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The Effects of Chronic Cadmium Exposure on Breast Cancer Progression

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By
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Certification of Approval

I certify that I have read *The Effects of Chronic Cadmium Exposure on Breast Cancer Progression* by Esmeralda Ponce, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California.

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ABSTRACT

Cadmium is an environmental contaminant that enters the body usually through diet or cigarette smoke. Exposure to cadmium has been associated with the development of breast cancer. Recent studies have suggested that cadmium functions as an endocrine disruptor and mimics the actions of estrogen in breast cancer cells by activating the receptor (ERα) to promote breast cancer cell growth. Although acute cadmium exposure is known to promote estrogen receptor-mediated gene expression associated with growth, the consequence of chronic cadmium exposure has been unclear. Since heavy metals are known to bioaccumulate, it is necessary to understand and elucidate the effects of prolonged, low-level cadmium exposure and its role in breast cancer progression. Therefore, this study aims to investigate the chronic effects of cadmium exposure on breast cancer progression. An MCF7 breast cancer cell line (MCF7-Cd) chronically exposed to 10^{-7}M $CdCl_2$ was developed and serves as a model system to facilitate studies on the effects of chronic cadmium exposure on breast cancer progression. The data presented here suggest that prolonged cadmium exposure results in the development of more aggressive cancer phenotypes– increased cell growth, migration, and invasion. Cells exposed to cadmium were shown to express higher levels of SDF-1— a chemokine known to contribute to cancer cell growth, migration and invasion. Results from this study demonstrated

that the expression of SDF-1 was not only dependent on ERα but also on c-jun and c-fos, and the interactions between ERα with c-jun and c-fos were significantly increased at both the protein and transcription levels in cells chronically exposed to cadmium. Additionally, the data showed that chronic cadmium exposure was able to disrupt cell-cell adhesion by down-regulating E-cadherin expression, which led to relocation of active β-catenin to the nucleus, and in turn led to increased metastasis and invasion. The work presented here provides a mechanistic link between chronic cadmium exposure, ERα, and breast carcinogenesis and demonstrates that prolonged cadmium exposure, even at low levels, contributes to the progression of breast cancer.

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ABBREVIATIONS

AP-1	Activating Protein-1
CdCl2	Cadmium Chloride
CDH-1	E-cadherin
ChIP	Chromatin Immunoprecipitation
CoIP	Co-Immunoprecipitation
CTD	Cathepsin D
DMEM	Dulbecco's Modified Eagle Medium
ER	Estrogen Receptor
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
GPR30	G-coupled protein receptor 30
MCF-7	Michigan Cancer Foundation-7
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SDF-1	Stromal Derieved Factor-1
siRNA	Silencing RNA

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CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer is one of the most common malignancies that occurs in women in the United States and accounts for 1 in 4 female cancers. It is the 2nd leading cause of cancer-related deaths in women after lung cancer (ACS 2012). In 2011 approximately 230,480 cases of invasive breast cancer were diagnosed compared to 57,650 in situ breast cancer cases (ACS 2011). Approximately 39,510 women and 410 men in 2012 are expected to die from breast cancer, with the majority of the cases categorized as invasive breast cancer (ACS 2012). Over the past several decades, the global incidence rates of breast cancer have increased continuously, and it is estimated that approximately 1 in 8 women in the United States will develop invasive breast cancer (ACS 2012).

Breast cancer is multi-factorial, which makes it difficult to pin-point a single cause or target for its initiation, and complicates the ability to treat and understand the disease (Richie and Swanson 2003, Althuis et al. 2005). Multiple risk factors are associated with breast cancer development, both biological and environmental. Biological risk factors include, but are not limited to, gender, age, family history, and age of menarche and menopause. Environmental risk factors include, but are not limited to, lifestyle habits and exposure to radiation, carcinogens, phytoestrogens, xenoestrogens, organohalogens and pesticides.

Epidemiological studies have shown that 90% of breast cancer cases are environmental in origin (Lipworth 1995, Harvey and Darbre 2004, Darbre 2006).

A large number of epidemiological and experimental studies have indicated a role for estrogens in the etiology of human breast cancer (Henderson et al. 1982, Henderson et al. 1988, Feigelson et al. 1996, Henderson and Feigelson 2000). This is why life-time exposures to estrogen are considered a major risk factor in breast cancer. Estrogens are produced in the female ovaries and play an important role in developmental processes, promoting growth and differentiation of sex tissues and reproductive organs. There are three circulating forms of estrogens in the body: estrone, estradiol, and estriol, with estradiol being the major and most active form of estrogen. Estriol is produced in large quantities by the placenta in pregnant women, and estrone is produced mainly in post-menopausal women. In addition, estrogens also play a pivotal role in brain function, bone maintenance, and accumulation of adipose tissue (Bryne et al. 2009). Finally, there is clear evidence that estrogen exposure is a key contributor to the development of breast cancer, partly through its ability to stimulate the growth of mammary epithelial cells (Eliassen et al. 2006, Henderson and Feigelson 2000, Henderson et al. 1988).

1.2 The Estrogen Receptor

The actions of estrogens in the breast are primarily mediated by two nuclear estrogen receptors, ERα and ERβ, and by GPR30 (GPER), a G-protein coupled receptor found in the membrane. Estrogen receptors are part of a super

family of nuclear hormone receptors that function as ligand-activated transcription factors. ERα and ERβ are isoforms and share more than 97% and 59% homology in their DNA binding and ligand binding domains, respectively; however, they are highly variable in their NH_2-terminal transactivation AF-1 domains (Mosselman et al. 1996, Paech et al. 1997, Aquino et al. 2012). The DNA binding domain contains well-conserved cysteine-rich regions which tetrahedrally coordinate with zinc ions to form two zinc finger motifs. The C-terminal domain is the most complex region, both structurally and functionally. It is responsible for receptor dimerization and ligand-specific gene transcription mediation (Yang et al. 2008, Montano et al. 1995, Koide et al. 2007).

While ERα and ERβ share similar ligand-dependent transactivation functions, it appears that they play distinct roles in the development and progression of breast cancer. Studies have suggested that ERα mediates the mitogenic actions of estrogen and ERβ mediates the anti-mitogenic effects (Pettersson and Gustafsson 2001). In support of this, Pettersson et al. studied mammary glands from ERα knockout mice and compared them to mammary glands from wild-type mice. Their results showed that the tissues in both animal types were indistinguishable, although the mammary glands from ERα knockout mice were found to be underdeveloped when compared to wild-type ERα mice. From his results, Pettersson concluded that ERα mediates mitogenic effects (Pettersson et al. 2001). The role of ERβ remains unclear. Some studies suggest that high levels of ERβ expression are indicative of more advanced breast cancer tumors while other studies show that ERβ might play a protective role in breast

cancer (Clarke et al. 2003, Bardin et al. 2004, Lazennec et al. 2001, Hodges-Gallagher et al. 2008, Jarvinen et al. 2000, Nair et al. 2012). Since ERα's role in mediating the mitogenic effects of estrogens is important in breast carcinogenesis, it serves as an important prognostic marker of the disease.

GPR30 is a seven-transmembrane receptor found in the plasma membrane and endoplasmic reticulum (Thomas et al. 2005, Filardo et al. 2006, Revankar et al. 2005). Activation of GPR30 upon binding to estrogen leads to rapid intracellular signaling by activation of a downstream signaling cascade, that includes activation of epidermal growth factor (EGFR) and subsequent increase of cyclic AMP, resulting in activation of transcriptional activity necessary for proliferation (Filardo et al. 2000, Filardo et al. 2006, Maggiolini et al. 2004). The role of GPR30 in breast cancer remains unclear due to contradictory findings. GPR30 has been shown to be expressed in invasive ductal carcinomas (Liu et al. 2009) and shown to induce breast cancer cell proliferation and migration *in vitro* (Pandey et al. 2009, Madeo and Maggiolini 2010). However, GPR30 has also been shown to be involved in apoptosis and cell cycle arrest (Teng et al. 2008, Wang et al. 2008, Ariazi et al. 2010).

Seventy percent of primary breast cancer tumors are ERα-positive, and two-thirds of these respond to endocrine therapy utilizing anti-estrogens (i.e. tamoxifen) (Hanstein et al. 2004, Giacinti et al. 2006). While a majority of the ERα positive breast cancers respond well to anti-estrogens, a fraction of the treated tumors become de-sensitized, resistant to tamoxifen. These resistant cancers are often more aggressive, and in some cases, tamoxifen treatments

have been shown to further stimulate cancer growth (Hiscox et al. 2005, Clarke et al. 1993).

ERα has been shown to interact with a group of proteins referred to as nuclear receptor regulators which include co-activators and co-repressors (Benecke et al. 2000, Robyr et al. 2000, McKenna and O'Malley 2002). Recruitment of steroid receptor co-activators is required for full transcriptional activity of ERα. Examples of coactivators include the p160 family of steroid receptor coactivators SRC-1, SRC-2, SRC-3, and p300/CBP (Chen et al. 1997, Han et al. 2009). Some of these co-regulators possess histone acetyltransferase activity which contributes to chromatin remodeling and thus can lead to further enhancement of ERα-mediated transactivator activity (Chen et al. 1997, Lu and Danielsen 1998, Kishimoto et al. 2005). A study by Kishimoto et al. further demonstrated the importance of co-activator recruitment for estrogen-induced expression of SDF-1, cathepsin D (CTD) and c-myc, and their results showed that coactivator binding is necessary for the expression of these ERα targets. Interestingly, these ERα genes (SDF-1, CTD and c-myc) are also involved in breast cancer proliferation, invasion, and metastasis (Kishimoto et al. 2005).

1.3 Endocrine disruptors and Metalloestrogens

The development of the breast from puberty through cycles of pregnancy and lactation is controlled by an intricate web of hormone signaling pathways (Darbre 2006). Among these hormone pathways, estrogen signaling is the major pathway controlling the development of the mammary gland. The importance of estrogens

in the etiology of breast cancer suggests that exposures to environmental or man-made compounds or chemicals (i.e. endocrine disruptors)— which mimic the effects of estrogen and alter the estrogen receptor signaling pathway— are potential risk factors for the disease (Colborn et al. 1993). Endocrine disruptors can be either man-made or naturally present in the environment, and some common endocrine disruptors include pharmaceutical estrogens found in contraceptives or hormone replacement therapy, phytoestrogens found in soybeans, xeno-estrogens (man-made chemicals with estrogenic activity), and metalloestrogens.

1.4 The Metalloestrogen Cadmium

Metalloestrogens are a group of heavy metals which mimic the actions of estrogen. Metalloestrogens include heavy metals and metalloids such as aluminum, nickel, cadmium, antimony, cobalt, copper, tin, vanadate, lead, mercury, arsenite, barium, chromium, selenite, nitrate and uranium (Garcia-Morales et al. 1994, Stoica et al. 2000a,b,c, Johnson et al. 2003, Martin et al. 2003, Darbre 2005, Choe et al. 2003, Raymond-Whish et al. 2007, Veselik et al. 2008). Exposure to some heavy metals has been associated with an increased incidence of breast cancer development (Choe et al. 2003, Martin et al. 2003).

Of all the metalloestrogens, cadmium is the best characterized. Cadmium is widely used in industry for the production of alloys, nickel-cadmium batteries, fertilizers, pigments and plastic stabilizers. Occupationally, individuals are exposed to cadmium through the production and processing of cadmium, the

refining and use of copper and nickel, fossil fuel combustion, and the recycling of electronic waste. Cadmium is found as a natural component of the Earth's crust and is present in the atmosphere; however, it is important to note that the production and processing of cadmium adds 3-10 times more cadmium to the atmosphere than natural sources (Irwin et al. 1997). Among non-occupationally exposed individuals, exposure to cadmium occurs primarily from cigarette smoke. For non-smoking, non-occupationally exposed individuals, exposure to cadmium arises mostly from dietary sources due to the high rates of transfer of cadmium from soils to plants (Clemens 2006, Franz et al. 2008, McLaughlin et al. 2006, Satarug et al. 2010). Therefore, exposure to cadmium occurs daily through contaminated food, water, and air.

Once absorbed into the body, cadmium is rapidly transported to various organs and has an estimated half-life of 15-20 years in humans (Jin et al. 1998, Satarug et al. 2003). Heavy metals like cadmium tend to accumulate in soft tissues of the body (Islam et al. 2007), and this accumulation often interferes with normal physiological functions by disrupting protein folding and/or activity. The chemical properties of cadmium allow it to combine easily with oxidized ligands, particularly those containing sulfur, which are a major component of many biological molecules including proteins. Additionally, due to the similar coordination chemistry of zinc and cadmium, zinc is often displaced by cadmium in many proteins and enzymes (Martelli et al 2006). Cadmium has also been shown to induce chromosomal aberrations, aneuploidy, sister chromatid exchanges, and to induce DNA strand breaks and DNA-protein crosslinks in

mammalian cells (Seoane and Dulout 2001, Ochi and Oshawa 1985, Lin et al. 1994, Ochi and Oshawa 1983, Misra et al. 1998, Fatur et al. 2002, Liu and Jan 2000). Furthermore, cadmium has been shown to inhibit DNA repair, activate proto-oncogenes (i.e. c-myc, c-jun, and c-fos) and genes associated with cell proliferation (i.e. cyclin D1), and inhibit apoptosis, all of which can contribute to the development and progression of breast cancer (Hartwig 1998, Abshire et al. 1996, Zheng et al. 1996, Shimada et al. 1998 Martelli et al. 2006, Siewit et al. 2010).

