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Cadmium Contributes to Breast Cancer Development by Influencing Cell Adhesion Network

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CADMIUM CONTRIBUTES TO BREAST CANCER DEVELOPMENT BY
INFLUENCING CELL ADHESION NETWORK

BY
ZHENGXI WEI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

Cadmium (Cd) is carcinogenic and a well-established human health risk. Epidemiological and experimental studies have demonstrated that Cd is associated with breast cancer. We reported previously that Cd promoted breast cancer cell growth. Whether Cd treatment also causes epithelial-mesenchymal transition (EMT) and progression to metastasis in breast epithelial cells is unknown. The dissertation described here investigated the effect of Cd in breast cancer progression.

Noncancerous breast epithelial MCF10A cells, non-metastatic HCC 1937 and HCC 38 cells, and metastatic MDA-MB-231 cells were treated with 1 or 3 μ M Cd for 4 weeks. The increase in the expressions of N-cadherin and vimentin but decrease in the expressions of E-cadherin and claudin-1 indicated that these epithelial cells were transformed to mesenchymal-like upon prolonged Cd treatment. Treatment of MCF10A-Zcad and MDA-MB-231-Zcad cells with 1 or 3 μ M Cd showed the suppression of E-cadherin transcription and an increase in the translation of ZEB-1, suggesting that transcription factors were involved in the EMT process. The accumulation of Snail in the nucleus of cells treated with Cd for 4 weeks confirmed that Cd affected the expression of the EMT regulator. Inhibition of transcription by actinomycin D eliminated the Cd-induced Snail accumulation. However, inhibition of nuclear export by leptomycin B had an additive effect on Cd-induced Snail expression. Based on these results, it appears that Cd induces EMT in breast epithelial cells by modulating the transcription of Snail.

The effects of Cd on metastasis-associated phenotypes were investigated in MDA-MB-231 cells. Treatment of the metastatic cells with 1 or 3 μ M Cd for 1-6 h

resulted in cell spreading. The activation of integrin $\beta 1$, FAK, Src, and Rac1 indicated the involvement of integrin signaling in Cd-induced cell spreading. Treatment with 1 or 3 μM Cd also inhibited GSK3 β and induced T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription, suggesting the involvement of Wnt/ β -catenin pathway. Furthermore, treatment with 3 μM Cd for 4 weeks increased β -catenin expression, resulting in enhanced TCF/LEF transcription. Prolonged Cd treatment elevated the expression of integrin $\alpha 5$ and $\beta 1$, paxillin, and vimentin. This indicated cytoskeleton remodeling leading to malignancy, as evidenced by enhanced matrix metalloprotease 2/9 (MMP2/9) secretion and cell invasion. Furthermore, prolonged Cd treatment increased cell growth rate. Together, these results suggest that Cd alters integrin and β -catenin signaling involved in regulation of cytoskeleton and enhances cancer cell migration, adhesion, invasion, and proliferation.

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PREFACE

This dissertation was prepared according to the manuscript format.

Two manuscripts will be submitted to Toxicology and Applied Pharmacology with the following tentative titles:

I. Epithelial-mesenchymal transition in breast epithelial cells upon treatment with cadmium

II. Cadmium enhances metastasis-associated phenotypes in breast cancer cells through integrin and β -catenin signaling

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CHAPTER 1

INTRODUCTION

1. Toxicity of cadmium

Cadmium (Cd) is a transition metal. Typical industrial uses for Cd are in batteries, alloys, electroplating, plastic stabilizers, and pigments. Cd and its compounds are highly toxic. Bioaccumulation of Cd in body interferes with normal physiological functions. The general population is exposed to Cd through dietary intake, smoking, and polluted air. Dietary Cd intake ranges from 0.5-55 µg/day (Cho et al., 2013). Absorbed Cd mainly binds to metallothionein or other cysteine-rich proteins in various organs, such as liver, kidney, lungs, prostate, spleen, mammary gland, and heart (Johnson et al., 2003; Waalkes, 2003). Therefore, acute or chronic exposure to Cd causes non-cancer disorder in cardiovascular, renal, bone, gastrointestinal, neurological, reproductive, immune, and respiratory systems. Moreover, occupational exposure to Cd through inhalation has been associated with lung cancer (Stayner et al., 1992). It raises the question, should Cd be considered as a carcinogen in the general population.

2. Epidemiological studies linking cadmium and cancer

A number of epidemiological studies have evaluated the association between cancer risk and Cd exposure in the general population in recent years (Adams et al., 2012b; Julin et al., 2012; Sawada et al., 2012; Nagata et al., 2013). Meta-analysis of

the data found that Cd exposure correlated to higher risks of breast, prostate, lung, kidney, and pancreas cancers (Cho et al., 2013).

