promoter. These data provide further evidence that each of the CRISPR/Cas9-edited clones lost wildtype ERα function.

To investigate how the complete loss of ERα affects the phenotypes of MCF7 and cadmium-adapted cells, we measured the doubling times for all the clones lacking ERα (ΔERα) compared to control cells by determining the total cell number after 0, 48, 72, and 96 hours. For statistical analysis, all three MCF7-ΔERα clones (C10, C22, and C24) served as biological replicates, while the three Cd7-ΔERα (C7, C9, and C11) and two Cd12-ΔERα (C16 and 17) clones were biological replicates of cadmium-adapted, ERα knock-out cells. The average doubling time of the MCF7-ΔERα group was about 37 hours, which was significantly longer than the 24 hour doubling time of normal MCF7 cells ($p<0.0001$). The average doubling time for the Cd-ΔERα clones was 28.2 hours compared to 21.4 hours for the cadmium-adapted group ($p<0.0001$; Figure 5A).



Doubling time (hr) of MCF7- and Cd-ΔERα Clones			
MCF7	MCF7-Ctrl	MCF7-ΔERα	
24.0±0.3	23.3±0.4	C10	39.2±2.0
		C22	38.1±1.6
		C24	33.8±0.8
Cd7	Cd7-Ctrl	Cd7-ΔERα	
20.9±1.4	21.8±1.5	C7	29.1±1.4
		C9	31.3±0.7
		C11	27.4±0.8
Cd12	Cd12-Ctrl	Cd12-ΔERα	
21.9±0.2	22.4±0.6	C16	24.7±0.3
		C17	28.6±0.8

Wound repair (%) of MCF7- and Cd-ΔERα Clones			
MCF7	MCF7-Ctrl	MCF7-ΔERα	
70.4±0.6	68.0±1.0	C10	42.7±3.1
		C22	28.8±7.8
		C24	37.1±3.1
Cd7	Cd7-Ctrl	Cd7-ΔERα	
72.1±2.0	70.6±2.4	C7	46.8±2.8
		C9	61.8±2.3
		C11	51.5±5.5
Cd12	Cd12-Ctrl	Cd12-ΔERα	
73.0±2.0	72.5±3.5	C16	51.9±2.1
		C17	58.0±1.0

Colonies formed by MCF7- and Cd-ΔERα Clones			
MCF7	MCF7-Ctrl	MCF7-ΔERα	
18.8±2.3	17.3±0.3	C10	0.67±0.3
		C22	4.7±2.7
		C24	2.2±1.2
Cd7	Cd7-Ctrl	Cd7-ΔERα	
21.7±1.0	18.5±1.6	C7	2.3±1.7
		C9	5.0±4.0
		C11	3.0±2.3
Cd12	Cd12-Ctrl	Cd12-ΔERα	
31.2±1.8	31.8±2.8	C16	6.8±4.8
		C17	4.2±1.2

Figure 5. Characterization of MCF7, Cd7, and Cd12 cells after ERα knockout. (A) Approximately $5x10^4$ cells were seeded in 6-well plates, and total cell number was counted 24, 48, 72, and 96 hours later to calculate the population doubling time of each cell line. MCF7-ΔERα included MCF7-C10, -C22, and -C24 clones, and Cd-ΔERα included Cd7-C7, -C9, -C11, Cd12-C16, and -C17. The data were derived from three independent experiments of triplicate samples (* = $p<0.05$; **** = $p<0.0001$). (B) Migration ability for each control and clone was quantified by comparing the surface area of scratch wounds at day 0 and day 4. The data represent the mean of three independent experiments of triplicate samples (** = $p<0.01$; **** = $p<0.0001$). (C) Representative images of MCF7, MCF7-ΔERα (ERα knockout), Cd, Cd-ΔERα cells at day 0 and 4 after being scratched with the wound outlined in green. (D) Anchorage-independent growth ability was measured using the colony formation assay in soft agar. Only colonies of approximately 100 cells or more were counted, and the results represent three independent experiments of triplicate samples (** = $p<0.01$; **** = $p<0.0001$). (E) Representative images of MCF7, MCF7-ΔERα, Cd, and Cd-ΔERα colonies growing in soft agar.

Consistent with previous data (65), results in figure 5A show that the cadmium-adapted cells grew faster than the MCF7 (24 vs 21.4 hours; $p<0.05$). Interestingly, despite the loss of ERα, Cd-ΔERα clones retained a significant growth advantage over the MCF7-ΔERα cells (28.2 vs 37 hours; $p<0.0001$) (Figure 5A).

To understand if depletion of ERα affects the ability of cadmium-adapted cells to migrate, we used a scratch wound assay. In brief, cells were grown to 80-90% confluence, a scratch wound was inflicted to the monolayer, and the ability of the cells to repair the wound was monitored over 4 days. The surface area of the wound was calculated using ImageJ software at day 0 and day 4, and the results in figure 5B and C show that both the wounds in the MCF7 and cadmium-adapted cells were almost fully healed by day 4, while this ability was reduced in Cd-ΔERα and even more significantly impaired in MCF7-ΔERα. More specifically, the MCF7 cells had a wound healing capacity of 70.4% compared to 36.2% for the MCF7-ΔERα cells ($p<0.0001$), while Cd-adapted cells had a wound healing capacity of 72.6% compared to 54% for the Cd-ΔERα cells ($p<0.0001$; Figure 5B-C). Although the slight increase in the migration potential of the cadmium-adapted cells relative to MCF7 cells was not significant, the wound healing capacity of the Cd-ΔERα clones was significantly greater than that of the MCF7-ΔERα clones ($p<0.01$; Figure 5B). As with cell growth, ERα is important for wound healing, though in the absence of ERα the cadmium-adapted cells still exhibit a greater migration potential.