1.5 Cadmium and Breast Cancer

The International Agency for Research on Cancer and the National Toxicology Program of the United States of America have classified cadmium as a category I human carcinogen (IARC 1993, NTP 2011) because increasing evidence shows both occupational and non-occupational exposure to cadmium are associated with cancers of the lung, prostate, pancreas, kidney, liver, stomach, and urinary bladder (Waalkes and Rehm 1994, Schwartz and Reis 2000, Pesch et al. 2000, Hu et al. 2002, Waalkes et al. 2000, Waalkes et al. 1999, Satarug et al. 2010, Akesson et al. 2008, Ursin et al. 1994). Cadmium exposure has also been correlated with an increase in the incidence of breast cancer (Cantor et al. 1994, Martin et al. 2003, Choe et al 2003).

Several studies have shown that cadmium has the ability to mimic biological functions of estrogen in breast cancer cells by activating the estrogen receptor (Martin et al. 2003, Bryne et al. 2009 Garcia-Morales et al. 1994, Stoica et al.

2000, Johnson et al. 2003, Siewit et al. 2010). Previous work from our lab and others has suggested that cadmium can promote breast cancer cell growth in MCF-7 cells via ERα (Siewit et al 2010, Martin et al. 2003, Garcia-Morales et al. 1994). Animal studies have suggested that cadmium can mimic the role of estrogen *in vivo* and promote neoplastic growth, increase uterine weight, induce changes in the uterine lining, and increase the density of mammary glands in rats and mice (Johnson et al. 2003, Hofer et al. 2009, Ali et al. 2010, Alonso-Gonzales et al. 2007). Collectively, these studies demonstrate that cadmium functions as a hormone-disruptor and metalloestrogen and is an important contributor to the development of breast cancer.

Recent studies have identified significantly higher levels of cadmium in tumor tissues as well as in biological samples from tumor patients (Ionescu et al. 2006, Strumylaite et al. 2008, Satarug et al. 2010; Strumylaite et al. 2011). Specifically, Strumylaite et al. measured and compared cadmium concentrations in biological samples from patients with breast cancer and benign breast tumors and found that patients with malignant cancer had significantly higher levels of cadmium concentrations when compared to patients with either benign tumors or estrogen receptor-negative cancers (Strumylaite et al. 2011), offering further support for a possible relationship between cadmium and ERα. Accumulating evidence links exposures of environmental estrogens to an increased incidence of cancer (Satarug et al. 2010, Akesson et al. 2008, Ursin et al. 1994, Cantor et al. 1994).

Most studies have only examined the effects of acute cadmium exposures, and few studies have focused on the effects of chronic cadmium exposure on breast cancer progression. Cancer progression is often marked by the development of more aggressive phenotypes (Hanahan and Weinberg 2000), and the role of chronic cadmium exposure in this process requires further investigation. A recent study has demonstrated that chronic cadmium exposure can transform normal breast epithelial cells, MCF10A cells, into a malignant-appearing form. These exposed MCF10A cells displayed more aggressive cancer-like characteristics, including an increased ability to grow in an anchorage-independent manner and the ability to invade the extracellular matrix through an increase in cellular expression of matrix metalloproteinase-9 (MMP-9), an enzyme which facilitates tumor cell invasion (Bembrahim-Tallaa, et al. 2009). Furthermore, when these transformed cells were implanted in nude mice, they formed highly aggressive tumors, further demonstrating the increased metastatic potential of cells chronically exposed to cadmium. Understanding the molecular mechanisms involved in the role of chronic cadmium exposure during breast carcinogenesis should provide further insights into how chronic environmental exposures may contribute to disease development and progression. Our lab is focused on investigating how chronic cadmium exposure contributes to the progression of breast cancer, specifically focusing on two genes, SDF-1 and CDH-1. Although there are studies on the relationship between SDF-1 and CDH-1 (Rhodes et al. 2011, Wang et al. 2011) and cancer progression, few to no studies have been done on the molecular connection

between cadmium and SDF-1 and cadmium and CDH1 (Park et al. 2008, Pearson and Prozialeck 2001).

1.6 SDF-1 and Breast Cancer Metastasis

Stromal derived factor-1 (SDF-1)— also known as CXCL12— is a novel estrogen receptor-regulated gene and a mediator of mitogenic effects. It can mimic the proliferative effects of estrogen in a dose-dependent manner in breast and ovarian cells (Hall and Korach 2003, Kishimoto et al. 2005). SDF-1 is one of the 40 to 50 human chemokines— small molecular weight proteins characterized by the ability to stimulate chemotaxis (Vacari and Caux 2002). Chemokines are considered pro-inflammatory and regulate cell trafficking and immune response to recruit macrophages and other immune cells to sites of infection (Vacari and Caux 2002). However, chemokines like SDF-1 have been reported to be expressed in many tumor cell types and have a direct tumor growth effect (Rollins 1997, Rossi and Zlontnik 2000, Vacari and Caux 2002). Additionally, SDF-1 and the family of chemokines are involved in growth, invasion and orchestrating events that result in primary tumor growth, invasion and metastasis (Vicari and Caux 2002). SDF-1 can induce the production of metalloproteinases (MMPs), enzymes which degrade the extracellular matrix to promote the invasive behavior of breast cancer cells (Williams et al. 2010, Jezierska and Motyl 2009), and facilitate metastasis. Metastasis occurs when a malignant cell has the ability to detach from its original tumor and travel to distant sites to form secondary

tumor foci. One mechanism that cancer cells can achieve this is by disrupting cell-to-cell adhesion complexes to facilitate malignant cell detachment.

1.7 E-Cadherin and Breast Cancer Metastasis

E-cadherin, otherwise known as CDH-1, is a Ca^{2+}-dependent transmembrane glycoprotein that plays a major role in cell-to-cell adhesion (Pearson and Prozialeck 2001, Baranwal and Alahari 2009, Jiang and Mansel 2000). The cell-to-cell adhesion complexes are composed of E-cadherin linked to the actin cytoskeleton through a group of molecules called catenins (i.e. β-catenin). β-catenin, a member of the E-cadherin/β-catenin complex, is not only involved in cell signaling and transcriptional regulation but is also a regulator of cell adhesion (Jiang and Mansel, 2000). E-cadherin a tumor suppressor protein and is often used as a prognostic marker for breast cancer (Heimann and Hellman 2000, Baranwal and Alahari 2009). Down-regulation of E-cadherin has been associated with more aggressive breast cancers and poor prognosis (Mohammadizadeh et al. 2009, Oka et al. 1993). A decrease or loss of E-cadherin expression could be due to genetic or epigenetic alterations. Genetic inactivation of E-cadherin is due to mutations and/or loss of heterozygosity in chromosome 16q22.1 and is reported to be associated with gastric carcinomas, gynecologic cancers, hepatocellular carcinomas, and breast carcinomas (Natt et al. 1989, Sato et al. 1990, Becker et al. 1994, Risinger et al. 1994, Kanai et al. 1994, Berx et al. 1995, Tsuda et al. 1990). Epigenetic inactivation of E-cadherin can also result from DNA methylation (Yoshiura et al. 1995, Graff et al. 1995).

Specifically, methylation of the E-cadherin promoter has been associated with many human cancer types and often results in decreased expression of E-cadherin and subsequent progression and metastasis of the disease (Nass et al. 2000, Strathdee 2002).

In this thesis, I will discuss how chronic cadmium exposure results in the deregulation of SDF-1 (Chapter 2) and E-cadherin (Chapter 3) and how this may contribute to chronic cadmium-induced breast cancer progression.

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CHAPTER 2

CHRONIC CADMIUM EXPOSURE STIMULATES SDF-1 EXPRESSION IN AN ERα DEPENDENT MANNER

2.1 Introduction

The majority of breast cancers initially develop as hormone-dependent, with estrogen receptors expressed in approximately 70% of breast cancer cases. The estrogen receptor is part of the super family of nuclear hormone receptors that function as ligand-gated transcription factors. Two isoforms of the ER exist— ERα and ERβ. These receptors play pivotal roles in cell differentiation, growth and homeostasis and function as molecular switches to modulate between states of transcriptional repression and activation, depending on the absence or presence of hormones. The two isoforms share considerable homology in their DNA binding domains and carboxyl terminal domains but are highly variable in their AF-1 amino terminal transactivation domains (Mosselman et al. 1996, Paech et al. 1997). Although both ERα and ERβ bind to 17β-estradiol with similar affinities and recognize the same consensus estrogen response element (ERE) in promoters of ER-regulated genes (Huang et al. 2002), their roles in breast cancer are quite different. While ERα has long been established as an important player in promoting the development and progression of breast cancer, less is known about the role of ERβ in breast cancer progression. The presence or absence of ERα is a key determinant of the prognosis of the disease as it determines whether the cancer will respond to

hormone therapy or not. ERα-positive breast cancers are often hormone-responsive and are typically treated with antiestrogens like tamoxifen. However, hormone-dependent breast cancer frequently progresses into more malignant cancer phenotypes that are often hormone-independent. In many of these cases, ERα is still present, and the role of ERα in hormone refractory breast tumorigenesis and its underlying mechanism is unclear. A potential mechanism involves metalloestrogens: heavy metals that mimic the actions of estrogen and function as endocrine disruptors.

As mentioned previously in Chapter 1, this study aims to further investigate the chronic effects of cadmium exposure on breast cancer progression. To this end, we developed a cadmium breast cancer cell line (MCF7-Cd) by chronically exposing MCF7 cells to 10^{-7} M $CdCl_2$ for over 6 months, and used it as a model system to study the effects of *chronic* cadmium exposure on breast cancer progression. This study provides several lines of evidence suggesting that prolonged cadmium exposure results in more aggressive cancer phenotypes such as increased cell growth, migration and invasion. These results also show that cells chronically exposed to cadmium express higher levels of SDF-1 and that this expression is not only dependent on ERα but also on c-jun and c-fos members of the activating protein (AP)-1 family of transcription factors. The ERα/c-jun/c-fos interactions were significantly increased in cells chronically exposed to cadmium, with higher levels of ERα, c-jun and c-fos occupying the SDF-1 promoter. In summary, these results suggest that chronic cadmium exposure promotes more aggressive cancer phenotypes

by modifying the molecular interactions of ERα with c-jun and c-fos thereby directly altering the expression of cancer-promoting genes like SDF-1.

2.2 Materials and Methods

Cell Culture

MCF7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF7-Cd was developed by exposing parental MCF7 cells to 10^{-7} M $CdCl_2$ for over 6 months. Parental MCF7 and MCF7-Cd were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin and streptomycin (P/S). Both cell lines were subcultured every 3-4 days.

Growth Assay

The MCF7 and MCF7-Cd cells were plated at a cell density of $2.0x10^5$ cells/well in six-well plates using hormone depleted media. Cells were counted in triplicate manually every 2 days using a hemocytometer. Data points represent three independent experiments.

Derivation of cadmium cell lines

To derive clonal cell lines chronically exposed to cadmium, MCF-7 cells were first exposed to 10^{-7}M $CdCl_2$ for over 6 months in order to mimic chronic exposures. Chronically exposed cells were plated on soft agar to allow single cells to develop into colonies. Briefly, a 1% agar solution made with complete media

(DMEM+10% FBS + 1% P/S) was used to create the bottom layer. Serial dilutions of single cell suspensions were mixed with enough complete media to make a 0.6% top agar that was added over the 1% bottom agar layer and allowed to solidify for 30 minutes at room temperature followed by incubation at 37°C. When colonies sizes reached about 100 cells or more, they were individually removed from soft agar under aseptic conditions and resuspended in complete media to permit further expansion.

Scratch Wound Assay

Approximately $2.0x10^4$ cells were plated in 6-well plates and allowed to grow to approximately 75-80% confluency in DMEM. A wound, or scratch, was created with a 200µL micropipette tip. Cells were allowed to incubate for an additional 3 days and images of the scratch area were captured digitally.

Migration and Invasion Assay

A modified Boyden chamber assay was used to quantitatively assess the cells' migration and invasion abilities. For the migration assay, cells were hormone-deprived, and $5.0x10^4$ cells were seeded in the upper chamber over polycarbonate membrane inserts and allowed to migrate toward the lower chamber filled with DMEM+ 10% FBS+ 1% P/S. The cells were incubated for 12-18 hours, fixed in formalin, and stained with crystal violet. The number of cells that migrated to the underside of the filter was counted in triplicate. For the invasion assay, a CytoSelect™ Cell Invasion Assay Kit (Cell Biolabs, Inc, San

Diego, CA) was used according to the manufacturer's protocol. Briefly, $5.0x10^4$ hormone-deprived cells were seeded in the upper chamber and the cells that invaded through the matrigel were fixed, stained and counted as previously described.