A case-control study in Japan reported that high dietary Cd intake increased the risk of breast cancer in postmenopausal women, specifically the ER⁺ subtype (Itoh et al., 2014). However, another retrospective cohort study conducted in the United States reported that dietary Cd was not associated with the risk of invasive breast cancer in postmenopausal women (Adams et al., 2012a). Moreover, a more recent meta-analysis of six studies linking dietary Cd and postmenopausal breast cancer reported no significant increase in breast cancer risk among postmenopausal women with higher dietary Cd intake (Van Maele-Fabry et al., 2016). One possible explanation for these contradictory conclusions could be the differences in Food Frequency Questionnaire (FFQ) that was used to estimate the dietary Cd intake level. Another prospective cohort study validated dietary Cd intake level through comparison with urinary Cd levels to avoid the bias or inaccuracy in FFQ design. In that study, the higher dietary Cd level was associated with higher breast cancer incidence, regardless of ER status. (Julin et al., 2012). Additionally, several studies of breast cancer incidence and cadmium exposure concluded that breast cancer risk was associated with higher Cd in urine or blood (McElroy et al., 2006; Wu et al., 2006; Gallagher et al., 2010; Rahim et al., 2013). Therefore, it seems that the method to assess Cd exposure affects the accuracy of the correlation study. EPA and IARC have categorized Cd as a probable human carcinogen since the controversial conclusions in epidemiological studies. It also motivated investigations into studying the carcinogenic effect of Cd in animal models and cell lines.

3. Carcinogenesis of cadmium in animals

Studies in mice and rats indicated that Cd exposure caused the development of different types of cancers. Depending on the exposure route, tumors developed in different organs. Oral administration resulted in leukemia and testicular tumors in the Wistar rats (Waalkes and Rehm, 1992). Exposure to Cd aerosol caused lung adenocarcinomas in the Wistar rats (Takenaka et al., 1983). Subcutaneous injection of CdCl₂ caused lymphoma in DBA/2NCR mice and local sarcomas in NFS/NCR mice (Waalkes and Rehm, 1994). Besides tumors development at target exposed tissues, Cd also caused cancers related to estrogen or androgen status, such as breast, prostate, and testicular cancers (Waalkes et al., 1999).

Studies in rats showed that low-level Cd treatment stimulated the growth of epithelium in the uterus and mammary glands (Johnson et al., 2003). Since then, Cd has been viewed as a xenoestrogen that may stimulate mammary tumor initiation and promotion (Schrauzer, 2008; Davis et al., 2013). Ramachandran et al. (2011) reported that an oral dose of 1 µg Cd /Kg upregulated luciferase expression in uterus and thymus in an estrogen response element-luciferase (ERE-luc) reporter mouse model. However, another study conducted in the ERE-luc mice showed that the estrogenic effect of Cd was exerted in an ER-independent way. Also, that study did not observe the ERE-luciferase activities in any reproductive or non-reproductive organ (Ali et al., 2010). To deplete endogenous estrogen, a study was conducted in ovariectomized Wistar rats (Hofer et al., 2009). In this study, an estrogenic effect was observed in the uterus upon intraperitoneal injection of Cd, but not with oral administration.

4. Cadmium induced Breast Cancer Initiation and Promotion

Extrinsic risk factors, such as environmental exposures and behaviors contribute to the development of 70 - 90 % cancer cases. The development of cancer is a multi-step process, including initiation, promotion, and progression (Berenblum and Shubik, 1947). Fig. 1 shows hallmark events in these three steps. When stimulated by carcinogens, normal epithelium transforms to hyperplastic tissue, which initiates tumorigenesis. The pre-neoplastic cells grow into a group of dysregulated tumor cells, promoting the formation of carcinoma in situ. Eventually, the tumor cells progress into metastatic carcinoma, which is an irreversible step. The detailed molecular mechanisms have been investigated in different aspects, such as DNA repair inhibition, induction of oncogenes, and activation of mitogenic pathways (Bertin and Averbeck, 2006; Waisberg et al., 2003).

Cd is defined as a tumor initiator since it causes DNA lesions in normal cells (Potts et al., 2003; Youn et al., 2005). The DNA lesions include DNA strand breaks and formation of 8-hydroxyguanine and 8-hydroxy-2'-deoxyguanosine (Dally and Hartwig, 1997; Mikhailova et al., 1997). Mechanistic studies revealed that Cd inhibited DNA damage repair enzymes, including inhibition of p53, base excision repair, nucleotide excision repair, and mismatch repair enzymes, resulting in genome instability (Porter et al., 1997; Zharkov and Rosenquist, 2002; Hirano et al., 1997; Giaginis et al., 2006). However, Waalkes (2003) pointed out that the direct interaction of Cd with DNA and DNA repair enzymes was minimal. Even though high concentrations of Cd initiated DNA damage, the high-level exposure was far beyond the environmentally relevant concentrations. Benbrahim-Tallaa et al. (2009) found that

low-level Cd treatment transformed normal human breast epithelial cells to basal-like cells, resulting in tumor formation in vivo. The increase in oncogenes k-ras and c-myc in Cd-treated cells further revealed the progress toward invasive cancer phenotype.