Given the differences observed in both growth and migration between MCF7-ΔERα and Cd-ΔERα clones, we assessed tumorigenic potential using a colony formation assay in soft agar. For each clone, approximately five hundred cells were seeded in soft

agar, and colonies of approximately one hundred cells were counted after two weeks. Results in figure 5D and E show an average number of 19 colonies formed by MCF7 cells which decreased to an average of 2.5 colonies in the MCF7-ΔERα clones ($p<0.0001$), while the Cd cells formed an average of 27 colonies in soft agar compared to 4.3 colonies formed by the Cd-ΔERα clones ($p<0.0001$). The Cd-adapted cells formed significantly more colonies than the MCF7 cells ($p<0.01$), which confirms previous findings (65) that prolonged cadmium exposure enhances the malignant characteristics of breast cancer cells. While the different number of colonies between Cd-ΔERα and MCF7-ΔERα cells was not statistically significant, it was trending towards significance with a p-value of 0.064 (Figure 5D). These findings confirm that chronic cadmium exposure increases tumorigenic potential of breast cancer cells. Collectively, the phenotypic analyses—growth, migration, and anchorage independency—demonstrate the importance of ERα for these cancer characteristics. However, despite the loss of ERα, the cadmium-adapted cells retained a growth and migration advantage over MCF7 cells, suggesting that chronic cadmium exposure enables breast cancer cells to better adapt to the loss of ERα.

Since our phenotypic assays highlighted significant differences in the characteristics of the cadmium-adapted cells upon permanent loss of ERα, we decided to further investigate how permanent loss of ERα in Cd-adapted cells affected the expression of ERα-regulated genes. Specifically, we analyzed the transcriptional expression of three classical (CTSD, pS2, and SDF1) and two non-classical (c-myc and cyclin D1) ERα-regulated genes using reverse transcriptase PCR (qRT-PCR). Results in figure 6A reveal that mRNA levels of CTSD, pS2, and SDF1 were significantly reduced

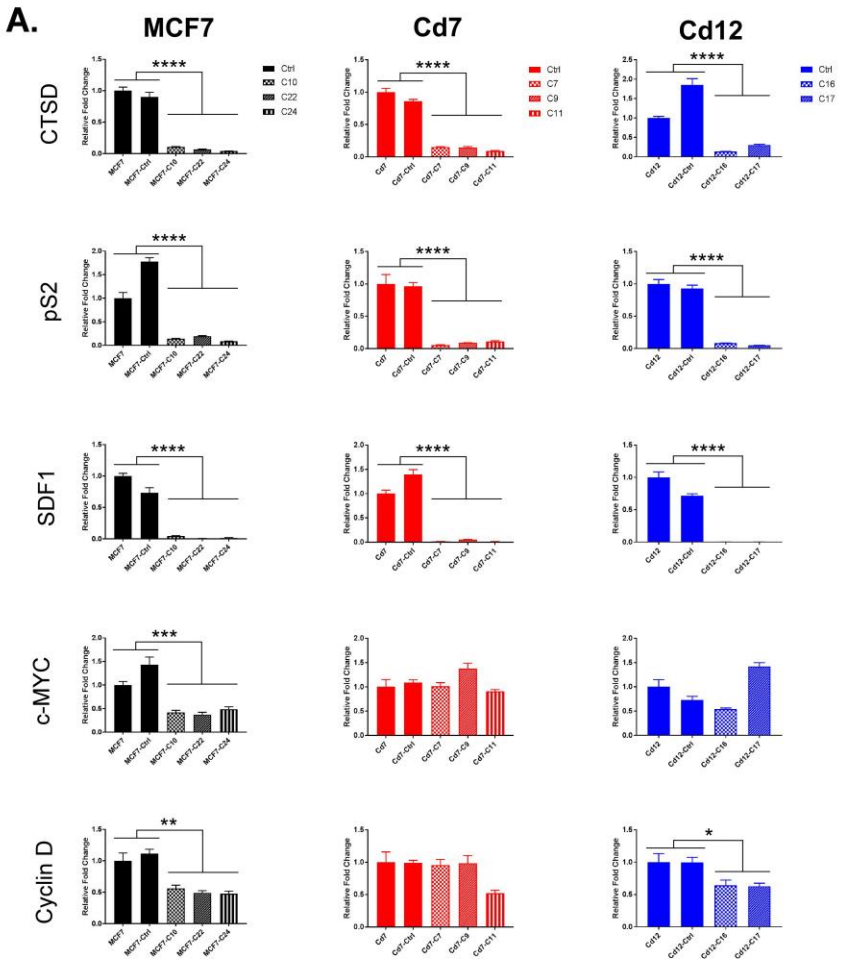


Figure 6. The effect of ERα knockout on expression of classical and non-classical ERα genes. (A) Gene expression of the classical ERα genes, CTSD, pS2, and SDF1, and the non-classical genes, c-myc and cyclin D1, were measured using qRT-PCR. Results represent the average of three independent experiments of quadruplicate samples (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$).

in all ΔERα (MCF7, Cd7, and Cd12) clones ($p<0.0001$; Figure 5A)—consistent with the transient ERα depletion (Figure 3). Curiously, although the results also show that the gene expression of c-myc and cyclin D1 were significantly downregulated in the MCF7-ΔERα cells ($p<0.01$), there was no significant difference in the expression observed with either gene in the Cd7-ΔERα cells (Figure 6A), which was not quite consistent with results in figure 3B. For the Cd12-ΔERα cells, c-myc expression was also not significantly altered, though a decrease in cyclin D1 expression was seen but not as significant compared to the decrease seen in MCF7-ΔERα cells ($p<0.05$ vs. $p<0.01$) (Figure 6A). Similar to earlier experiments, these findings demonstrate that ERα, whether transient (Figure 3) or permanent (Figure 6A) loss, is critical for the expression of classical ERα genes in all three cells lines; however, the cadmium-adapted cells appear to have an increased ability to continue expressing non-classical ERα genes after permanent loss ERα, whereas this was not observed under transient reduction of ERα.

2.4 Discussion

As stated previously, epidemiological reports have found a link between cadmium and breast cancer risk and malignancy (47, 49, 50). Animal models have also shown cadmium promotes early signs of cancer development in the mammary gland and uterus (58, 59, 63). Although multiple *in vitro* studies have shown that acute levels of cadmium can mimic the effects of estrogen and activate ERα to alter expression of target genes (60-62, 65), whether or not ERα is activated in response to the relatively low, environmental levels of cadmium is unclear. Since our lab previously demonstrated that chronic cadmium exposure is associated with a distinct gene expression "signature"—which includes differences in estrogen responsive genes—we further investigated the

role of ERα in mediating cadmium-induced gene expression and breast cancer progression. Results from this study extend our earlier observations that chronic cadmium exposure alters expression of ERα-regulated genes, including PRSS23, CTSD, c-myc, and SDF1 (66), and upon transient reduction of ERα by antiestrogen treatment, the expression of these genes was decreased (Figure 2A and B). Additionally, over half of all the DE genes in response to ICI were shared in the three cell lines suggesting ERα plays a critical role in gene regulation even after prolonged exposure to cadmium (Figure 2B).