Western Blot

Cells were lysed in 1% SDS-HEPES buffer and the total protein concentration was normalized using the Bio-Rad Dc Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Protein expression was monitored using protein-specific antibodies: α-ERα (NeoMarkers, Fremont, CA), α-cyclin D_1 (Santa Cruz Biotechnology, Santa Cruz, CA), α-c-myc (Santa Cruz Biotechnology), α-cyclin A (Santa Cruz Biotechnology), α-cyclin E (Santa Cruz Biotechnology), α-cdk2 (Santa Cruz Biotechnology), α-cdk4 (BD Transduction Laboratories, San Jose, CA), α-p21 (Santa Cruz Biotechnology), α-p-27 (Santa Cruz Biotechnology), α-CDH1 (Santa Cruz Biotechnology), α-β-catenin (Cell Signaling Technology, Danvers, MA), α-GAPDH (Cell Signaling Technology).

SDF-1 ELISA

Cells were plated at a density of $1x10^5$ cells per well in 6-well plates. Cell culture media was collected after 48 hours and stromal cell-derived factor-1 (SDF-1) levels were measured with the enzyme-linked immunosorbent assay (ELISA)

SDF-1 kit (RayBiotech, Inc, Norcross, GA) according to the manufacturer's instructions. Briefly, 100µL of sample was added to 96-well plates containing immobilized antibodies specific for human SDF-1 and allowed to incubate overnight with shaking at 4°C. Wells were then washed and a biotinylated anti-human SDF-1 antibody was added, followed by another wash and subsequently by the addition of HRP-conjugated streptavidin. Following a final wash, tetramethylbenzidine (TMB) substrate for color development was added and samples were read at 450nm after the addition of a stop solution.

siRNA Transfection

Approximately $1x10^5$ cells/well were plated in 6 well plates and transfected with siRNA (Santa Cruz Biotechnology) targeting either ERα, c-jun or c-fos using siRNA transfection reagents (Santa Cruz Biotechnology). A scrambled siRNA was transfected as a control. Five hours after transfection, the medium was changed to DMEM medium containing 10% FBS and 1% P/S. Cells were harvested 48 hours later for gene and/or protein expression analysis using semi-quantitative reverse transcription-polymerase chain reactions (RT-PCR) or quantitative RT-PCR (qRT-PCR), and Western blot analysis, respectively.

Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

Total RNA was isolated from cells using TRI-Reagent (Zymo Research Corporation, Irvine, CA) and columns from the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's protocol. Three micrograms (μg) of

total RNA were used for the reverse transcription reaction with oligo-dT_{18} primers and Moloney Murine Leukemia Reverse Transcriptase (M-MLV RT, Promega, Madison,WI). Gene expression was monitored using semi-quantitative PCR for 28 cycles. PCR products were separated by agarose gel electrophoresis, and visualized using Chemi-Doc (Biorad). Gene expression was quantified using gene specific primers in a quantitative real-time RT-PCR procedure with SYBR green (SA Biosciences, Valencia, CA). All primers were synthesized by Integrated DNA Technologies, Inc. (IDT; San Diego, CA).

RT-PCR Primer Sequences:

SDF-1_F : GTCAGCCTGAGCTACAGATGC (Boudot et al. 2011)
SDF-1_R: CACTTTAGCTTCGGGTCAATG (Boudot et al. 2011)
ERα_F: ATGACCCTCCACACCAAAGCAT
ERα_R: ACTGGCCAATCTTTCTCTGCCA
c-myc_F: CTCCACACATCAGCACAACT
c-myc_R: GTTTCCGCAACAAGTCCTCT
cyc$D1_F$: AATGTGTGCAGAAGGAGGTC
cyc$D1_R$: GAGGGCGGATTGGAAATGAA
$GAPDH_F$: GAAATCCCATCACCATCTTCCAG
$GAPDH_R$: ATGAGTCCTTCCACGATACCAAAG

Coimmunoprecipitation (CoIP) Assay

Cells were plated in 10 cm tissue culture plates and harvested 48 hours later. Cells were lysed in 0.5% NP-40 lysis buffer and sonicated three times for 30 seconds. Cell lysates were immunoprecipitated with α-ERα, α-c-fos, α-c-jun or normal rabbit IgG (Santa Cruz Biotechnology) for 2 hours followed by incubation with either protein A or G agarose beads for 1 hour. Protein complexes were separated with SDS-PAGE followed by Western blot analysis with ERα, c-jun, c-fos, and normal rabbit IgG antibodies.

Chromatin Immunoprecipitation (ChIP) Assay

Cells were plated in 10cm plates and harvested by fixation with formaldehyde. Cells were lysed and sonicated in lysis buffer to fragment the chromatin into 1-2 kb fragments. Chromatin mixtures were immunoprecipitated with antibodies specific for ERα, c-fos, and c-jun (Santa Cruz Biotechnology). Antibody complexes were purified using protein A agarose beads (Pierce, Rockford, IL). The complexes were reverse-cross-linked to release the chromatin fragments by incubation at 65°C for 4-6 hours. DNA fragments were purified using QIAQuick columns (Qiagen, Germantown, MD). Occupancy at a specific promoter was determined with PCR using promoter specific primers. PCR products were detected with agarose gel electrophoresis and quantitated using Quantity One ® (Biorad, Hercules, CA).

ChIP Primer Sequences:

SDF1-ChIP$_F$: CACCATTGAGAGGTCGGAAG (Boudot et al. 2011)
SDF1-ChIP$_R$: AATGAGACCCGTCTTTGCAG (Boudot et al. 2011)
cyclin D1-ChIP$_F$: CATTCAGAGGTGTGTTTCTCCC
cyclin D1-ChIP$_R$: CTCAGCGACTGCATCTTCTTTC
c-myc-ChIP $_F$:GACACATCTCAGGGCTAAACAG
c-myc-ChIP $_R$: GAGAGTGGAGGAAAGAAGGGTA
U6RNA-ChIP$_F$: GAGGGCCTATTTCCCATGATTC
U6RNA-ChIP$_R$:GAATTTGCGTGTCATCCTTGC

ChIP re-ChIP

For the re-ChIP assay, protein A-antibody complexes resulting from the first ChIP assay were extracted twice after incubation with 100μl of 10mM DTT for 20 minutes at 37°C. The supernatants were pooled and diluted 10 times with Re-

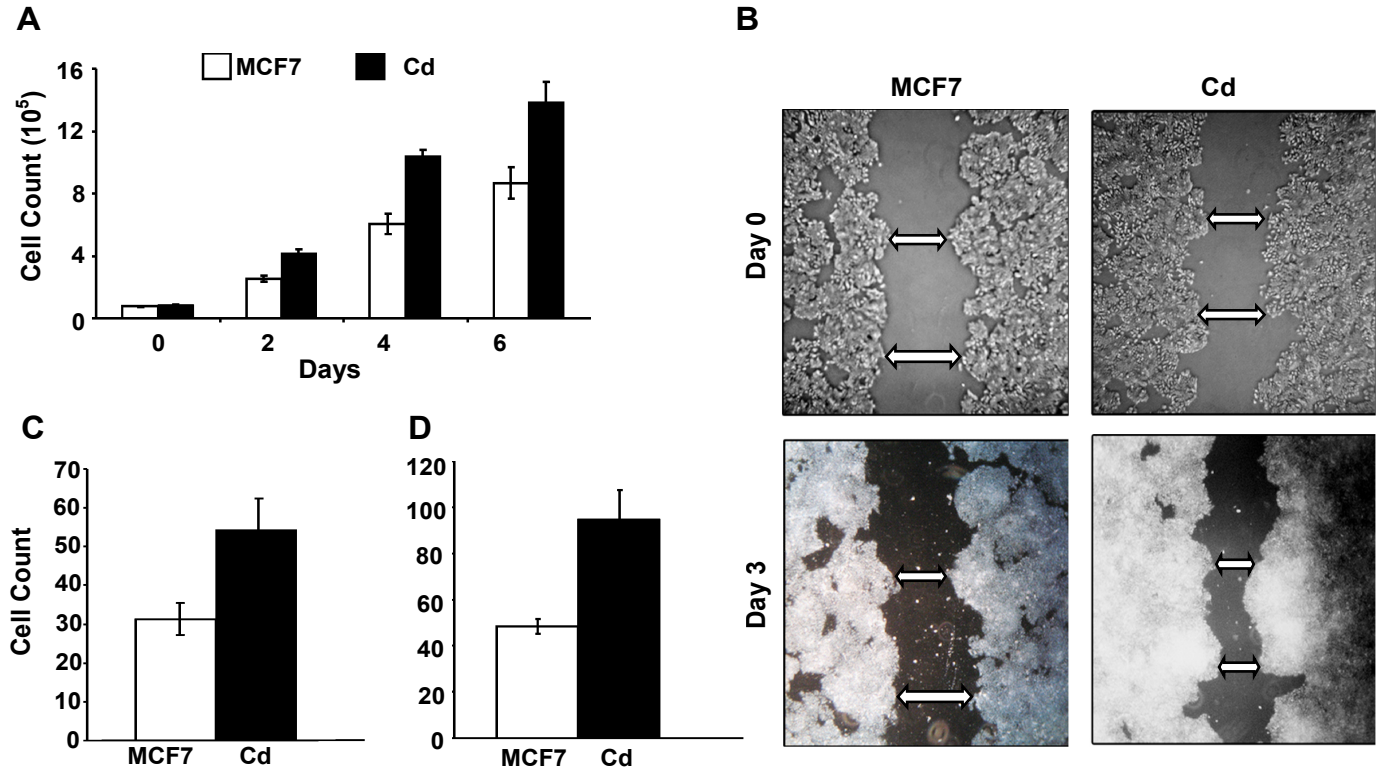

Figure 1: Chronic cadmium exposure induces more aggressive cancer phenotypes: cell growth, migration and invasion. (A) MCF7-Cd cells (black bars) and parental MCF-7 cells (white bars) were plated in 6 well plates under hormone-deprived conditions and cell growth was monitored 2, 4, and 6 days after plating by counting in triplicate. Experimental results are averages of three independent experiments ($P<0.01$). (B) A scratch wound assay was used to assess cell migration ability. MCF-7 and MCF7-Cd cells were plated in 6-well plates and allowed to reach 70-80% confluence. A scratch was created using a p-200 pipette tip and a digital image was captured on Day 0. Cells were then allowed to grow and migrate for 3 days and images of the wound were captured 3 days later. (C) Modified Boyden chamber assays were performed to measure the migration abilities of the cells. MCF7 (white bars) and MCF7-Cd (black bars) cells were seeded into upper chambers and cells were allowed to migrate to lower chamber for 15-18 hours. Data is representative of 3 independent experiments performed in triplicate ($P< 0.01$). (D) An invasion assay was performed by seeding either MCF7 (white bars) or MCF7-Cd (black bars) cells in the upper chambers. Cells were allowed to invade through matrigel-coated membranes for 18-24 hours.Data is an average of 3 independent experiments done in triplicate ($P<0.01$).

ChIP Buffer (20 mM Tris- HCl, pH 8.1, 2 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100). The chromatin mixture was re-ChIPed with the second antibody: α-ER, α-c-jun, α-c-fos or normal rabbit IgG, (Santa Cruz Biotechnology). Antibody complexes were purified using protein A beads, and the complexes were reverse-cross-linked to release the chromatin fragments after an incubation at 65^{o}C for 4-6 hours. The DNA was purified with QIAQuick and analyzed as previously described.

Luciferase Reporter Assay

The reporter gene assay was performed by sequentially transfecting MCF7 cells with siRNA targeting ERα (Santa Cruz Biotechnology) followed by the SDF1 promoter-Luc reporter plasmid. Five hours after siRNA transfection, the second transfection with the SDF-1 Luc reporter plasmid along with the pRL-SV40 *Renilla* luciferase plasmid (Promega) was accomplished using Fugene HD (Promega) according to the manufacturer's protocol. The medium was changed to DMEM media containing 10% FBS and penicillin and streptomycin, and cells were harvested 48 hours post-transfection and analyzed using a dual luciferase assay kit (Promega). All reporter gene assays were performed in quadruplicate, with the entire experiment repeated at least three times. SDF-1-Luc was purchased from Genecopoeia (Rockville, MD).

2.3 Results

We previously found that acute cadmium exposures increase growth rates

of three ERα positive breast cancer cell lines— MCF7, T-47D and ZR-75-1— and increases the expression of genes associated with growth (Siewit et al. 2010). The observation that acute cadmium exposure stimulates breast cancer cell proliferation led us to question the effects of chronic cadmium exposure on breast cancer progression. To understand how chronic cadmium exposure affects the progression of ER-positive breast cancer, a cell line chronically exposed to cadmium was developed by exposing cells to low concentrations of cadmium (10^{-7} M) for over six months. Since cancer progression often results in the acquisition of more aggressive phenotypes, including an increased growth rate (Hanahan and Weinberg 2000), the effects of prolonged cadmium exposure on cancer cell growth was assessed. The growth properties of cells chronically exposed to cadmium (MCF7-Cd) were analyzed in the presence or absence of cadmium and compared to parental MCF7 cells. Approximately $2.5x10^4$ cells were plated in 6-well plates under hormone-deprived conditions and treated with 10^{-7} M cadmium chloride ($CdCl_2$) or mock treated with phosphate buffered saline (PBS). Cell growth was monitored 2, 4 and 6 days after treatment. The MCF7-Cd cells displayed a significantly faster growth rate than the parental MCF7 cells in both the presence and absence of cadmium, suggesting that cells exposed to prolonged periods of cadmium have accelerated growth (Fig. 1A).