Cd enhances the expressions of several oncogenes, including c-myc, c-jun, and c-fos which are transcription factors regulating cell growth (Jin and Ringertz, 1990; Angel and Karin, 1991; Epner and Herschman, 1991). Cd alters cellular homeostasis in various aspects, which may be the mechanism of induction of oncogenes. Cd increases reactive oxygen species through inactivating glutathione, which induces the expression of oncogenes as a protective mechanism under oxidative stress (Joseph et al., 2001). The increase in Ca^{2+} enhances the response element binding with promoter regions for transcription of oncogenes (Hardingham et al., 1997). Alternatively, Cd activates cell survival signaling, which stabilizes mRNA of oncogenes (Tsai et al., 2016).

Cd has been perceived as a tumor growth promoter in animal studies that are described above. Although there is no agreement on the estrogenicity of Cd, it has been accepted as a tumor growth stimulant. Most of the in vitro studies reported that Cd induced breast cancer cell proliferation (Garcia-Morales et al., 1994; Martin et al., 2003; Brama et al., 2007; Zang et al., 2009). Garcia-Morales et al (1994) first reported that Cd stimulated breast cancer MCF7 cell proliferation and induced transcription of ER-regulated genes for cell growth. Thus, the effect of Cd was attributed as an estrogenic effect. In our prior work, Cd was found to stimulate triple-negative breast cancer cell growth through EGFR-mediated cell cycle progression (Wei et al., 2015). Yu et al. (2011) reported that Cd stimulated cell growth via G protein coupled receptor

30 in an ER α negative breast cancer cell line SKBR3. Although researchers reported that Cd acted as a xenoestrogen since its effects were similar to those of estrogen, based on the observation in cells lacking ER α , Cd can also promote cell proliferation in an ER-independent manner.

In summary, Cd has been considered as a weak mutagen in bacterial and yeast system (Rossman et al., 1992). It initiates cancer through affecting genome stability (Waalkes, 2003). Cd induces dysregulation of apoptosis and cell cycle, rendering it the properties of a tumor growth promoter. Considering the multistep nature of cancer development, Cd may act at all stages of carcinogenesis. It is important to investigate the molecular events that contribute to Cd-induced carcinogenesis.

5. Breast cancer progression

Breast cancer development is a long-term process. It may take years or decades for initiated breast tumor to progress into malignant breast cancer. Initiation and promotion of breast cancer have been discussed above. This section addresses on breast cancer progression, which is the process of tumor cells migrating to distant organs and growing into metastases.

The multiple-step process entails invasion, intravasation and extravasation of primary tumor cells, and colonization of metastases (Fig. 1). Adhesion molecules play important roles in cancer progression (Okegawa et al., 2004). Cadherin and integrin are adhesion molecules on the cell membrane that mediate interaction between cells, or with the extracellular matrix (ECM). Epithelial-mesenchymal transition (EMT) is the first hallmark of tumor cell movement, which is associated with reduced E-

cadherin expression (Creighton et al., 2010). The reduced cell-cell adhesion allows tumor cells to disseminate from the original site and intravasate into the blood vessels and lymph system. When tumor cells are arrested in distant organs, integrins mediate adherence to the endothelium of capillary vessel walls. Increased cell-ECM interaction allows the cells to extravasate to the new microenvironment. The next sections describe the regulation of EMT and elucidate the role of cell adhesion molecules in metastatic cell adhesion and growth.

Fig. 1. Overview of breast cancer development: initiation, and promotion of primary tumor, and progression into metastases.

6. EMT and cancer progression

EMT is a process in which epithelial cells lose tight junctions and transform to mesenchymal-like cells. It is an important step during development and embryogenesis (Yang and Weinberg, 2008). Recently, this concept has been extended to tumor progression and metastasis, since accumulating evidence in clinical samples showed that EMT is associated with advanced stages of cancers (Willipinski-Stapelfeldt et al., 2005). EMT is associated with a decrease in expression of epithelial

markers such as E-cadherin, claudin-1, cytokeratin, and occludin, and an increase in mesenchymal markers such as N-cadherin, vimentin, and fibronectin (Sarrio et al., 2008). Switch to mesenchymal phenotype relates to changed cell morphology and enhances motility (Kokkinos et al., 2007). With the enhanced motility, tumor cells migrate and metastasize to distant organs. The switch from epithelial to mesenchymal-like is mediated by key transcription factors, including Snail/Slug, ZEB and Twist. Their expression and functions are finely regulated at the translational and post-translational levels (De Craene and Berx, 2013).

A number of substances have been identified as EMT inducers. The most potent ones are growth factors and cytokines, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor β (TGF β), and fibroblast growth factor (FGF) (Grotegut et al., 2006; Lo et al., 2007; Shirakihara et al., 2011; Patel et al., 2015). Some external factors, such as nicotine, deferoxamine, cyclosporine A, chromium, arsenic, and multi-walled carbon nanotube induce the expressions of some mesenchymal markers related to EMT (Berzal et al., 2012; Ding et al., 2013; Wang et al., 2013; Zhang et al., 2014; Polimeni et al., 2016; Zhang et al., 2016). Although Cd has not been reported as an EMT inducer, it decreased E-cadherin expression in breast cancer cells and kidney tubular epithelial cells, and increased fibronectin expression in lung epithelial cells (Chakraborty et al., 2010a; Chakraborty et al., 2010b; Ponce et al., 2015). These observations suggest that treatment with Cd may cause EMT in breast cancer cells.