While it is expected that ERα modulation would affect the expression of estrogen responsive genes (99), a larger number of genes were impacted by the decrease in ERα levels mediated by ICI in the cadmium-adapted cells. The RNA-seq results also revealed that there was more variability in how ERR genes were impacted in the cadmium-adapted cells in response to the reduction in ERα levels with only 53.6% of the ERR genes altered in the same direction in all three cell lines as compared to 76.7% of ERE genes (Figure 2C-D). Notably, Cd7 and Cd12 had 89 and 98 ERR genes, respectively, that were differentially expressed after ICI treatment and not shared by any other cell line compared to only 17 in parental MCF7 cells. We speculate that prolonged cadmium exposure may have deregulated ERα and expanded its function. Since cadmium has been shown to displace other divalent metals, like zinc and calcium—both of which are involved in ERα function—cadmium could bind to ERα and affect its transcriptional capabilities (93, 103). Furthermore, difference in ERα function were also observed in Cd7 and Cd12 (Figure 3), which is expected since these cell lines were derived from single cells from a pooled population of MCF7 cells chronically exposed to cadmium.

Future experiments to examine ERα transactivation in the pooled population of cadmium-adapted MCF7 cells and/or additional Cd lines would provide more insight into whether this difference is unique to Cd7 and Cd12. Given the gene expression alterations in the cadmium-adapted MCF7 cells, future studies to determine if global ERα promoter occupancy in breast cancer cells is altered by chronic cadmium exposure would be interesting.

Of course, it is possible that these differences in gene expression are not dependent on direct interactions between cadmium and ERα. Since ERR genes are co-regulated by other transcription factors (i.e. AP-1, Sp-1) in partnership with ERα (78, 79), alterations in the expression or activity of these transcriptions factors in the cadmium-adapted cells could explain the differential response of ERR genes to ERα depletion. Hart and colleagues reported that during cadmium adaption in lung epithelial cells, the DNA binding activities of AP-1, EGR-1, and NFkB were increased, whereas the binding activity of Sp-1 was reduced (104). Cadmium is also known to induce oxidative stress, which can alter the expression of stress response genes, such as metallothioneins, heat shock proteins, glutathione, and various transcription factors (105, 106). Similar to our lab's results with chronic cadmium exposure (65), Mahalingaiah *et al.* found that chronic oxidative stress upregulated pro-metastatic genes and increased the tumorigenicity of breast cancer cells (107). Thus, overall gene expression in the cadmium-adapted cells might respond differently to antiestrogen treatment because their ability to react to stress has been enhanced by chronic cadmium-induced oxidative stress.

In addition to the molecular changes, phenotypic differences in how MCF7 and Cd-adapted cells respond to the loss of ERα were also observed. Consistent with past

results, this study confirmed that the cadmium-adapted MCF7 cells grew faster than parental MCF7 cells (Figure 5A). However, this study showed only minute differences in migration between MCF7 and cadmium-exposed MCF7 which were not significant as we had shown previously (65). This discrepancy may be explained by the fact that the prior study was performed on a pooled population of MCF7 cells after chronic cadmium exposure, and migration was examined after three days of growth; whereas clonal, single-cell derived lines were analyzed in this study and migration was assessed after a 4-day period.

To further examine the impact of ERα loss on the malignant characteristics of both MCF7 and Cd-adapted cell lines, a colony formation assay was used. After weeks of growing in soft agar, Cd-adapted cells displayed a higher frequency of colony formation in comparison to MCF7 cells (Figure 5D-E). Our results further establish that Cd-adapted cells possess a greater tumorigenic potential with an increased ability to grow in an anchorage-independent manner. While the loss of ERα negatively impacted the tumorigenic potential of both MCF7 and Cd-adapted cells, the impact was slightly less on the Cd-adapted cells. Our results confirmed and extended the observation—made by our lab and others—that low levels of cadmium over prolonged periods of time induces cancer progression (43, 44, 65, 108, 109). We speculate that chronic cadmium exposure may also increase the ability of breast cancer cells to better adapt to stresses like ERα loss. While more in-depth molecular and biochemical studies are necessary to uncover the mechanistic underpinnings, initial gene expression analysis demonstrate that the levels of the non-classical ERα-regulated genes, c-myc and cyclin D1, were less affected by deletion of ERα in the cadmium-adapted cells in comparison to MCF7 cells (Figure

6). Interestingly, c-myc and cyclin D1 expression were downregulated in MCF7, Cd7, and Cd12 cells after transient modulation of ERα expression using either ICI or RNAi (Figure 3A-B). This difference may be attributed to either the lack of a complete loss of ERα under the transient condition or the fact that transient reduction of the receptor does not allow for the cells to adapt to the change, unlike the permanent knockout of ERα using CRISPR/Cas9.

Although our findings establish that ERα remains critical for the maintenance of cadmium-induced malignant phenotypes in MCF7 cells, the cadmium-adapted breast cancer cells seem to have developed an additional mechanism by which they can partially circumvent the loss of ERα and continue to thrive. Confirmation of this hypothesis is found in a study by Benbrahim-Tallaa *et al.*, in which the authors concluded that the estrogenic effects of cadmium were not necessary for carcinogenesis after prolonged cadmium exposure malignantly transformed MCF10A cells, an immortalized normal breast epithelial cell line that does not express ERα (64). In line with previous observations (110-113), our study does not dispute that cadmium induces changes independent of ERα, but also offers support that when present, ERα plays a critical role in cancer progression. Further investigation of how chronic cadmium exposure may change and expand the function of ERα could potentially identify additional mechanisms by which cadmium functions as a metalloestrogen. Our findings that chronic cadmium exposure stimulates the expression of estrogen-responsive genes and increases the malignancy of breast cancer cells demonstrate that cadmium likely promotes breast cancer progression through both estrogen-dependent and -independent pathways. In conclusion, this study shows that chronic cadmium exposure reduced their dependency

on ERα and increased the adaptability of breast cancer cells, which could have important implications regarding the use of antiestrogen therapy against ERα+ breast cancers with higher levels of cadmium.