In addition to increased growth rates, one of the characteristics that cancer cells acquire as they become more malignant is the ability to metastasize (Hanahan and Weinberg 2000). This is characterized by an increased ability to migrate and invade the extracellular matrix. Therefore, the migration ability of

cells chronically exposed to cadmium was analyzed and compared to parental MCF7 cells by using a scratch wound assay (Fig. 1B). Again, cells were plated in a 6-well plate, and when cells reached 70% confluence, a wound was created using a p200 pipette tip. Cells were rinsed with PBS and migration was observed 3 days later. The data suggest that MCF7-Cd cells display a greater migration ability than the parental MCF7 cells (Fig. 1B).

To confirm the qualitative observations in Figure 1B, a modified Boyden Chamber assay was used to quantify the effects of chronic cadmium exposure on breast cancer cell migration (Fig. 1C). In this assay, $5x10^4$ cells of each cell type (MCF7 or MCF7-Cd) were plated in the upper chamber and allowed to migrate for 16 hours through an 8μM polycarbonate membrane. Cells that have the ability to migrate were able to attach to the underside of the membrane whereas non-migratory cells stayed in the upper chamber. Cells on the underside of the membrane were fixed and stained with crystal violet, and a microscope was used to determine the number of cells migrated in each well. The results suggest that cells chronically exposed to cadmium display an increased ability to migrate (31 vs. 54, $p < 0.05$, Fig. 1C). The invasive ability of the MCF7-Cd cells was also investigated using a similar assay, but the membranes in the chambers were coated with matrigel in order to mimic the extracellular matrix (Fig. 1D). Again, $5x10^4$ cells (MCF7 or MCF7-Cd) were added to the upper chamber, and cells with the ability to invade were able to digest the matrigel and attach to the underside of the chamber. Cells were allowed to invade for 18 hours and then counted as in the migration assay. Consistent with the results from the migration

assay, MCF7-Cd cells also displayed a greater ability to digest the matrigel and invade (48 vs 94, $p < 0.05$).

To understand the molecular alterations that occur in the cells exposed for prolonged periods to cadmium, clonal cell lines (MCF7-Cd2 to Cd12) were derived from MCF7-Cd cells, and differentially expressed genes were analyzed using both a metastasis-specific PCR array (Superarray RT-Profiler) and microarray. Both array studies identified SDF-1, also known as CXCL12, as being up-regulated in a majority of the cadmium-adapted cells. Further verification using semi-quantitative RT-PCR demonstrated that about 70% of the cadmium adapted cell lines expressed higher levels of SDF-1 (Fig. 2A), with Cd7 and Cd12 expressing the highest levels. The levels of SDF-1 protein present in the Cd7 and Cd12 cells and secreted into the media were also examined using Western blot analysis and ELISA, respectively. Consistent with the gene expression data, levels of SDF-1 in both the protein lysates and in the media were elevated, as shown in Figures 2B and C.

Interestingly, SDF-1 has been shown to be an estrogen receptor (ERα) regulated gene (Hall and Korach 2003). Since cadmium functions as a metalloestrogen, we questioned whether cadmium could induce the expression of SDF-1. To determine if the expression of SDF-1 is regulated by acute cadmium exposure, parental MCF7 cells were treated with either 10^{-6} M $CdCl_2$ or 10^{-7} M 17β-estradiol. The results in figure 3 indicate that both acute cadmium and estrogen can increase SDF-1 expression by 6 and 12 fold, respectively (Fig. 3 A-B). Estrogen-induced SDF-1 expression continued to increase for up to 24

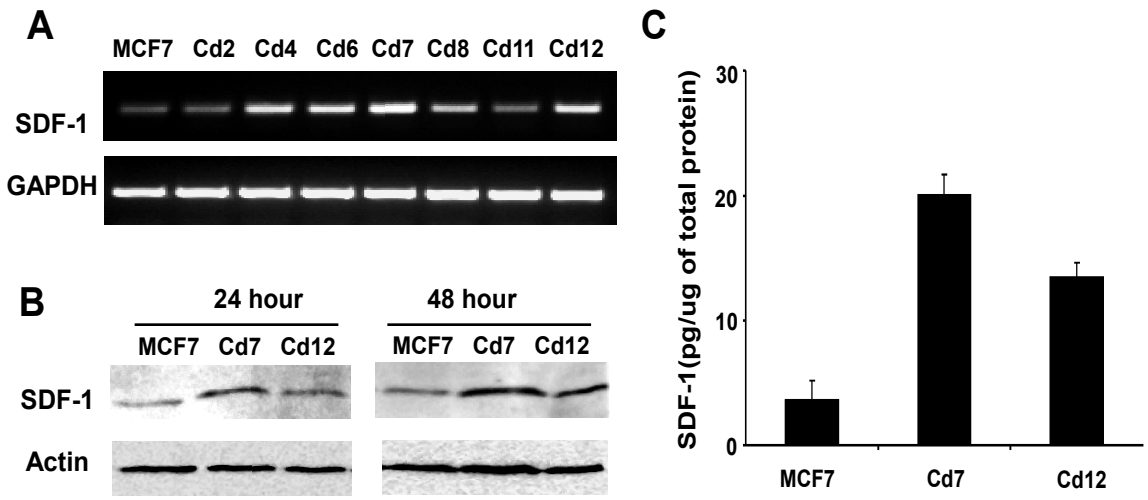

Figure 2: Chronic cadmium exposed cells express and secrete higher levels of SDF-1. (A) MCF7 and MCF7-Cd clonal derivatives (Cd-2, -4, -6, -7, -8, -11 and -12) were plated in 6 well plates and total RNA was isolated for gene expression analysis using semi-quantitative PCR. GAPDH was used as a loading control. (B) SDF-1 protein expression in MCF7, Cd7, and Cd12 cells was analyzed with Western blot analysis. Actin was used as a loading control. (C) Conditioned media was collected in the same experiments shown in B after 24 hours, and the levels of SDF-1 were measured with an enzyme-linked immunosorbent assay (ELISA). Data is representative of several experiments done in triplicate ($P < 0.01$).

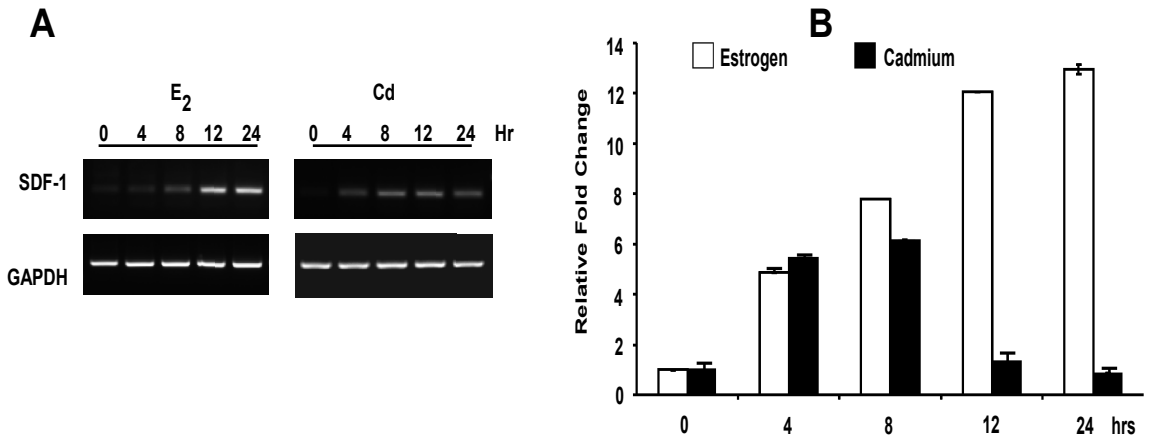

Figure 3: SDF-1 is induced by acute cadmium and estrogen exposure. MCF7 cells were hormone deprived and treated with either 10-7 M E2 (white bars) or 10-6 M CdCl2 (black bars). Cells were harvested for gene expression analysis using (A) quantitative and (B) semi-quantitative RT-PCR. Quantitative PCR data is presented as relative SDF-1 fold increases.

hours, whereas cadmium-induced SDF-1 expression peaked after 8 hours of exposure. Although, cadmium induction was not as robust as 17β-estradiol induction, these results clearly demonstrate that cadmium has the ability to stimulate SDF-1 expression within hours of exposure.

Given that SDF-1 is an ER-target gene, SDF-1 expression was further analyzed in the Cd-cell lines to see if it was dependent on ERα by depleting endogenous levels of ERα (Fig. 4). To mediate ERα silencing (Ei), siRNA was transfected into parental MCF7, MCF7-Cd4,-Cd7 and -Cd12 cells, and the effects of decreased ERα levels on SDF-1 expression were examined. Cells transfected with scrambled siRNA were used as controls (Ci). At 48 hours after transfection, cells were collected for both protein and gene expression analyses using Western blot and semi-quantitative RT-PCR, respectively (Fig. 4A-B). Gene expression analysis was also confirmed by quantitative RT-PCR (qRT-PCR) (Fig. 4C). As expected, the successful depletion of ERα by siRNA resulted in lower levels of SDF-1 in parental MCF7 cells. Interestingly, a similar effect was observed in cells chronically exposed to cadmium, suggesting that SDF-1 expression in the cadmium-adapted cells is also dependent on ERα. To determine if ERα plays a direct role in regulating the transcription of SDF-1, a luciferase reporter assay was used in the presence and absence of ERα (Fig. 4E). MCF7, Cd7 and Cd12 cells were transfected with either a control siRNA (Ci) or one that targets ERα (Ei). The depletion of ERα, as shown by the Western blot, significantly decreases the transactivation of the SDF-1 promoter in all three

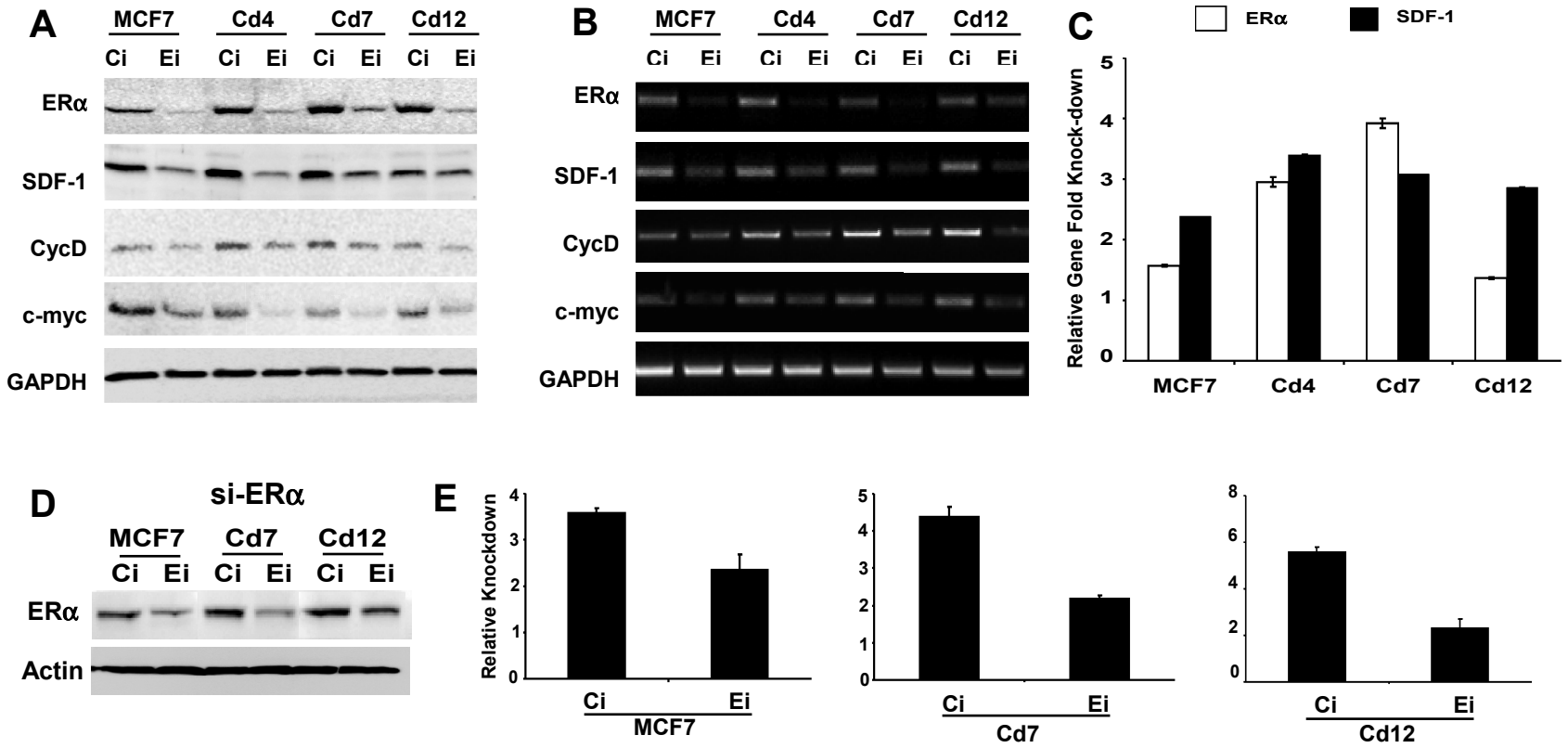

Figure 4: SDF-1 expression in cells chronically exposed to cadmium is dependent on ERα. MCF7, Cd4, Cd7 and Cd12 cells were plated in 6-well plates and transfected with either si-ERα (Ei) or si-control (Ci), and collected 48 hours after treatment for protein and gene expression analyses using (A) Western blot and (B) RT-PCR, respectively. Changes in gene expression were also measured using (C) quantitative RT-PCR, and fold knockdown was expressed as Ct^{Ei}/ Ct^{Ci}. Fold knockdown of ERα and SDF-1 are represented in white and black, respectively. Data is representative of at least three independent experiments done in triplicate. GAPDH was used as a control. MCF7, Cd7 and Cd12 were also transfected with siRNA targeting ERα and the transactivation of the SDF-1 promoter measured using a luciferase reporter assay. Following transfection, cells were collected for (D) Western blot analysis and (E) reporter gene assay. Data represents at least three independent experiments done in quadruplicate. Actin was used as a loading control for the Western blot.

cell lines (Fig. 4E), further establishing the role of ERα in regulating the expression of SDF-1.