6.1 Regulation of EMT markers by transcription factors

Snail, Twist, and ZEB are transcription factors that regulate the expression of EMT markers. They decrease epithelial markers and increase mesenchymal markers through transcriptional regulation (Batlle et al., 2000; Cano et al., 2000). Snail directly binds to the three E-box regions at CDH1 promoter and functions as a transcription suppressor. It also induces histone modification at the CDH1 promoter through the recruitment of HDACs (Peinado et al., 2004). HDAC further recruits the histone methyltransferase complex to increase histone-specific methylation, thereby activating mesenchymal genes such as those encoding vimentin and N-cadherin (Wu et al., 2011). Similar to Snail, Twist directly binds to E-box regions at CDH1 promoter and suppresses E-cadherin transcription (Vesuna et al., 2009; Vesuna et al., 2008). Twist also enhances the activity of N-cadherin promoter through binding to the E-box cis-element in the first intron of the CDH2. Also, the nuclear accumulation of Twist is dependent on integrin β 1 (Alexander et al., 2006). ZEB cooperates with deacetylase SIRT1 at the CDH1 promoter leading to reduced binding of RNA polymerase II which results in suppression of E-cadherin expression (Byles et al., 2012). There is increasing interest in deciphering epigenetic modifications at the CDH1 and genome-wide epigenetic profiling during EMT have been undertaken (McDonald et al., 2011).

Until now, there is no thorough understanding of the cooperative regulation of different EMT transcription factors. Mostly, they are studied individually. Nevertheless, knockdown of only one transcription factor is sufficient to partially or fully block EMT and metastasis in several cancer cell lines (Murakami et al., 2011).

6.2 Regulation of Snail

Snail plays a critical role in physiologic EMT. Snail knockout mice fail to gastrulate because suppressed EMT process leads to an abnormal mesodermal layer that maintains E-cadherin expression (Sienel et al., 2003). The expression and function of Snail are regulated at transcriptional and post-translational levels (De Craene et al., 2005; Serrano-Gomez et al., 2016). Activated growth factor receptors and Wnt pathway increase Snail transcription, and Wnt/ β -catenin signaling stabilizes Snail subcellular localization to exert its function (Katoh and Katoh, 2006; Medici et al., 2008; Zhou et al., 2004).

Transforming growth factor β (TGF β) is the best-characterized EMT inducer. It binds to TGF β receptors and activates Smads, and crosstalk with MAPK and PI3K pathways (Peinado et al., 2003). In Smad-dependent pathway, activated-Smad complex translocates into the nucleus. High-mobility group A2 (HMGA2) integrates Smads, TGF β , MAPK, and PI3K signals and induces Snail transcription (Shirakihara et al., 2011). Additionally, induction of hyaluronan synthase 2 (HAS2) by TGF β mediates Snail transcription in a Smad-independent manner (Sullivan et al., 2009).

Most of Wnt target genes play important roles in EMT (Vincan and Barker, 2008). Snail transcription is induced by TCF/LEF complex in an axin 2-dependent activation of canonical Wnt pathway (Yook et al., 2006). Also, a kinase in Wnt/ β -catenin pathway, GSK3 β , regulates Snail degradation. GSK3 β phosphorylates Snail at two consensus motifs to dually regulate Snail degradation and export from the nucleus to the cytoplasm. Inhibition of GSK3 β stabilizes Snail in the nucleus where it exerts its function (Zhou et al., 2004).

7. Cell adhesion network

Physical interaction of different cells and ECM components are mediated by cell adhesion molecules at the cell membrane. The interaction exists in various modes, such as coupling of adhesion receptors, convergent signaling or shared signaling pathway. Despite the complexity, cell-ECM and cell-cell interactions together perform a simple role, connecting the internal cytoskeleton to cell exterior and maintaining tissue integrity. This role is carried out mainly by two groups of cell adhesion molecules, cadherin, and integrin (Weber et al., 2011).

7.1 Cell-cell interaction - cadherin

Cell-cell adhesion is critical in maintaining tissue integrity. It supports cell communications through physical contacts. Cadherins are Ca^{2+} dependent glycoproteins, which serve as the primary molecule in cell-cell adhesion, and play a role in tissue differentiation. The most studied are E- and N-cadherins (Takeichi et al., 1990a). In epithelial cells, E-cadherins are abundantly expressed at adherent junctions to hold epithelial sheets together (Takeichi et al., 1990b). The amount of cadherins affects cell-cell adhesion and individual cell movement. Alteration in E-cadherin indicates either tissue differentiation or disease. Chen et al. (2012) demonstrated that loss of E-cadherin promoted colorectal cancer metastasis.