3. Chapter 3

3.1 Introduction

Chronic cadmium exposure has been shown to transform normal breast epithelial cells and enhance the progression of malignant tumors (44, 106, 108, 109, 114, 115). Abshire *et al.* demonstrated that when transformed rat myoblast cells were repeatedly exposed to cadmium and then inoculated into immunodeficient mice, the resultant tumors were more malignant and invasive compared to unexposed controls (108). Another study showed that human fibrosarcoma cells chronically exposed to cadmium displayed more aggressive behavior by readily invading reconstituted basement membranes (109). In rats, repeated cadmium exposure at low doses resulted in more malignant and metastatic tumors than rats treated with higher, acute dosages of cadmium (44). Cadmium exposure has also been found to alter host-tumor interactions (115) and promote epithelial-to-mesenchymal transition (EMT) (113), both of which may increase the invasiveness of tumors, often observed after prolonged cadmium exposure. Additionally, our lab has shown that breast cancer cells chronically exposed to cadmium for over six months have higher growth rates, increased migratory ability, and enhanced invasiveness (65). These findings not only suggest that cadmium can promote tumor progression, but that human cadmium exposure may have important implications regarding cancer malignancy and treatment.

Cadmium exposure may alter the effectiveness of certain anticancer drugs (116, 117). For example, Asara *et al.* demonstrated that treatment of normal MCF7 cells with 5-fluouracil (5-FU), an inhibitor of DNA synthesis and common breast cancer chemotherapeutic, induced morphological signs of cytotoxicity, including swollen

mitochondria, cell nuclei degeneration, and pore-like formations in the cell membrane (116). However, when these cells were treated with an acute dose of cadmium (5 μM) in addition to 5-FU, these morphological changes were no longer observed, suggesting that cadmium may alter the response of MCF7 to 5-FU-treatment (116). In a follow-up study, this group found that cadmium-exposed MCF7 cells had higher levels of *bcl2* and lower expression levels of p53, *bax*, and caspase-8 and -9 in response to 5-FU treatment, while the reversed effect was observed in MCF7 cells treated with 5-FU but not exposed to cadmium (117).

Although these findings indicate that acute cadmium exposure may inhibit the cytotoxic effects of 5-FU, the effect of prolonged exposure to cadmium at low environmental levels on the efficacy of certain chemotherapeutic drugs is not known. In this chapter, we investigate the effects of chronic cadmium exposure on the sensitivity of breast cancer cells to different classes of anti-cancer drugs, including antimetabolites, anthracyclines, and taxanes. Our results suggest that breast cancer cells chronically exposed to cadmium display a decreased response to the taxane drugs while exhibiting little to no difference in response to doxorubicin and gemcitabine.

3.2 Materials & Methods

Materials

Cells were obtained or derived as described previously in Chapter 1. The following anticancer drugs were obtained: cabazitaxel (Selleck Chemicals, Houston, TX), docetaxel (Tocris, Bristol, UK), doxorubicin (Tocris), gemcitabine (Tocris), and 5-fluouracil (Tocris). Drug stocks were diluted to 10^{-2} M or 10^{-3} M in either ethanol or DMSO according to manufacturer's protocol.

Drug Response Assay

Approximately 1,000 cells were seeded in 96-well plates and treated the following day with different concentrations of compounds: docetaxel (10^{-7}-10^{-11} M), cabazitaxel (10^{-7}-10^{-11} M), doxorubicin (10^{-6}-10^{-10} M), and gemcitabine (10^{-6}-10^{-10} M) for 48 hours. Cell growth was measured indirectly using tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Invitrogen) or [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS] (Promega). GI_{50} values (concentrations that inhibited 50% of cell growth) were plotted on a non-linear regression of log-transformed data using GraphPad Prism v.7.02 (GraphPad Software, Inc, La Jolla, CA) and expressed as mean ± SE.

Western Blot Analysis

Approximately 2 x 10^5 cells were seeded in 6-well plates and treated the following day with varying concentrations of docetaxel or cabazitaxel for 24 hours. Cells were lysed and samples processed as described previously in Chapter 1. Protein expression was determined by incubation of the membrane with the following specific antibodies at dilutions ranging from 1:500 to 1:1000—PARP (Cell Signaling Technology), p53 (Ab-6) (Calbiochem, San Diego, CA), p21(12D1; Cell Signaling Technology), alpha-tubulin (11H10; Cell Signaling Technology), and beta-tubulin (T4026; Sigma Aldrich). HRP-goat anti-mouse and -rabbit secondary antibodies (Invitrogen) were used at a concentration of 1:2000 and Clarity Western ECL Substrate (Biorad) was used for detection. Images were captured and analyzed using the iBright CL1000 imager (Invitrogen).

Annexin V Apoptosis Assay

Cell death was measured using the RealTime-Glo™ Annexin V Apoptosis assay (Promega) according to the manufacturer's protocol. Briefly, approximately 2,500 cells were plated in 96-well plates, and the following day triplicate samples were treated with concentrations of cabazitaxel ranging from 10^{-7} to 10^{-11} M. The assay reagents were added to each well immediately following drug treatment and incubated at 37ºC. Luminescent readings were measured 36 and 48 hours later using the Fluorstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Half maximal effective concentration (EC_{50}) was determined with Graphpad Prism using a non-linear regression curve fit algorithm.

Immunofluorescence

Approximately 1.5 x 10^5 cells were plated onto sterilized coverslips placed in 6-well plates and treated the following day with cabazitaxel, gemcitabine, or vehicle control for 20 hours. Cells were fixed with acetone then permeabilized with 0.05% Saponin/PBS and blocked with a 10% FBS/PBS solution. Anti-beta tubulin antibody (Sigma) was used at a 1:300 dilution and was incubated for 1.5 hours. Alexa Fluor 488 secondary antibody (Invitrogen) was used at a 1:200 dilution and was incubated for 1.5 hours. Slides were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen) and imaged using a fluorescent microscope (Leica Microsystems, Inc., Deerfield, IL).