Although SDF-1 is an ERα-regulated gene, it is unclear whether ERα regulates SDF-1 alone or in collaboration with other transcription factors. To determine if ERα interacts with other transcription factors, a co-immunoprecipitation experiment was used to identify potential transcription factors that may interact with ERα in cells chronically exposed to cadmium (Fig. 5). Cells were grown for 48 hours, and cell lysates were immunoprecipitated with α-ERα antibodies. The presence of specific transcription factors in the immunoprecipitated complexes was determined with Western blot analysis. Results in figure 5A indicate that a greater fraction of c-fos and c-jun co-precipitated with ERα in the Cd7 and Cd12 cells when compared to the parental MCF7 (M) cells, whereas similar levels of Sp-1 and β-catenin— two transcription factors known to interact with ERα— were found in both parental MCF7 cells and cells chronically exposed to cadmium (Fig. 5A). Changes in the interaction of ERα with c-jun and c-fos were further confirmed by reciprocal co-immunoprecipitation with antibodies against c-jun and c-fos (Fig. 5B).

Despite the observation that ERα co-precipitates with both c-jun and c-fos, it is unclear whether these AP-1 transcription factors are directly involved in the regulation of SDF-1. To understand the role of c-jun and c-fos in mediating the expression of SDF-1, the expression of SDF-1 cells was analyzed following siRNA-mediated silencing of c-jun and c-fos (Fig. 5C). MCF7, Cd7 and Cd12 cells were transfected with siRNA to target either c-jun (Ji) or c-fos (Fi), and cells

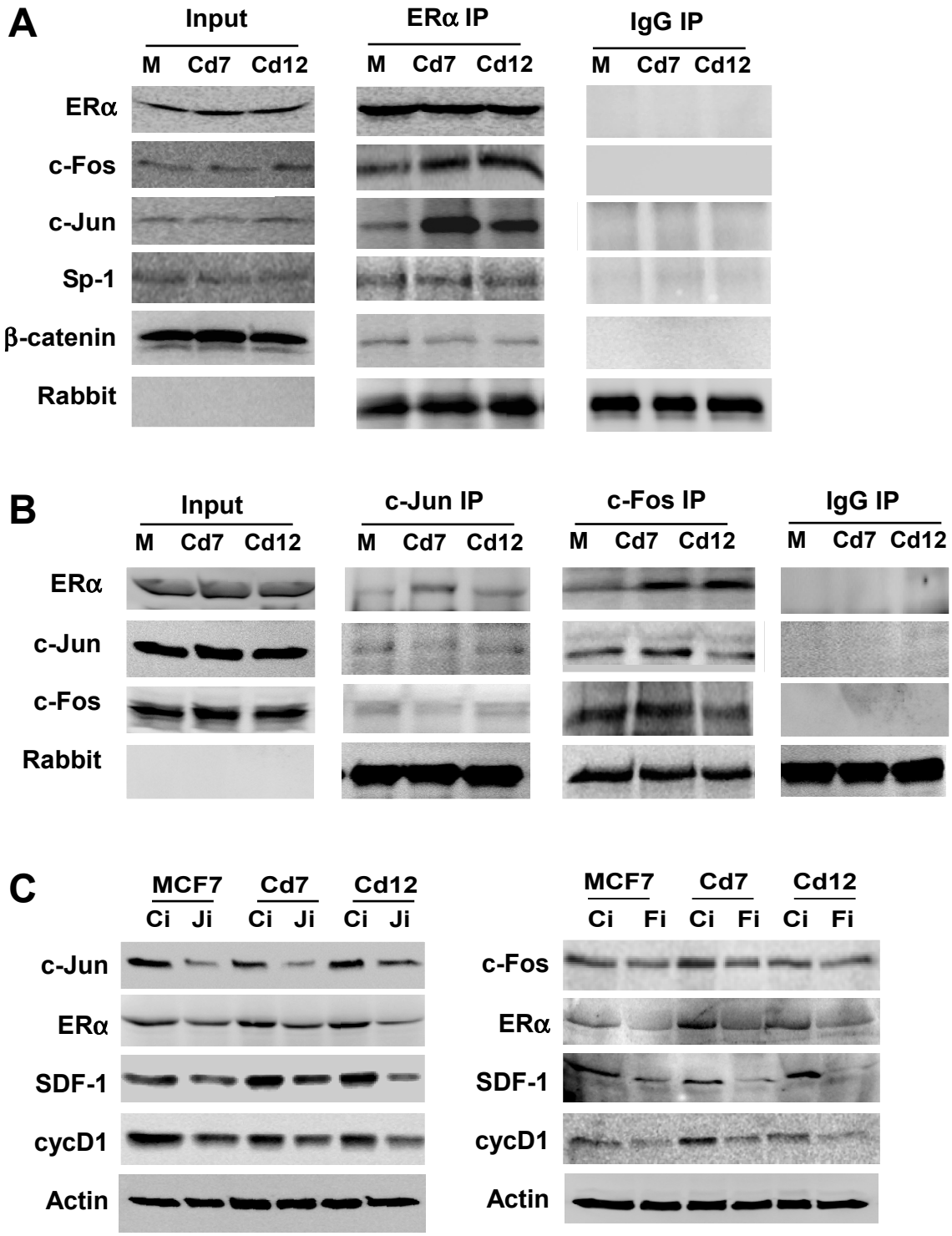

Figure 5: Prolonged exposure to cadmium enhances the interactions of ERα with c-jun and c-fos. (A) MCF7, Cd7 and Cd12 cell lysates were immunoprecipitated with either α-ERα or normal rabbit IgG. Proteins interacting with ERα were analyzed with Western blot analysis. (B) Reverse co-IP was performed with α-c-Jun, α-c-Fos, or normal rabbit IgG. (C) MCF7, Cd7 and Cd12 were plated in 6-well plates and transfected with siRNA targeting either c-jun (Ji), c-fos (Fi) or a scramble siRNA control (Ci) and collected 48 hours later for protein analysis.

were harvested 48 hours later for Western blot analysis (Fig. 5C). While lower levels of c-jun and c-fos resulted in a significant reduction of SDF-1 expression, these lower levels of c-jun and c-fos apparently led to decreased levels of ERα, complicating the determination of whether c-jun and c-fos directly regulate SDF-1 expression.

Since the regulatory region of SDF-1 consists of only one full ERE site located 234 bp upstream of the promoter, five half ERE sites located within the proximal promoter, and multiple AP-1 and Sp-1 sites on the promoter (Boudot et al 2011), it is probable that ERα may interact with other transcription factors to regulate SDF-1 expression. Therefore, a chromatin immunoprecipitation (ChIP) assay was used to determine whether a greater fraction of c-jun and c-fos is recruited to the SDF-1 promoter in cells chronically exposed to cadmium. Cells were plated for 48 hours and harvested for ChIP analysis. The recruitment of ERα, c-jun and c-fos was analyzed by PCR. Results in Figures 6A and B indicate that cells chronically exposed to cadmium (Cd7 and Cd12) had higher levels of ERα recruited to the SDF-1 promoter in comparison to parental MCF7 (M) cells (27% and 47% vs. 15%, respectively). Similarly, the occupancy of c-jun and c-fos on the SDF-1 promoter were significantly elevated in Cd7 and Cd12 cells when compared to parental MCF7 cells, with c-jun showing at least a 2-fold increase (Fig. 6A-B). The occupancy of all three transcription factors (ERα, c-jun, and c-fos) was also found elevated in the cyclin D1 and c-myc promoters of cells chronically exposed to cadmium (Fig. 6A-B). In contrast, Sp-1 was only found elevated on the c-myc promoter of cells chronically exposed to cadmium (Cd7

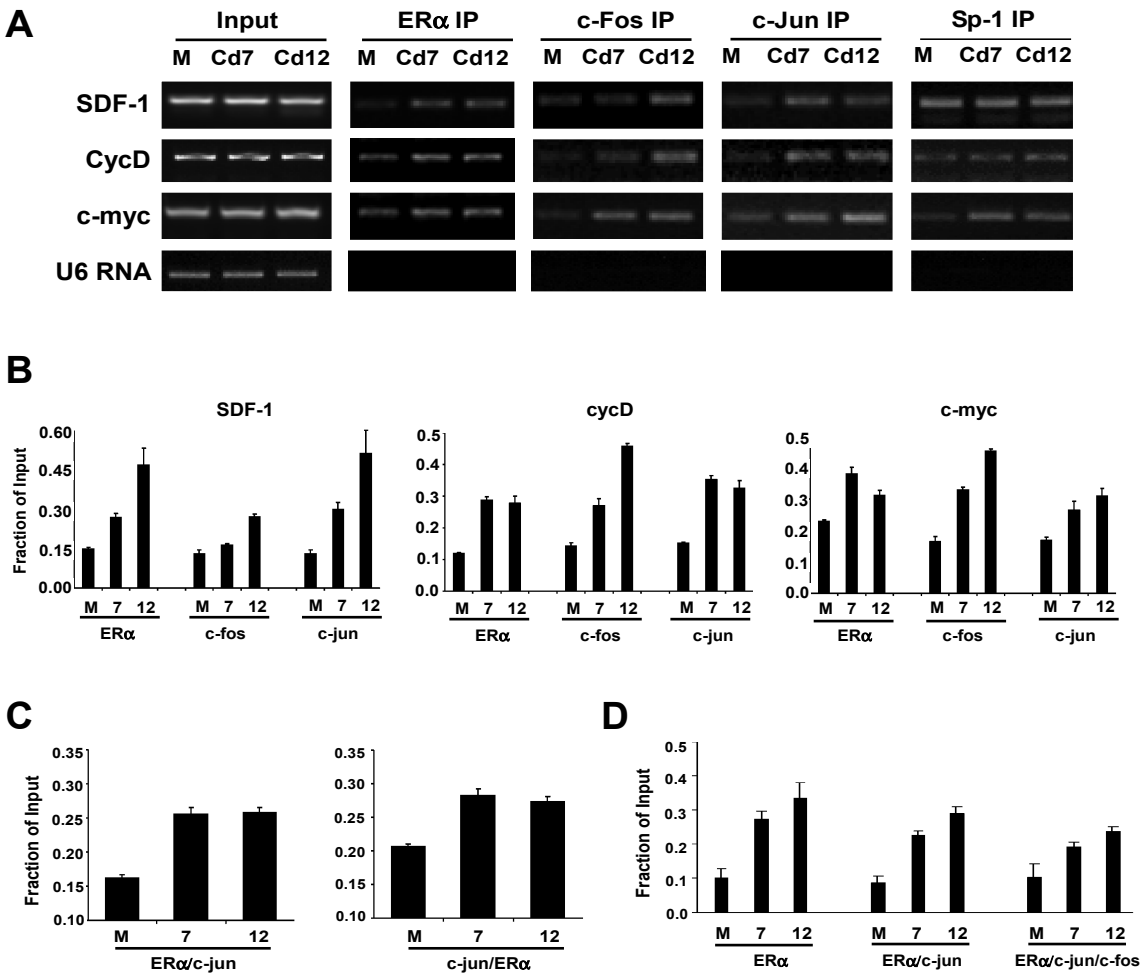

Figure 6: ERα, c-fos and c-jun are recruited to SDF-1 promoter. (A) MCF-7, Cd7 and Cd12 cells were harvested for chromatin immunoprecititation (ChIP) analysis. ChIP analysis was done with α-ERα, α-c-Fos, α-c-Jun, and α-Sp1, and recruitment of proteins to the SDF-1, cyclin D1 and c-myc promoters was determined using promoter specific primers and semi-quantitative PCR. (B) Band intensities of PCR products for SDF-1, cycD, and c-myc were quantified and normalized to input using Quantity One (Bio-Rad) ($P<0.001$). (C) In the ChIP re-ChIP assay, DNA/protein complexes from the first ChIP assay were extracted and re-ChIPed with a second antibody. The occupancy of ERα/c-jun or c-Jun/ERα complexes on the SDF-1 promoter was analyzed as in B ($P<0.001$). (D) To confirm the presence of c-fos in the ERα/c-jun complex on SDF-1, reChIPed DNA containing ERα/c-jun complexes were immuno-precipitated with α-c-fos antibody, and the resulting DNA fragments were analyzed as in B ($P<0.001$).

and 12); no significant difference in Sp-1 levels was observed on the SDF-1 and cyclin D1 promoters among the different cell lines (Fig. 6A).