E-Cadherin not only functions as a structural protein at the adhering junction, but is also involved in Wnt/ β -catenin pathway (Thievessen et al., 2003). The cytosol domain of E-cadherin attaches to β -catenin, which is the most important molecule in Wnt/ β -catenin pathway. Disruption of E-cadherin triggers the release of β -catenin.

Increasing nuclear translocation of β -catenin activates Wnt-responsive genes, which foster carcinogenesis (Prange et al., 2003).

7.2 Cell-ECM interaction and role of integrins

Integrins are transmembrane glycoprotein receptors that mediate cell-ECM interaction. They form heterodimers in different combinations of α and β subunits. Integrins mediate anchorage and migration of cells via recognition of variable ECM molecules and other ligands. The cytoplasmic tail of integrin associates with scaffold proteins. At the focal adhesion site, the scaffold proteins talin and vinculin provide a hub for kinases recruitment and linkage to actin filaments (Legate et al., 2009).

The activation of kinases at focal adhesion site is an important downstream signaling of integrin-mediated cell-ECM interaction (Huvneers and Danen, 2009). When integrins encounter ECM or other ligands, focal adhesion kinase (FAK) is autophosphorylated at Tyr 397 site. It provided a binding site for Src and activates it at Tyr 416 site. In turn, Src phosphorylates other tyrosine residues of FAK (Brunton et al., 2005). The FAK/Src complex maximizes the adhesion signaling to induce the assembly of focal adhesion. FAK/Src complex further recruits and phosphorylates other adhesion proteins, like paxillin, and potentially controls Rho/Rac activation, which directly regulates polymerization of microtubules. The spatiotemporal activation of focal adhesion kinase determines cell adhesion, membrane protrusion and further modulates cell motility (Ohta et al., 2006). In addition, activated integrin-signaling crosstalk with mitogenic pathways, stimulating cells growth (Vial et al.,

2003). Cell adhesion, migration, and proliferation are key factors in the formation of secondary tumor at distant organs.

Treatment with low concentrations of Cd stimulated actin filament formation in lung epithelial cells (Go et al., 2013). Choong et al. (2013) described that Cd induced the phosphorylation of FAK (Tyr 397/Tyr 975), and Src (Tyr 416) in rat mesangial cells. These phosphorylation patterns indicate that Cd affects FAK-Src complex. This complex is an important regulatory complex in focal adhesion site, which is downstream of integrin signaling. It is possible that Cd interferes with membrane integrin and alters cytoskeleton for cell migration.

Summary

Many in vitro studies have illustrated how low concentrations of Cd contribute to breast cancer initiation and promotion. Nevertheless, few studies address its effect on cancer progression. Cd may accelerate breast cancer progression to fulfill the role as a carcinogen, which is aligned with our current understanding in cancer biology.

The concept of EMT has emphasized the importance of plastic changes in tumor cell migration. Integrins and ECM interaction further facilitates metastasized cancer cell adhesion and growth. Investigating the effects of Cd on EMT and integrin will help illustrate the role of Cd in cell adhesion and migration. This dissertation reports the results of such an investigation in Chapters 2 and 3.

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CHAPTER 2

EPITHELIAL-MESENCHYMAL TRANSITION IN BREAST EPITHELIAL CELLS UPON TREATMENT WITH CADMIUM

by

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Running Title: Cadmium and breast cancer

Abstract

Epidemiological and experimental studies have implicated cadmium (Cd) in breast cancer development. Cancer initiation and promotion have been described previously. The present study examined cancer progression upon Cd treatment. Specifically, it examined whether Cd treatment causes epithelial-mesenchymal transition (EMT), a hallmark of cancer progression. Four human breast epithelial cell lines, consisting of non-cancerous MCF10A, non-metastatic HCC 1937 and HCC 38, and metastatic MDA-MB-231 were treated with 1 or 3 μ M Cd for 4 weeks. Among these prolonged Cd-treated epithelial cells, MCF10A cells showed different morphology, which was accompanied with the increased expression of E-cadherin and a decreased of N-cadherin at cell borders. Furthermore, the Cd-treated cells exhibited an increase in vimentin, and a decrease in claudin-1 level. These changes in EMT markers indicate that Cd caused EMT. MCF10A and MDA-MB-231 cells labeled with Zcad, a dual sensor for tracking EMT, demonstrated a decrease in the epithelial marker E-cadherin, and an increase in a mesenchymal marker ZEB-1. In order to further explore the mechanism of Cd-induced EMT, Snail, which is a transcription factor involved in regulating EMT, was investigated. The results revealed an increase in Snail level in the nucleus. Inhibition of transcription by actinomycin D eliminated the Cd-induced Snail accumulation. However, inhibition of nuclear export by leptomycin B had an additive effect on Cd-induced Snail expression. Based on these results, it appears that Cd induces EMT in breast epithelial cells by modulating the transcription of Snail.

Keywords: Cadmium, Epithelial-mesenchymal transition, Snail.