3.3 Results

To understand the effects of chronic cadmium exposure on drug sensitivity, a panel of chemotherapies typically used in the treatment of breast cancer was selected. This panel included microtubule-stabilizing agents, cabazitaxel and docetaxel (taxanes),

the anthracycline doxorubicin, and antimetabolites gemcitabine and 5-fluorouracil. To assess the potency of these drugs, we performed growth assays on control MCF7 and two cadmium-adapted MCF7 clones (Cd7 and Cd12) to determine the drugs' GI_{50}, or the drug concentration required to inhibit cell growth rate by 50%. The GI_{50} values for doxorubicin were 429 nM, 282 nM, and 369 nM for MCF7, Cd7, and Cd12, respectively ($p>0.05$), and for gemcitabine were 24.7 nM, 17.1 nM, and 19.4 nM, respectively ($p>0.05$) (Fig. 7A). The GI_{50}'s for 5-fluorouracil could not be determined because the values were apparently above the highest concentration of the drug tested (10^{-5} M) for all three cell lines. These results indicate no significant differences in how the three cell lines responded to doxorubicin and gemcitabine. On the other hand, control MCF7 cells and the cadmium-adapted cells showed significant differences in their responses to the taxanes. More specifically, the GI_{50} values for cabazitaxel were 3.31 nM, 9.94 nM ($p<0.001$), and 9.98 nM ($p<0.001$) for MCF7, Cd7, and Cd12, respectively, and 3.86 nM, 11.4 nM ($p<0.0001$), and 12.27 nM ($p<0.0001$) for docetaxel, respectively (Fig. 7A-B). These findings indicate that chronic cadmium exposure alters the cells' ability to respond to taxanes and may promote resistance.

Since docetaxel and cabazitaxel are cytotoxic drugs that induce cell cycle arrest and apoptosis, we analyzed the expression of proteins involved in these pathways to evaluate if the cadmium-adapted cells are more resistant to taxane-induced apoptosis. Results in figure 8A-B show the protein expression of poly(ADP-ribose) polymerase-1 (PARP1), p53, and p21—which are signals of either apoptosis or cell cycle arrest—in MCF7, Cd7, and Cd12 cells 24 hours after treatment with increasing concentrations of docetaxel and cabazitaxel (Fig. 8A-B). There were significantly higher levels of cleaved

A.

Potency of different classes of chemotherapeutic drugs GI_{50} in nM (Mean ± SEM)				
Drug	Class	MCF7	Cd7	Cd12
Cabazitaxel	Taxane	3.31 ± 0.71	9.94 ± 0.66	9.98 ± 1.39
Docetaxel	Taxane	3.86 ± 1.57	11.4 ± 3.52	12.27 ± 2.53
Doxorubicin	Anthracycline	$4.29x10^2 \pm 1.05x10^2$	$2.82x10^2 \pm 0.69 x10^2$	$3.69x10^2 \pm 0.64x10^2$
Gemcitabine	Antimetabolite	24.7 ± 7.5	17.1 ± 6.3	19.4 ± 6.8
5-Fluouracil	Antimetabolite	$> 1x10^5$	$> 1x10^5$	$> 1x10^5$

B.

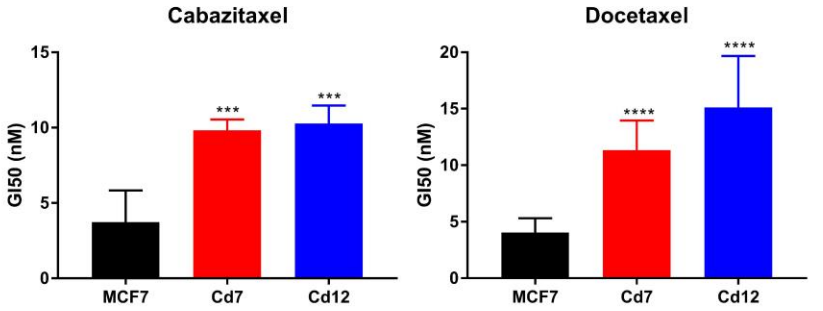


Figure 7. Chronic cadmium exposure increases resistance to taxane drugs. Approximately 1,000 MCF7, Cd7, or Cd12 cells were seeded onto 96-well plates and treated with varying concentrations of cabazitaxel, docetaxel, doxorubicin, gemcitabine, and 5-fluorouracil for 48 hours. (A) Cell growth after treatment was measured indirectly using MTT and compared to the mock treated control to determine the GI_{50} value for each drug. Each drug concentration and the control were done in replicates of six, and the data are representative of three independent experiments. (B) Bar graphs comparing the GI_{50} values for the taxane drugs (*** = $p<0.001$; **** = $p<0.0001$).