While the results from the ChIP assay strongly support the notion that ERα, c-jun and c-fos all regulate SDF-1 expression, it is unclear whether these transcription factors work independently or together as one regulatory complex. To fully understand the components of the transcription complex regulating SDF-1 expression, a ChIP re-ChIP assay was performed. Following the first ChIP assay with either ERα or rabbit IgG as a control, the DNA-protein complexes were extracted and re-ChIPed with a second antibody (c-jun, or rabbit IgG). Data from both forward and reverse ChIP re-ChIP assays display higher levels of ERα/c-jun complex bound to the SDF-1 promoter in Cd7 and Cd12 cells when compared to parental MCF-7 cells (Fig. 6C). Since previous studies have suggested that ERα and c-fos do not interact directly (Teyssier et al. 2001), we evaluated the presence of c-fos in the ERα/c-jun complex using a triple-ChIP assay (three sequential chromatin IPs), in which the re-ChIPed DNA containing ERα/c-jun complexes was immunoprecipitated with a third antibody (c-fos or rabbit IgG) to verify the presence of c-fos in the complex. Results in Figure 6D not only confirm the occupancy of ERα/c-jun complex on the SDF-1 promoter, but also demonstrate that c-fos is involved in transcriptional regulation of SDF-1 in both parental and cadmium-adapted cell lines.

2.4 Discussion

Often referred to as an endocrine disruptor, cadmium is an environmental

contaminant that has been shown to have the ability to bind to the estrogen receptor and alter the expression of various estrogen receptor target genes (Martin et al. 2003, Garcia-Morales et al. 1994, Siewit et al. 2010, Yu et al. 2010). While epidemiological and animal studies (Martin et al. 2003, Johnson et al. 2003, Hofer et al. 2009, Alonso-Gonzalez et al. 2007, Ali et al. 2010, Waalkes et al. 1999, Gunn et al. 1964, Poirier et al. 1983) have implicated cadmium as a carcinogen, there is no direct evidence suggesting cadmium promotes breast cancer in humans. Despite this, the International *Agency for Research on Cancer (*IARC) has classified cadmium as a potential human carcinogen. Although multiple studies— including work from this lab— have demonstrated that acute cadmium exposures can stimulate breast cancer cell growth and can activate the estrogen receptor to mediate the expression of genes associated with cell growth (Stoica et al. 2000, Martin et al. 2003, Garcia-Morales et al. 1994, Choe et al. 2003, Liu et al. 2008, Brama et al. 2007), less is known about how chronic exposures of cadmium may contribute to the development and progression of breast cancer. Studies on prostate cancer and sarcomas have indicated that chronic exposures to cadmium can be associated with more malignant tumors (Waalkes et al. 2000, Achanzar et al. 2001, Haga et al. 1996). Additionally, significantly higher levels of cadmium have been found in breast tumor tissues in comparison to non-tumor tissues (Antila et al. 1996, Strumylaite et al. 2011, Strumylaite et al. 2008, Romanowicz et al. 2011, Ionescu et al. 2006). The chronic effects of cadmium on the progression of breast cancer and the molecular alterations induced by cadmium have never before been fully

elucidated.

Results from this study confirm that chronic cadmium exposure does promote the development of more aggressive cancer characteristics, including increased cell growth and an increased ability to migrate and invade (Fig. 1). To understand the impact of chronic cadmium exposures at the molecular level, clonal cell lines were developed from the original MCF7-Cd cell line (MCF7-Cd-2 to 12), and it was found that SDF-1, one of the genes identified in microarray expression analysis, was elevated in more than 70% of the cadmium-adapted cell lines (Fig. 2A). In addition to expressing higher levels of SDF-1 at the mRNA level, cells chronically exposed to cadmium (MCF7-Cd7 and Cd12) also expressed and secreted more SDF-1 protein into the media (Fig. 2B-C), consistent with its function as a chemokine, a factor that promotes cell migration and metastasis (Mukherjee et al. 2013).

Interestingly, past studies have also shown SDF-1 to be regulated by ERα (Hall and Korach 2003, Boudot et al. 2011, Zhu et al. 2006). Consistent with these findings, SDF-1 mRNA levels were indeed shown to increase within hours of the cells being treated with 17β-estradiol (Fig. 3A-B). Strikingly, our results demonstrate that the metalloestrogen cadmium can also stimulate SDF-1 gene expression, but to a lesser extent than 17β-estradiol (6 fold vs. 12 fold, Fig. 3). To establish a link between chronic cadmium-induced SDF-1 expression and ERα, siRNA was used to reduce intracellular levels of ERα in parental MCF7, MCF7-Cd4, -Cd7, and -Cd12 cells. Results showed that suppressing ERα expression subsequently inhibited the expression of SDF-1 in both MCF7 cells

and in cells chronically exposed to cadmium, suggesting that elevated SDF-1 expression is in part dependent on ERα (Fig. 4). In order to determine whether ERα regulates the transcription of the SDF-1 gene, a luciferase reporter assay was used with SDF-1-*luc* and co-transfected siRNAs to silence ERα. Results in Figure 4E suggest that depletion of ERα, as indicated by Western blot analysis (Fig. 4D), significantly reduces transcriptional activation of the SDF-1 promoter. Similarly, silencing ERα in cadmium-adapted and parental MCF-7 cells also decreased other ERα target genes, specifically cyclin D1 and c-myc (Fig. 4). This further indicates that the estrogen receptor signaling pathway is intact and may have a heightened response in cells chronically exposed to cadmium.

To determine if chronic cadmium exposure enhances the interaction of ERα with other transcription factors, co-immunoprecipitation experiments were performed. Results in Figure 5A show that a greater fraction of c-jun and c-fos co-precipitated with ERα in the cadmium-exposed cells. These interactions were confirmed by reciprocal co-immunoprecipitation (co-IP) with either c-jun or c-fos (Fig. 5B). While the interaction of ERα with c-jun is well-documented (Strumylaite et al. 2008, Romanowicz et al. 2011, Ionescu et al. 2006, Hanahan and Weinberg 2000), the direct interaction between ERα and c-fos has not been documented. More specifically, a study by Teyssier et al. demonstrated that ERα binds directly to c-jun, but not to c-fos, in pull-down assays (Teyssier et al. 2001). Although our co-IP results show an ERα/c-fos interaction, it is important to note that this interaction may be mediated indirectly by c-jun, especially since c-jun and c-fos often function as heterodimers (Bjornstrom et al. 2005). Consistent with

the results presented in this study, previous studies have demonstrated that both cadmium and arsenic can increase the interactions of ERα with c-jun under acute exposures (Siewit et al. 2010, Qi et al. 2004).

Though promising, the increased interactions between ERα, c-jun and c-fos do not prove that these complexes are directly involved in regulating the expression of SDF-1 in cells chronically exposed to cadmium. We attempted to show this via siRNA-mediated silencing of c-jun and c-fos, which did result in a reduction of SDF-1. Unfortunately, the data could not clearly demonstrate whether the decrease in SDF-1 was due solely to c-jun or c-fos since the down regulation of c-jun and c-fos also decreased ERα levels, which is already known to affect SDF-1 levels (Fig. 5C).

To confirm the roles of ERα, c-jun and c-fos in the transcriptional regulation of SDF-1 in cells chronically exposed to cadmium, chromatin immunoprecipiation (ChIP) assays were then used to determine the occupancy status of each transcription factor on the SDF-1 promoter. In line with previous reports by Zhu et al. and Boudot et al., our results demonstrate that ERα is recruited to the SDF-1 promoter and furthermore, the promoter occupancy by ERα is elevated in cells chronically exposed to cadmium (Fig. 6A-B) (Boudot et al. 2011, Zhu et al. 2006). A greater fraction of c-jun and c-fos were also found on the SDF-1, cyclin D1, and c-myc promoters after chronic cadmium exposure. In contrast, the levels of transcription factor Sp-1 were only elevated on the c-myc promoter.

Whether ERα regulates the expression of SDF-1 by binding to classical ERE sites or via interactions with other transcription factors was uncertain. However, based on the occupancy patterns of c-jun and c-fos on the SDF-1 promoter, it seemed conceivable that SDF-1 is regulated in a similar manner as cyclin D1 and c-myc— both of which are regulated by the interaction of ERα with c-jun/c-fos heterodimers (Klein et al. 2008, Cicatiello et al. 2004, Castro-Rivera et al. 2001, Wang et al. 2011, Shang et al. 2000). Since the interaction between ERα and c-fos is likely mediated indirectly by c-jun (Bjornstrom et al. 2005), further studies were carried out using both ChIP re-ChIP and triple-ChIP assays to determine if ERα regulates SDF-1 via interactions with c-jun/c-fos heterodimer. Data from both forward and reverse ChIP re-ChIP assays suggested that cells chronically exposed to cadmium (Cd7 and Cd12) exhibited higher levels of ERα/c-jun complexes on the SDF-1 promoter (Fig. 6C). The increase in ERα/c-jun interactions further emphasizes the importance of ERα and c-jun in regulating SDF-1 expression, especially in cells chronically exposed to cadmium. Consistent with these results, c-jun has been shown to regulate SDF-1 expression in other cell types (Florin et al. 2004, Florin et al. 2005). These new results also mirrored those from our previous study, which had demonstrated that the ERα/c-jun interaction was important in modulating the expression of genes in response to *acute* cadmium exposure (Siewit et al. 2010). Interestingly, the ERα/c-jun interaction has also been shown to be important in cells exposed to arsenic stress (Qi et al. 2004), suggesting this may serve as a common metal-response mechanism.

In summary, the results from this study demonstrate for the first time that chronic cadmium exposure promotes the acquisition of more aggressive cancer phenotypes (e.g. growth, migration and invasion) by stimulating the expression of SDF-1, and altering the molecular interactions between ERα, c-jun and c-fos. Additionally, the data in this study suggest that the molecular dynamics of cells chronically exposed to cadmium are significantly different from cells that are not exposed to cadmium. We speculate that the molecular interactions of ERα with other transcription factors, including other members of the AP-1 family and members of E2F and NFkB families are also affected by chronic cadmium exposure, and likely in a promoter-specific manner (Marino et al. 2006, Klinge et al. 2004). Since estrogen receptor is known interact with many coactivators and corepressors (Shang et al. 2000, Florin et al. 2004, Florin et al. 2005, Marino et al. 2006, Klinge et al. 2004), it would also be interesting to determine how chronic cadmium exposure may alter the molecular interactions between ERα and its coregulators in future studies. The results presented in this study offer molecular insights into how mammary tumors containing significant levels of cadmium can become more aggressive and underscore the importance of studying the molecular effects of *chronic* cadmium exposure in breast carcinogenesis.

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CHAPTER 3

CHRONIC CADMIUM EXPOSURE DISRUPTS CELL-TO-CELL ADHESIONS BY DOWN-REGULATING E-CADHERIN

3.1 Introduction

As stated in the previous chapter, cells exposed to cadmium for prolonged periods of time have been shown to display more aggressive cancer phenotypes that include an increased ability to grow in an anchorage-independent fashion and the capacity to invade surrounding tissue (Benbrahim-Tallaa et al. 2009, Achanzar et al. 2001). Invasion and subsequent metastasis require the detachment of cells from the primary tumor site. One way this can be achieved is through loss of cell adhesion molecules such as E-cadherin and β–catenin. E-cadherin is a transmembrane glycoprotein which plays a key role in hemophilic Ca^{2+}-dependent cell-to-cell adhesion (Pearson and Prozialeck 2001). Specifically, E-cadherin associates with actin microfilaments via β–catenin, a key protein coordinating cell-to-cell adhesions. Studies have shown that cadmium may replace calcium in the E-cadherin cell-to-cell junctions, thus disrupting the cadherin/catenin complex (Prozialeck and Niewenhuis 1991, Prozialeck and Lamar 1997), but such studies have not demonstrated a link between this cadmium-induced disruption and the development of invasive/metastatic phenotypes in breast cancer.