Highlights

- Prolonged Cd treatment induces EMT in breast epithelial cells.
- The EMT is associated with accumulation of Snail in the nucleus.
- Cd affects Snail expression and regulates the post-transcriptional activity of

ZEB-1

Introduction

Cadmium (Cd) is an environmental pollutant and a well-established human health risk. Epidemiological and experimental studies have established its carcinogenic potential (Waalkes et al., 1994; Adams et al., 2012). The International Agency for Research on Cancer has categorized Cd as a group 1B carcinogen. Waisberg et al. (2003) summarized studies implicating Cd in breast cancer initiation and promotion. As the second leading cancer in women, most breast cancer deaths are due to tumor progression into advanced stages (Siegel et al., 2016). Therefore, investigating environmental factors that could potentially contribute to breast cancer progression provides insight into breast cancer prevention. The study described herein aims to further investigate the effect of Cd on breast cancer progression.

Progression of a tumor in situ to an invasive phenotype requires an increase in tumor cell plasticity (Park et al., 2010). EMT is a hallmark of increased cell plasticity and has been associated with invasive breast cancer progression (Fuchs et al., 2002). EMT is marked by the loss of epithelial markers such as E-cadherin and claudin-1, and the gain of mesenchymal markers such as N-cadherin, fibronectin and vimentin (Willipinski-Stapelfeldt et al., 2005). Treatment with Cd has been reported to induce

expressions of two mesenchymal markers such as vimentin, and fibronectin in kidneys of mice and in lung epithelial (Chakraborty et al., 2010; Person et al., 2013). Results reported by Ponce et al. (2015) showed that E-cadherin expression was downregulated in MCF7 cells treated with Cd for six months. Benbrahim-Tallaa et al. (2009) reported transformation of the normal breast epithelial MCF10A cells to a basal-like cancer phenotype upon treatment with Cd for 40 weeks. Thus, Cd appears to induce EMT, the mechanism remains to be elucidated.

EMT is a continuous process of epithelial cells transforming into motile mesenchymal cells. There are several transcription factors that regulate the expression of EMT markers. Snail is one such marker and is a zinc-finger transcription factor, it represses E-cadherin expression through binding to the three E-box consensus sequences at CDH1 promoter region (Batlle et al., 2000; Cano et al., 2000; Peinado et al., 2004). Similarly, zinc-finger E-box-binding homeobox (ZEB) binds to E-boxes and suppresses E-cadherin expression through cooperation with co-repressors (Postigo et al., 2003; Sanchez-Tillo et al., 2010). Thus, it is important to assess the effect of Cd on the expressions of Snail and ZEB.

The study described herein investigated the process of EMT in normal and breast cancer cells upon treatment with Cd.

Materials and Methods

Materials

MEGM, DMEM, RPMI 1640, and trypsin-versene were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Atlanta

Biologicals (Flowery Branch, GA). CdCl₂, PMSF, and protease inhibitor were purchased from Sigma-Aldrich (Dallas, TX). Bis-acrylamide powder, supersignal west femto maximum sensitivity substrate, and BCA kit were purchased from Thermo-Fisher Scientific (Pittsburgh, PA). Phosphatase inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Laemmli buffer and blotting buffers were obtained from Bio-Rad (Hercules, CA). Actinomycin D (ACD) and leptomycin B (LMB) was purchased from Cayman Chemical (Ann Arbor, Michigan). Primary antibodies in epithelial-mesenchymal transition kit, lamin A/C, and HRP-conjugated secondary antibody were from Cell Signaling Technology (Danvers, MA). GAPDH primary antibody and slide chamber were purchased from EMD Millipore (Billerica, MA). CF5.5 goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Dallas, TX). IRDye 680 goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Licor (Lincoln, NE). Nuclear Extract Kit was purchased from Active Motif (Carlsbad, CA).

Cell lines and cell culture

MCF10A, MDA-MB-231, HCC 1937 and HCC 38 cell lines were obtained from American Type Culture Collection (Manassas, VA). MCF10A-Zcad and MDA-MB-231-Zcad cells stably transfected with the Zcad sensor were generously provided by Dr. Jeffery Rosen of Baylor College of Medicine. The MCF10A and MCF10A-Zcad cells were cultured in MEGM containing insulin, hydrocortisone, and EGF. MDA-MB-231 and MDA-MB-231-Zcad cells were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 U/mL). HCC 1937 and HCC

38 were cultured in RPMI 1640 supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 U/mL). Cells undergoing prolonged Cd treatment were maintained in corresponding medium for 4 weeks. The cells were maintained in a humidified incubator (37°C) with 5% CO₂. Cell morphology was observed under a Nikon phase contrast microscope.

Immuno-fluoresce staining

Control and Cd-treated MCF10A cells were seeded in slide chambers in MEGM. For specimen preparation, cells were fixed by treatment with 4% paraformaldehyde in PBS for 15 min followed by permeation with 0.3% Triton X-100 in 1% BSA-supplemented PBS. Cells were then blocked in 5% BSA-supplemented PBS. Finally, cells were incubated with primary antibody for E- or N-cadherin overnight. After washing with PBS for 5 min, three times, cells were stained using secondary antibody CF5.5 goat anti-rabbit IgG. Nuclei were stained with DAPI. Images of the stained cells were taken with a Nikon NE2000 fluorescence microscope and processed using NIS Elements acquisition software.