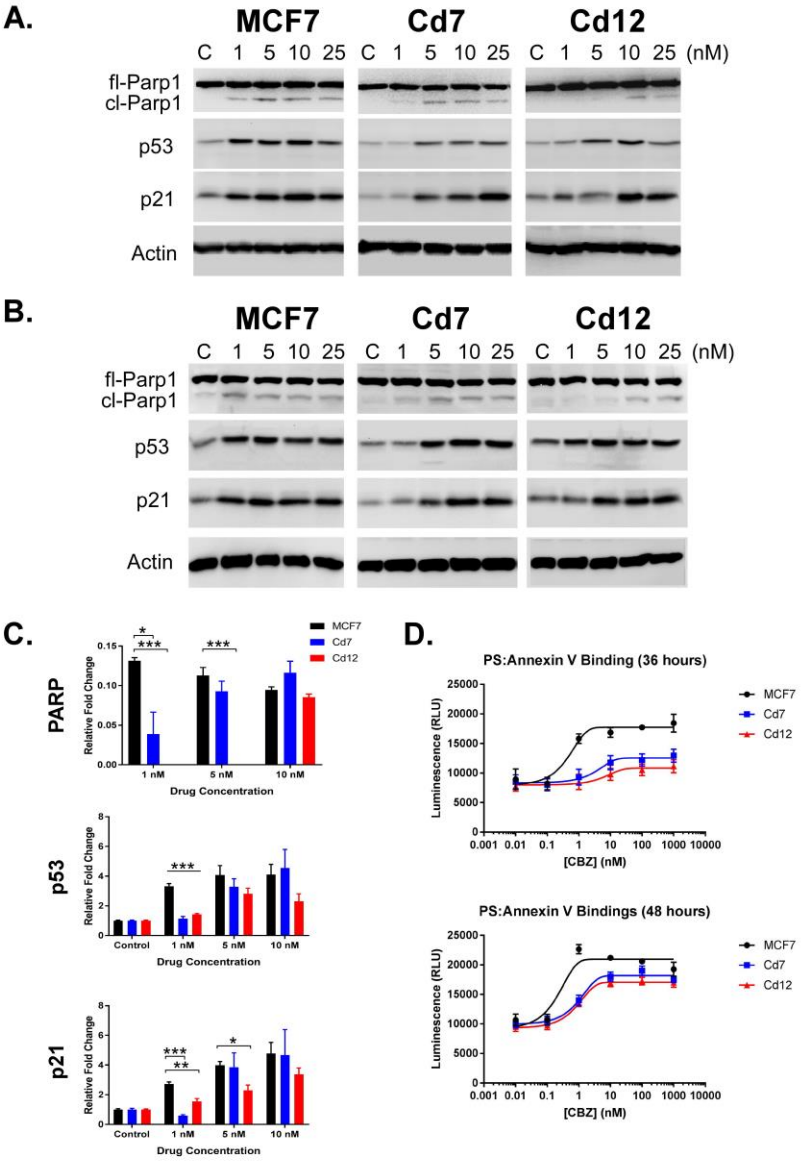


Figure 8. Chronic cadmium exposure promotes resistance to taxane-induced apoptosis. Approximately $2x10^5$ cells were seeded in 6-well plates and treated with varying concentrations of docetaxel (A) and cabazitaxel (B) for 24 hours before being collected for protein expression analysis using western blot and actin was used as a loading control. (C) Protein expression data for both drugs was quantified using iBright Image software, and the relative fold changes for both drugs were averaged together for each concentration and are representative of at least 4 independent experiments. Apoptosis was measured using a luciferase-tagged recombinant protein to detect annexin V binding after treatment with cabazitaxel (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). (D) Luminescent readings were collected at 36 and 48 hours. EC_{50} values represent three independent experiments; n=3.

PARP1, a marker for apoptosis, in MCF7 cells treated with 1 nM of either drug compared to Cd7 ($p<0.05$) and Cd12 cells ($p<0.001$) (Fig. 8A-C). Significant levels of PARP1 cleavage did not occur in the cadmium-exposed clones Cd7 and Cd12 until the cells were treated with 5 nM and 10 nM, respectively, of the drugs (Fig. 8A-C). Additionally, in MCF7 control cells, protein expression of p53 and p21, both of which are involved in cell cycle arrest, increased by an average of 3.3- and 2.7-fold, respectively, in response to 1 nM of the taxane drugs. These increases were significantly higher than the 1.3- and 0.6-fold change for Cd7, and the 1.4- and 1.6-fold change for Cd12 cells in p53 and p21 expression, respectively, after treatment with either docetaxel or cabazitaxel (Fig. 8A-C). Consistent with the growth analysis, these results indicate that a higher concentration of taxanes is necessary to promote the expression of proteins necessary to induce apoptosis and cell cycle arrest in MCF7 cells chronically exposed to cadmium in comparison to unexposed MCF7 cells.

As cells undergo apoptosis, the phospholipid phosphatidylserine (PS) translocates from the inner to the outer leaflet of the cell membrane, and this can be detected in real-time using a luciferase-tagged annexin V recombinant protein. We analyzed the levels of annexin V binding in order to provide a more quantitative measurement of apoptosis. To assess the effects of the taxane cabazitaxel in promoting apoptosis in cadmium-adapted cells, real-time exposure of PS on the outer leaflet of the cell membrane was observed 36 and 48 hours after treatment (Figure 8D). Since both cabazitaxel and docetaxel were found to have similar effects on Cd7 and Cd12 cells and are known to have a similar mechanism of action on breast cancer cells (118), we chose to focus on just cabazitaxel. MCF7, Cd7, and Cd12 cells were treated with various concentrations (0.01 nM to 1 μM)

of cabazitaxel, and the amount of PS translocation was detected by measuring luminescence at 36 and 48 hours. Figure 8D shows an overall higher luminescent value for MCF7 and a significant increase in PS translocation and annexin V binding at 1 nM cabazitaxel after 36 hours, while an increase was not observed until 48 hours in cadmium-adapted cells (Figure 8D). These data suggest that a larger number of MCF7 cells undergo apoptosis in response to cabazitaxel treatment compared to Cd7 and Cd12. To determine the cells' sensitivity to taxane-induced apoptosis, the concentration required to induce 50% of PS and annexin V binding—or the half maximal effective concentration (EC_{50})—was calculated based on the results shown in Figure 8D. After 48 hours of cabazitaxel treatment, the EC_{50} values were 0.27±0.1 nM, 1.02±0.08 nM ($p<0.01$), and 0.90±0.07 nM ($p<0.05$) for MCF7, Cd7, and Cd12 cells, respectively (Figure 8D). Collectively, these results support our growth and protein expression results and demonstrate that chronic cadmium exposure promotes resistance to cabazitaxel and necessitates a higher concentration to induce apoptosis.

Given that the mechanism of taxanes involves binding to microtubules to prevent their disassembly, we evaluated the expression of alpha- and beta-tubulin, the monomer components of microtubules, to understand how cadmium may alter the sensitivity to taxanes (Fig. 9A). After 24 hours of cabazitaxel treatment, no significant alterations in the expression levels of alpha- or beta-tubulin proteins were observed in MCF7, Cd7, or Cd12 cells (Figure 9A). Since there was no significant difference in the expression of α- and β-tubulin, we questioned whether the organization of β-tubulin was altered by chronic cadmium exposure to promote the taxane-resistant phenotype. The microtubule network of MCF7, Cd7, and Cd12 cells was visualized by immunofluorescent (IF)