This study aims to understand how chronic cadmium exposure contributes

between E-cadherin and β–catenin. We provide several lines of evidence that suggest that prolonged cadmium exposure disrupts the cell-to-cell adhesion molecules E-cadherin and β–catenin. Our results demonstrate that MCF7 breast cancer cells chronically exposed to cadmium have decreased E-cadherin expression and subsequently a diminished number of E-cadherin/β-catenin complexes. In short, these results suggest that chronic exposure to heavy metals like cadmium disrupts inter- and intracellular adhesion protein complexes to facilitate breast cancer progression by mediating molecular changes that alter the cancer cells' ability to migrate and invade (Chapter 2).

3.2 Materials and Methods

Cell Culture

MCF7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF7-Cd was developed by exposing parental MCF7 cells to 10^{-7} M $CdCl_2$ for over 6 months, and clonal cell lines Cd7 and Cd12 were derived from this line. Parental MCF7 and MCF7-Cd7 and -Cd12 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT) and antibiotics. Both cell lines were subcultured every 3-4 days.

Western Blot

Cells were lysed in 1% SDS-HEPES buffer and total protein concentration was normalized using the Bio-Rad Dc Protein Assay kit (Bio-Rad, Hercules, CA).

Total protein was separated via SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA). Protein expression was monitored using protein-specific rabbit polyclonal antibodies: α-CDH-1 (Santa Cruz Biotechnology, Santa Cruz, CA), α–β–catenin (Santa Cruz Biotechnology, Santa Cruz, CA), and α-GAPDH (Cell Signaling Technology, Danvers, MA.

Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

Total RNA was isolated from normal MCF7, MCF7-Cd4, Cd7, and Cd12 using TRI-Reagent (Zymo Research Corporation, Irvine, CA) and columns from the Direct-zol RNA MiniPrep kit (Zymo Research) according to manufacturer's protocol. Three micrograms (μg) of total RNA were used for the reverse transcription reaction with oligo-dT_{18} primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, Madison, WI). Gene expression was monitored using semi-quantitative PCR for 28 cycles. PCR products were separated by agarose gel electrophoresis and visualized using the Chemi-Doc gel documentation system (Bio-Rad, Hercules, CA). Gene expression was quantified by quantitative real-time RT-PCR using the SYBR Green real-time PCR master mix (SA Biosciences, Valencia, CA). All primers were synthesized by Integrated DNA Technologies, Inc. (IDT; San Diego, CA). Primers used and their sequences are as follows:

$CDH\text{-}1_F$: CATTCTACACGTAGCAGTGACG
$CDH\text{-}1_R$: CTGGAGAACCATTGTCTGTAGC
$\beta\text{-catenin}_F$: TACCTCCCAAGTCCTGTATGAG
$\beta\text{-catenin}_R$: CTTATTAACCACCACCTGGTCC

$GAPDH_F$: GAAATCCCATCACCATCTTCCAG
$GAPDH_R$: ATGAGTCCTTCCACGATACCAAAG

Coimmunoprecipitation (CoIP) Assay

MCF7, MCF7-Cd7 and -Cd12 were plated in 10cm plates and harvested 48 hours later. Cells were lysed in 0.5% NP-40 lysis buffer and sonicated three-times for 30 seconds. Total protein cell lysates were treated with anti-E-cadherin antibody (Santa Cruz Biotechnology) for 2 hours and then incubated with either protein A or G agarose beads for 1 hour. Protein complexes were separated by SDS-PAGE followed by Western blot analysis. Membranes were probed for E-cadherin (Santa Cruz Biotechnology), β-catenin (Santa Cruz Biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology).

Immunofluorescence Microscopy

MCF-7, MCF7-Cd7 and -Cd12 were plated on 6-well plates containing sterile cover slips. Cells were fixed 24 hours later in 10% formalin. Protein expression was analyzed using monoclonal antibodies to β-catenin (Santa Cruz Biotechnology), polyclonal antibodies to E-cadherin (Cell Signaling Technology), or polyclonal antibodies to active β-catenin (Cell Signaling Technology). Secondary antibodies conjugated to Alexa-488 (green) and Alexa-647 (red) (Molecular Probes, Life Technologies, Grand Island, NY) were used to detect protein expression and images were visualized using a fluorescent microscope (Leica Microsystems, Inc, Deerfield, IL).

3.3 Results

Previous data from our lab suggest that MCF7 cells chronically exposed to cadmium (MCF7-Cd) display increased metastatic phenotypes—including changes in migration, cell adhesion and invasion—all of which are characteristics of cancer progression. Other studies have also implicated E-cadherin in many of these processes. To understand how chronic cadmium exposure affects the expression of E-cadherin, clonal cell lines (MCF7-Cd7, Cd12) derived from cells chronically exposed to cadmium were used for expression analysis and compared to those of parental MCF7 cells. Gene and protein expression levels were analyzed by semi-quantitative RT-PCR and Western blot, respectively (Fig. 1 A-B). Results shown in Figure 1A suggest that cells chronically exposed to cadmium (Cd7 and Cd12) express similar levels of E-cadherin at the transcription level as the parental MCF7 cells. There is also no significant difference in β-catenin gene expression among the cadmium-adapted and parental MCF7 cells. However, cells chronically exposed to cadmium did express lower levels of E-cadherin at the protein level (Figure 1B). This effect was not uniformly observed with β-catenin, as one cadmium-exposed clone demonstrated a significant decrease in expression (Cd12) while another showed no appreciable change in β-catenin expression (Cd7) when compared to the parent cell line. To determine if the cadmium-induced decrease of E-cadherin is specific to only chronic exposures, we treated parental MCF7 cells with 10^{-6} M cadmium chloride ($CdCl_2$) and compared it to cells treated with 10^{-7} M estrogen under acute conditions. Cells were then harvested 0, 1, 2, 4, 8, 12 and 24 hours after treatment, and

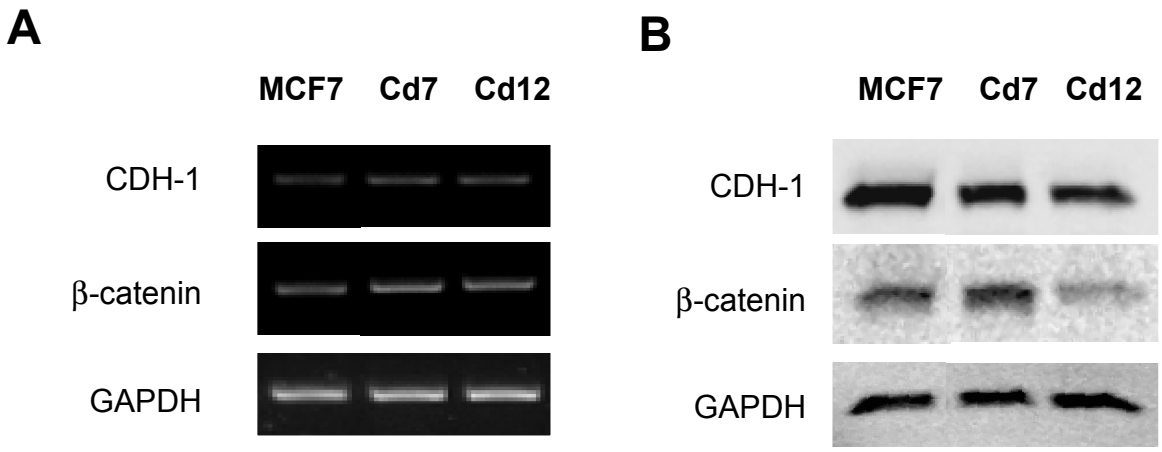

Figure 1: Cells chronically exposed to cadmium express lower protein levels of E-cadherin. (A) MCF7, MCF7-Cd7 and -Cd12 were plated in 6 well plates and total RNA was isolated for gene expression analysis using semi-quantitative PCR. (B) Protein expression in MCF7, Cd7, and Cd12 cells was analyzed by Western blot analysis.

protein expression was analyzed using Western blot analysis. Results in Figure 2 show that both cadmium and estrogen are able to promote down-regulation of E-cadherin protein expression under acute conditions. In contrast, the expression of total β-catenin was elevated in response to both acute cadmium and estrogen treatment. Other than the membrane-localized form of β-catenin that normally interacts with E-cadherin to mediate cell-cell interaction, β-catenin also exists in a dephosphorylated or active form that functions as a transcription factor to regulate gene expression in the nucleus. We also evaluated the levels of active β-catenin and found that— similar to total β-catenin levels— the active form was also increased in response to cadmium and estrogen (Fig. 2).

Since E-cadherin and β-catenin often function in a complex to mediate cell-cell adhesion, we question whether the cadmium-induced decrease of E-cadherin may alter this interaction. To determine if the interaction between E-cadherin and β-catenin are disrupted in cells chronically exposed to cadmium, cells were collected for co-immunoprecipitation assays. Total cell lysates of both cadmium-adapted (Cd7 and Cd12) and parental MCF7 cells were immunoprecipitated with antibodies against E-cadherin. Results in Figure 3 show a decrease of β-catenin protein complexed to E-cadherin in cells chronically exposed to cadmium (7 and 12) in comparison to the parental MCF7 cells (M).

To verify that fewer E-cadherin/β-catenin complexes exist within cells chronically exposed to cadmium, we also examined the expression of this protein complex using immunofluorescence microscopy. Results in Figure 4 show that both parental (MCF7) and cadmium-adapted cells (Cd7 and Cd12) express E-

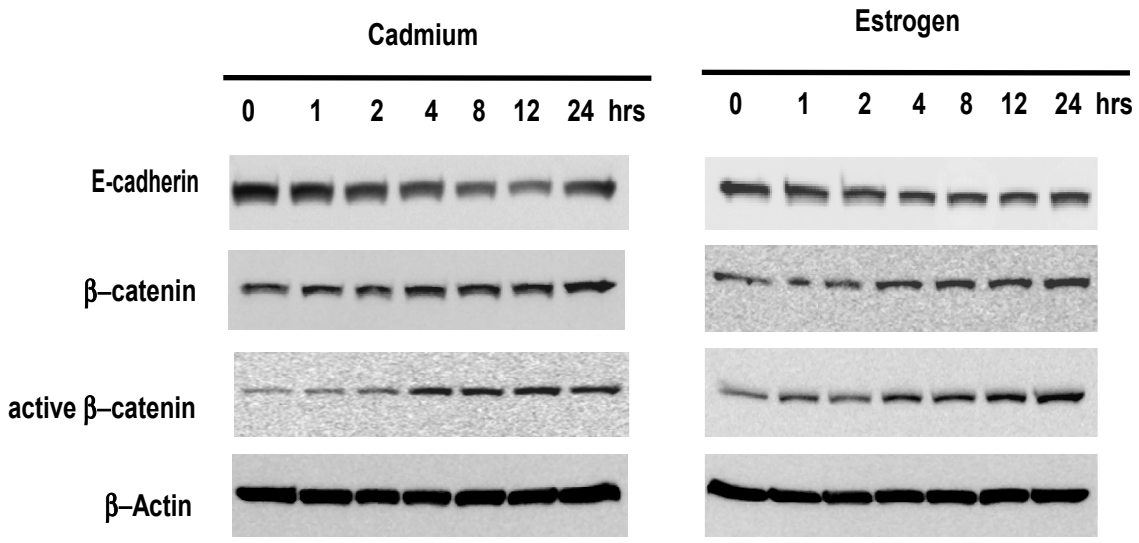

Figure 2: Acute cadmium and estrogen exposures promote the down-regulation of E-cadherin. MCF7 cells were plated in 6-well plates and treated with 10^{-6} M $CdCl_2$ or 10^{-7} M E_2. Cells were harvested 0, 1, 2, 4, 8, 12, and 24 hours after treatment for protein expression analysis by Western blot.

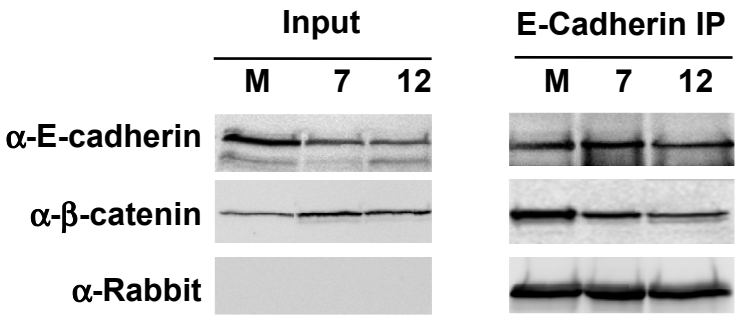

Figure 3: Chronic cadmium exposure alters E-cadherin/β-catenin complex interactions. MCF7 (M), Cd7 (7) and Cd12 (12) were harvested forco-immunoprecipitation (co-IP) and total cell lysates were immunoprecipitated with α-E-cadherin. β-catenin/E-cadherin interactions were analyzed by Western blot analysis.

cadherin (green) and β-catenin (red); however, the distribution of the two proteins is dramatically different. In parental MCF7 cells, both E-cadherin and β-catenin are primarily co-localized to the plasma membrane (merged- yellow) with low levels found in the cytoplasm; in Cd7 and Cd12 cells, lower levels of E-cadherin and β-catenin are found in the plasma membrane. The β-catenin that *is* found near the plasma membrane is not as well-organized as that which is observed in the parental MCF7 cells. Consistent with these observations, the Cd7 and C12 cells are morphologically different, with individual cells farther apart and displaying fewer cell-cell interactions in comparison to parental cells (Fig. 4).