Evaluation of Zcad expression

MCF10A-Zcad and MDA-MB-231-Zcad cells were seeded in 6-well plates and cultured in MEGM or DMEM, respectively. Evaluation of E-cadherin expression and ZEB-1 activity in Cd- or TGFβ-treated Zcad cells was performed using a BD FACSVerse. The intensities of RFP and GFP were analyzed in FITC and PE channels,

respectively. Quantification of cells expressing E-cadherin or ZEB-1 was performed by measuring the relative change in RFP or GFP intensities.

Western blot

Whole cell lysates were prepared in RIPA buffer. Nuclear and cytoplasmic fractions were prepared using an Active Motif nuclear extraction kit. Protein concentration in cell lysates was determined using the BCA kit. Proteins were denatured by heating the lysate to 95°C in Laemmli buffer containing 5% β -mercaptoethanol. Proteins in the samples were then separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with 5% non-fat milk for 1 h followed by incubation with the primary antibody overnight. The membranes were rinsed in PBS containing 0.5% Tween 20, for 5 min, three times, and incubated with the IRDye or HRP-conjugated secondary antibody for 1 h. Following incubation, the membranes received three washings in PBS containing 0.5% Tween 20 and scanned in a Licor Odyssey Imager. The optical densities of protein bands were quantified by Odyssey Image Analysis Software.

Data analysis

All experiments were repeated at least three times. One-way ANOVA was performed by Prism with Tukey's post hoc test, and $p < 0.05$ was considered statistically significant.

Results

Cell morphology and expressions of cadherins

Treatment with 1 μ M Cd for 4 weeks transformed the morphology of normal breast epithelial cells (Fig. 1). The untreated MCF10A cells displayed regular epithelial sheets, indicating tight cell-cell adhesion. In contrast, cells treated with Cd appeared spindle-like and had reduced intercellular adhesion.

Intracellular adhesion is mediated through cadherins. Thus, cells were stained for E- and N-cadherin, respectively. The immunofluorescence pattern indicated the expression and location of cadherins. As shown in Fig. 2, the normal MCF10A cells displayed strong E-cadherin, but faint and diffuse N-cadherin expression in the cytoplasm. Treatment with 1 μ M Cd for 4 weeks significantly changed the fluorescence pattern of cadherins. In most Cd-treated cells, less E-cadherin (epithelial marker) was visible, but N-cadherin (mesenchymal marker) was apparent at well-defined cell borders. Overall, these morphological and phenotypical changes indicated that prolonged Cd treatment transformed the breast epithelial MCF10A cells to a more mesenchymal-like phenotype.

Expressions of EMT markers

To further examine the EMT, four human breast epithelial cell lines, consisting of normal MCF10A, non-metastatic HCC 38 and HCC 1937, and metastatic MDA-MB-231 were treated with 1 or 3 μ M Cd for 4 weeks. As shown in Fig. 3, in MCF10A cells, treatment with 1 μ M Cd reduced the expression of E-cadherin 84% and increased the expression of N-cadherin and vimentin 1.6- and 2.6-fold, respectively. Although MDA-MB-231 cells expressed very low level of both E- and N-cadherin

(Blick et al., 2008), their expression of vimentin was upregulated 5.1-fold (Fig 3). In HCC 1937 cells, treatment with 3 μ M Cd decreased E-cadherin and claudin-1 expression 55 and 63%, respectively. The HCC 38 cells expressed high basal levels of E- and N-cadherins. In these cells, the Cd-induced reduction in E-cadherin was not statistically significant, but claudin-1 level was reduced 59%, and the expression of N-cadherin was enhanced 2.7-fold over the control cells. Thus, in all four types of breast epithelial cells, generally, prolonged Cd treatment caused an increase in mesenchymal markers and a decrease in epithelial markers, further confirming its effect on EMT.

Further confirmation of EMT by Zcad sensor

Transition of epithelial to mesenchymal-like phenotype is a gradual process. Thus, to track the change in expressions of E-cadherin and its transcription suppressor ZEB-1, the Zcad sensor was utilized. The Zcad sensor is comprised of an Ecad-RFP plasmid containing RFP driven by the CDH1 promoter, and the ZEB-GFP plasmid containing destabilized GFP indicates ZEB-1 translation (Toneff et al., 2016). Therefore, RFP intensity measures E-cadherin expression, and GFP intensity indicates ZEB-1 expression. Stably transfected MCF10A and MDA-MB-231 cells were treated with Cd or TGF β for up to 4 weeks. As expected, the TGF β -treated MCF10A-Zcad cells expressed less E-cadherin as indicated by a marked reduction (45%) in RFP intensity in UL area than the control cells after 4 weeks of treatment. Similar, but less pronounced effect was observed in the 1 μ M Cd-treated cells which was a 13% decrease in RFP intensity in UL area (Fig. 4A). The MDA-MB-231-Zcad cells which had higher ZEB-1 activity, showed strong GFP intensity. As shown in Fig. 4B,