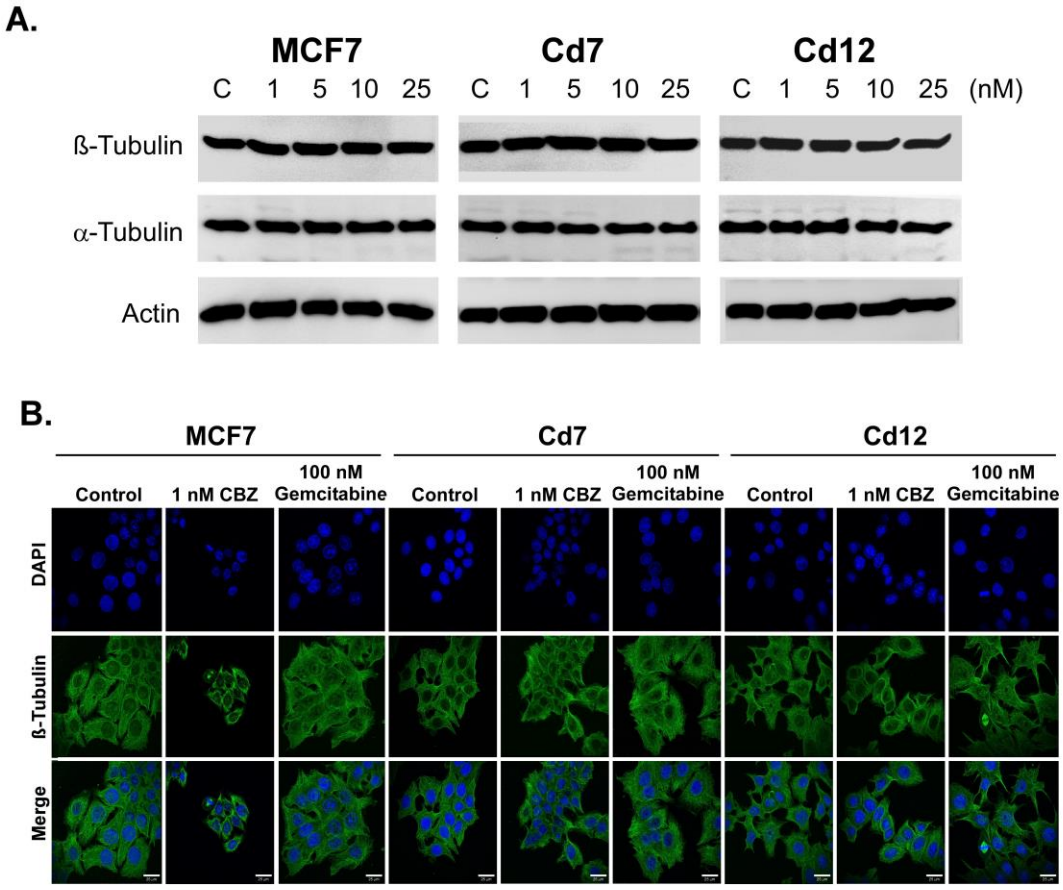


Figure 9. Microtubules of cadmium-adapted cells are less affected by cabazitaxel treatment. (A) Protein expression of α- and β-tubulin in MCF7 and cadmium-adapted MCF7 cells after 24 hours of cabazitaxel treatment. Results represent three independent experiments. (B) MCF7, Cd7, and Cd12 cells were treated with 1 nM cabazitaxel, 100 nM gemcitabine, or vehicle for 20 hours before being fixed with 100% acetone and stained with a β-tubulin specific antibody. DAPI (4′,6-diamidino-2-phenylindole) was used to stain total nuclei. Fixed cells were imaged at 63X magnification using a confocal fluorescent microscope and the experiment was repeated a total of three times (β-tubulin: green; DAPI: blue). Scale bar represents 25 μm.

staining of β-tubulin (Fig. 9B). Cells were treated with 1 nM cabazitaxel, 100 nM gemcitabine, or the vehicle control for 20 hours before being chemically fixed and stained with β-tubulin-specific antibodies. During taxane-induced cell death, free tubulin becomes overpolymerized to form shorter, highly bundled microtubules in a sheet-like pattern (119). The IF images revealed that under normal conditions all three cell lines had an expansive network of microtubules that branched throughout the cell body, but after treatment with 1 nM cabazitaxel, the microtubule network of the MCF7 cells was largely reduced compared to untreated cells, and β-tubulin was concentrated around the nuclear periphery (Figure 9B). The effect on the microtubules was not as striking in the Cd7 and Cd12 cells treated with 1 nM cabazitaxel. Instead, the microtubules of the cadmium-adapted cells were well-distributed throughout the cell body similar to their controls and did not shrink or localize around the nucleus to the same extent observed in MCF7 cells (Figure 9B). Gemcitabine was used as a control since it has the same growth inhibitory effects on all three cell lines and is known to block cancer growth via a mechanism independent of microtubules. Results show a similar increase in cell size for the MCF7 and cadmium-exposed cells in response to gemcitabine, but no differences in the distribution and branching of microtubules were observed, as expected (Figure 9B). These results indicate that the differential effects observed in MCF7 versus Cd7 and Cd12 with cabazitaxel were specific and not associated with cytotoxicity or inhibition of cell growth. These findings suggest that the microtubules of the Cd7 and Cd12 cells were less impacted by cabazitaxel treatment and that chronic cadmium exposure enables the cells to be less sensitive to the microtubule-stabilizing effects of the taxane drugs.

3.4 Discussion

Animal and cell culture models have shown that prolonged cadmium exposure can promote cancer progression (44, 49, 58, 64, 65), and acute exposure can inhibit the activity of the anticancer drug 5-FU (116, 117). In this study, we investigated the effects of chronic cadmium exposure on the sensitivity of breast cancer cells to different chemotherapeutic drugs, including antimetabolites, anthracyclines, and taxanes. Our results indicate that breast cancer cells chronically exposed to cadmium displayed a decreased response to the taxane drugs, while exhibiting little to no difference in their response to doxorubicin and gemcitabine (Figure 7). Although cadmium is recognized as a carcinogen and associated with occupational cancer risk (106, 120, 121), its role in the development and progression of breast cancer is still not fully understood, and epidemiological associations between cadmium and breast cancer have been inconsistent (47, 52, 55, 57). This current study demonstrates a potentially new role for chronic cadmium exposure in promoting resistance to anticancer drugs and further highlights the possible risks of prolonged environmental cadmium exposure in breast cancer.