As mentioned earlier, in addition to the membrane-localized form of β-catenin, the active form of β-catenin is primarily localized to the nucleus where it regulates gene expression. To examine how changes in the E-cadherin protein levels and distribution alter the active form of β-catenin, we used immunofluorescence to monitor the localization of the active form of β-catenin in the various cells. Results in Figure 5 show that cadmium-adapted cells (Cd7 and Cd12) display elevated levels of active β-catenin (green) in the nucleus in comparison to parental MCF-7 cells, suggesting that changes in expression of genes associated with chronic cadmium exposure may be associated with the redistribution of β-catenin from the plasma membrane to the nucleus.

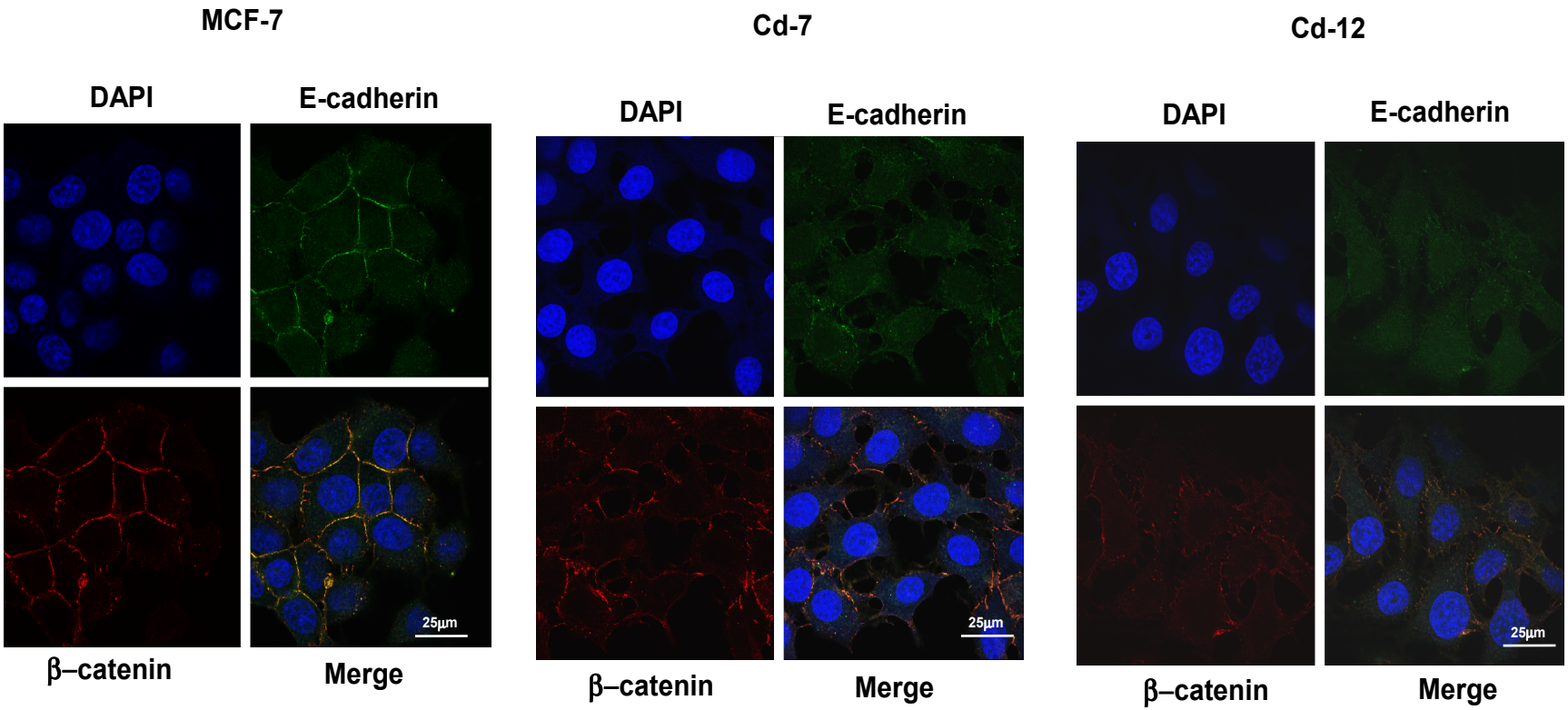

Figure 4: Prolonged cadmium exposure disrupts E-cadherin/β-catenin complex. Protein localization of E-cadherin and β-catenin was analyzed in MCF-7, Cd7 and Cd12 cells using immunofluorescence microscopy. E-cadherin (FITC), β-catenin (Texas Red), and nucleus (DAPI).

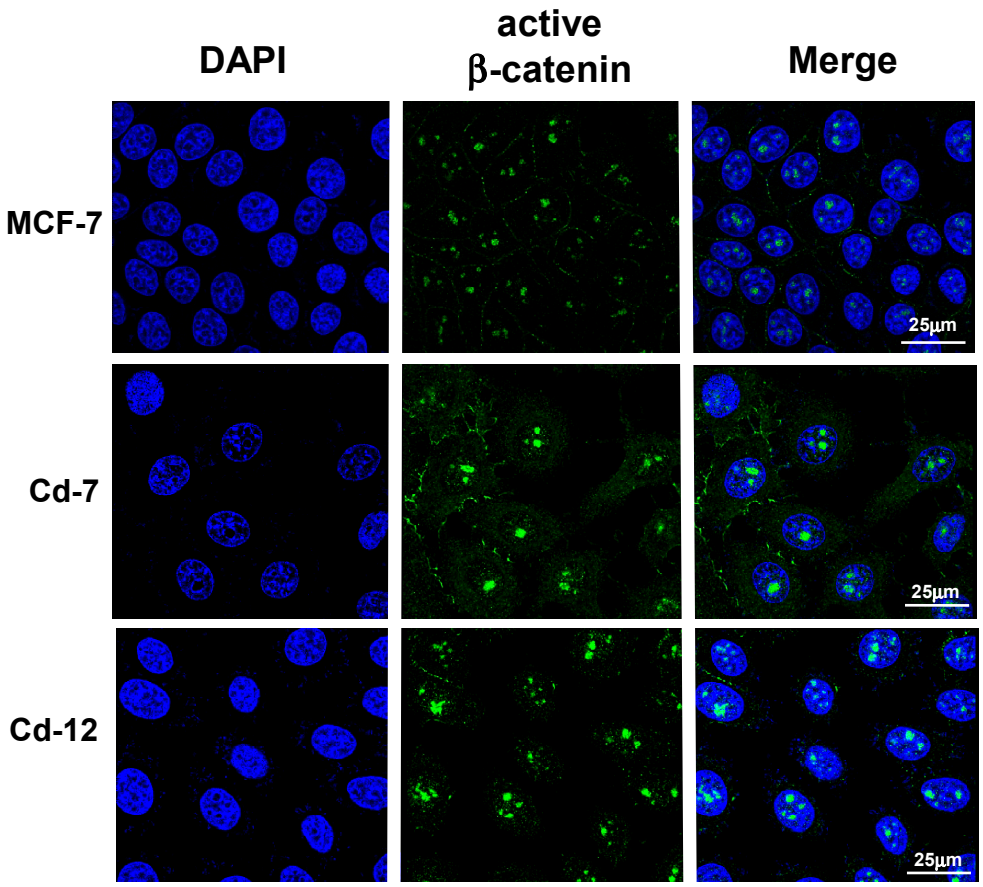

Figure 5: Active β-catenin localizes in the nucleus of prolonged cadmium exposed cells. Protein localization of active β-catenin was analyzed in MCF-7, Cd7 and Cd12 cells using immunofluorescence microscopy. Active β-catenin (FITC) and nucleus (DAPI).

3.4 Discussion

E-cadherin is known as a tumor suppressor protein and is used as a prognostic marker for breast cancer (Heimann et al. 2000, Baranwal and Alahari 2009). Results from this study show that cadmium disrupts cell-to-cell adhesion by down-regulating E-cadherin at the protein level (Fig. 1). Down-regulation of E-cadherin has been associated with more aggressive breast cancers and poor prognosis (Mohammadizadeh et al. 2009, Oka et al. 1993). Consistent with a recent study by Chakraborty et al. which showed that acute treatments of cadmium were able to disrupt E-cadherin/β-catenin junction complex and degrade E-cadherin in kidney tubule cells (Chakraborty et al. 2010), we demonstrated that both acute cadmium and estrogen exposure lead to the down-regulation of E-cadherin protein (Fig. 2). Several studies have shown that estrogen can induce the down-regulation and degradation of E-cadherin and the process has been shown to be mediated through ERα (Oesterreich et al. 2003, Cardamone et al. 2009, Potter et al. 1996, Park et al. 2008). Since cadmium functions as a metalloestrogen, the observation that both acute and chronic cadmium exposures likewise down-regulate E-cadherin is not surprising.

Most importantly, we showed that chronic cadmium exposure promotes the down-regulation of E-cadherin and significantly alters the interaction of E-cadherin with β-catenin. Using co-immunoprecipitation assays, results show that indeed fewer β-catenin molecules were complexed with E-cadherin in cadmium cells (Cd7 and Cd12) in comparison to the parental MCF7 cells. Once E-cadherin and β-catenin are no longer complexed, we speculate that increased amounts of

β-catenin may translocate to the nucleus and cross-talk with other transcription factors to modulate expression of genes (Chakraborty et al. 2010, Lin et al. 2000, He et al. 1998, Li et al. 1999, Mann et al. 1999), including c-myc and cyclin D1, which been shown to be elevated in cadmium exposed cells (Siewit et al. 2010, Chakraborty et al. 2010, Benbrahim-Tallaa et al. 2009). Immunofluorescence microscopy supported this speculation by revealing altered distribution patterns of E-cadherin and β-catenin in cells chronically exposed to cadmium. Lower levels of E-cadherin and β-catenin were found in the plasma membrane of cadmium-treated cells, and there were elevated levels of active β-catenin in the nucleus. We contend that β-catenin, once freed from the E-cadherin adhesion complex, can localize into the nucleus where it regulates transcription by interacting with transcription factors such as the T-cell factor-4 (TCF-4) or lymphoid enhancer factor-1 (LEF-1), thus activating target genes and the Wnt signaling pathway (Lin et al. 2000, Chakraborty et al 2010).

In short, we propose that results from this study demonstrate that prolonged cadmium exposure disrupts cell-cell adhesion by down-regulating E-cadherin expression and relocating active β-catenin to the nucleus. This in turn mediates the increased invasive and metastatic properties associated with chronic exposures to cadmium.

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CHAPTER 4

CONCLUSION

Our research has provided an original contribution to the field of breast cancer progression. Specifically, we have demonstrated two possible mechanisms of cadmium carcinogenesis (Chapters 2 and 3). Unlike most studies that have focused on the effects of acute cadmium exposure on breast cancer, our study largely focused on the effects of chronic cadmium exposure on breast cancer progression. In order to begin to understand the effects of chronic cadmium exposure on breast cancer progression, we developed several MCF7 clonal cell lines that were exposed to low concentrations of cadmium for a prolonged period of time (6 months). Using this model system, we evaluated the impacts of this exposure on breast cancer progression. Results from this work not only demonstrated that prolonged exposure to cadmium increases cell proliferation through the activation of ERα — as seen in most acute studies— but also results in the acquisition of more aggressive cancer phenotypes.

Our findings in Chapter 2 show that cells chronically exposed to cadmium display an increased ability to proliferate, migrate and invade when compared to the parental MCF7 cell line. Further analysis revealed that chronic cadmium exposure stimulated cells to express higher levels of SDF-1, a chemokine often associated with tumor invasion and metastasis. We also provided several lines of evidence suggesting that the expression of SDF-1 is not only dependent on the levels of ERα, but also on its interaction with c-jun/c-fos heterodimers. The

findings presented here suggest that chronic cadmium exposure promotes migration and invasion by altering the molecular interactions of ERα, c-jun and c-fos and subsequent transcriptional machinery to up-regulate SDF-1 expression.

While SDF-1 is an important contributor of metastasis, there are likely other factors that participate in this process and facilitate the transformation to a more malignant phenotype. In Chapter 3, we revealed that chronic exposure to cadmium also alters the expression of two crucial cell-cell adhesion molecules, E-cadherin and β-catenin. More specifically, cells chronically exposed to cadmium expresses lower levels of E-cadherin at the protein level. Since no changes are observed at the transcriptional level, this suggests that cadmium exposure promotes the down-regulation of the E-cadherin protein. The consequence of losing E-cadherin is two fold— (1) decrease in cell-cell adhesion and (2) redistribution of β-catenin into the nucleus where it alters gene expression to stimulate breast cancer progression.

In summary, the research presented here has demonstrated that chronic cadmium exposure facilitates the progression of breast cancer by mediating molecular changes that alter cancer cells' ability to migrate and invade. While this work has made significant advancements in the understanding of how environmental contaminants such as cadmium promote breast cancer progression, it also warrants the need for further research in this area to help us truly understand cadmium carcinogenesis.