In contrast to the cytotoxicity caused by antimetabolites and anthracyclines through the inhibition of DNA and RNA synthesis, taxane cytotoxicity is mediated by the drug binding to microtubules and preventing their disassembly (118, 122). Results in figure 9A show no differences in the protein levels of either α- or β-tubulin, suggesting that the mechanism of cadmium induced-taxane resistance is unlikely due to changes in the expression of the microtubule subunits. Consistent with our findings, a previous study by Ledda and colleagues demonstrated that cadmium exposure does not affect the overall levels of α- and β-tubulin but rather induced tubulin posttranslational

modifications (123). While this was not explored in our study, increased levels of tyrosinated α-tubulin and differential expression of β-tubulin subtypes were found in paclitaxel-resistant MCF7 cells (124), suggesting that the effect of chronic cadmium exposure on posttranslational modifications should be evaluated further. We also visualized β-tubulin localization before and after cabazitaxel treatment using IF (Figure 9B), and although no significant structural differences were observed between MCF7 and cadmium-exposed cells in the absence of drugs, the microtubules of the untreated MCF7 cells were localized to the nuclear periphery, and the extensive branching throughout the cytoplasm was decreased after cabazitaxel treatment (Figure 9B). This effect was not as dramatic in the Cd7 or Cd12 cells, which suggests that chronic cadmium exposure may decrease the sensitivity of microtubules to the stabilizing effect of cabazitaxel.

While our study shows that the response of microtubules to taxane treatment is altered in cadmium-adapted cells, the mechanism of how cadmium mediates this effect is unclear. O'Brien *et al.* demonstrated that calcium accelerates the rate of GTP hydrolysis within the microtubule cap to destabilize growing microtubule ends without modulating free tubulin levels (125). Interestingly, cadmium is known to behave similarly as other divalent metals, like zinc, iron, and calcium (126), and multiple studies have shown that cadmium destabilizes microtubules at concentrations of 10 μM and above (127). However, these high levels are toxic to cells, causing alterations in cell morphology and reduced cell fitness (128). An *in vivo* report found the microtubules of kidney cells were highly irregular and diminished after repeated exposure to cadmium (129). Thus, higher than normal intracellular cadmium caused by prolonged, chronic exposure may promote microtubule disassembly by mimicking calcium, which could counteract the microtubule

stabilizing effect of taxanes thus making breast cancer cells less sensitive to these drugs. Lung cancer cells resistant to paclitaxel, another common taxane drug, had increased microtubule dynamicity at rates 57-167% greater than paclitaxel-sensitive lung cancer cells, suggesting that microtubule dynamics play a role in taxane-resistance (130).

Cadmium-transformed cells display diminished apoptosis (110, 131), and thus the ability to suppress apoptosis is thought to be important in cadmium carcinogenesis (132, 133). While Cd7 and Cd12 less were less susceptible to taxane-induced cell cycle arrest and apoptosis than control MCF7 cells (Figure 8), other studies indicate that apoptosis resistance does not appear to be critical to cadmium-induced drug resistance, as the cadmium-adapted cells are still sensitive to the apoptosis-inducing drugs doxorubicin and gemcitabine (134-136).

Cadmium exposure and chemotherapy are both known to induce oxidative stress (137-140), and chronic levels of oxidative stress have been shown to increase the tumorigenic potential of breast cancer cells (107). Similarly, chronic cadmium exposure may also promote breast cancer progression by persistently inducing sub-lethal levels of oxidative stress, which may enable cells to adapt and tolerate other forms of stress, such as anticancer drugs. Additionally, increased expression of metallothioneins, which are metal-binding proteins upregulated in response to cadmium exposure (66, 141), has been associated with resistance to certain drugs. We, too, have previously shown that chronic cadmium exposure does increase expression of metallothioneins (66). Kelley *et al.* found that overexpression of metallothionein-IIA conferred resistance to several antineoplastic agents, but not 5-FU and vincristine (142). Since vincristine has a similar mechanism to taxanes in that it targets the microtubules of the mitotic spindle to block cell division, this

suggests that higher metallothionein levels may not play a prominent role in the resistance to taxanes.

Contrary to our findings, Asara *et al.* demonstrated that cadmium exposure can protect against the cytotoxic effects of 5-FU (116). However, this study used a cadmium concentration 50-fold higher than the concentration we used, and unlike chronic exposure, acute doses do not allow for the cells to adapt to cadmium. The study by Asara and colleagues also did not evaluate differences in the growth rate of MCF7 cells treated with 5-FU plus cadmium versus 5-FU alone. Since we were unable to determine the GI_{50} values for 5-FU in MCF7 and the cadmium-adapted cells (see Fig. 7A), further investigation of the effects of cadmium on 5-FU response is definitely warranted.

In summary, the results of this study show for the first time that chronic cadmium exposure increases resistance to taxane drugs. Specifically, cadmium-adapted cells are less sensitive to taxane-induced apoptosis, cell cycle arrest, and microtubule stabilization. Although the exact mechanism is still not clear, we speculate that higher intracellular cadmium levels might decrease taxane potency by promoting microtubule disassembly to counteract the stabilizing effects of the drug. However, it is likely that other pathways are also involved, as chronic cadmium exposure causes MCF7 cells to become more adaptable to stress (Chapter 1). Regardless of the mechanism, our findings do indicate that breast cancer cells with a higher cadmium burden may be less responsive to taxanes, and therefore these drugs may not be effective in treating ERα+ breast cancers that no longer respond to hormone therapy. Patient cadmium levels can be measured through blood, urine, hair, and tissue samples and have been shown to correlate with breast cancer risk, tumor malignancy, and metastasis frequency, all which demonstrates cadmium's

potential as a biomarker in cancer (47, 49, 50). Fortunately, though chronic cadmium exposure decreases the sensitivity of breast cancer cells to microtubule stabilizing agents like taxanes, it appears to have little effect on the efficacy of drugs that target and block DNA replication or RNA synthesis, indicating these might be better therapeutic options for hormone-refractory breast cancer. Considering that taxanes are frequently prescribed to breast cancer patients, the consequences of chronic cadmium exposure on taxane resistance may have implications in predicting treatment success and the appropriate selection of chemotherapeutic regimens in the future.

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