treatment with 3 μ M Cd for 4 weeks caused a 1.7-fold increase in GFP intensity (LR area) in the MDA-MB-231-Zcad cells. The positive control, TGF β caused a 3.7-fold increase. The increase in GFP expression in Zcad cells indicated enhanced translation of ZEB-1. ZEB-1 is a direct E-cadherin transcription suppressor (Sanchez-Tillo et al., 2010). Enhanced ZEB-1 activity predicts a more mesenchymal-like property, and indicates that Cd treatment suppress the transcription of E-cadherin.

Expression of transcription factor Snail

Snail primarily functions as an EMT regulator (Peinado et al., 2007). When HCC 1937, HCC 38, and MDA-MB-231 cells were treated with 3 μ M Cd for 4 weeks, the Snail levels were increased 3.5-, 3.4-, and 2.1-fold in these cells, respectively (Fig. 5).

Snail is formed in cytosol and imported to nucleus to exert its function. Therefore, the distribution of Snail in these compartments following Cd treatment was analyzed in the HCC 1937 and HCC 38 cells after treatment with 3 μ M for 4 weeks. As shown in Fig. 6, the Snail expression was barely detectable in the cytosol. However, in the nuclear fraction of HCC 1937 and HCC 38 cells, there was a 2.5- and 3.4-fold increase, respectively, in Snail levels as compared to the control cells. Therefore, Snail accumulation in the nucleus indicates that the breast cancer cells undergo Snail-mediated EMT upon prolonged Cd treatment.

Time-course of Snail expression

The HCC 1937 and HCC 38 cells were treated with 1 μ M Cd for up to 8 h and Snail levels were determined in the whole cell extract. As shown in Fig. 7A, the Snail

expression was gradually upregulated to 3-fold during 8 h of treatment with 1 μ M Cd. To evaluate whether the upregulation was due to increased transcription, ACD, a transcription inhibitor, was used as a co-treatment with 1 μ M Cd for 8 h. Treatment with TGF β served as positive control. In the absence of ACD, Cd and TGF β treatments resulted in a 2.1- and 2.4-fold increase in Snail expression, respectively (Fig. 7B). Co-treatment with ACD completely abolished this effect. This finding indicates that Snail accumulation in the nucleus could be through the increase in transcription.

The half-life of Snail is 30 min. Snail is exported from the nucleus into the cytoplasm where it is degraded by proteasomes (Zhou et al., 2004). Therefore, Snail accumulation in the nucleus could also be due to inhibition of nuclear export. To further evaluate this possibility, the HCC 1937 cells were treated with Cd and LMB, a nuclear export inhibitor. As shown in Fig. 8, while Cd and LMB treatments individually resulted in a 2.7- and 2.5-fold Snail expression, respectively. The co-treatment had an additive effect in Snail expression. This finding further indicates that Cd regulates the expression of Snail in a transcriptional-related manner, and not by inhibition of its nuclear export.

Discussion

Prozialeck's group reported that Cd reduced E-cadherin expression at cell membrane in kidney tubular cells, causing cell-cell dissemination (Prozialeck and Niewenhuis, 1991; Prozialeck and Lamar, 1997; Zimmerhackl et al., 1998; Prozialeck, 2000; Prozialeck et al., 2003). The loss of E-cadherin expression in breast and prostate

carcinomas is correlated with tumor aggressiveness and metastasis (Umbas et al., 1992; Morita et al., 1999; Kowalski et al., 2003; Jeschke et al., 2007). In the present study, E-cadherin level was suppressed upon prolonged Cd treatment in breast epithelial cells. Such alteration was attributed to EMT based on the following observations.

The primary key finding in the present study is that prolonged treatment of Cd changes the expression of epithelial and mesenchymal markers E- and N-cadherins, vimentin, and claudin-1. These markers are hallmarks of EMT (De Craene and Berx, 2013). The initiation or enhancement of EMT was not restricted to the stage of cancer cells and occurred in normal MCF10A cells, non-metastatic HCC 1937 and HCC 38 cells, and metastatic MDA-MB-231 cells (Fig. 3). Furthermore, In Zcad transfected cells, expressions of RFP and GFP represented expression of E-cadherin and ZEB-1, respectively. MCF10A-Zcad and MDA-MB-231-Zcad cells exhibited a decrease in RFP and increase in GFP intensities upon Cd treatment, indicating suppression of E-cadherin transcription and active ZEB-1 translation (Fig. 4).

The use of Zcad cells also revealed related mechanism in Cd-induced EMT. Zcad is a dual fluorescence sensor which consists of ZEB-GFP and Ecad-RFP plasmids. According to the construct sequence provided by Toneff et al. (2016), ZEB-GFP plasmid is designed based on the knowledge that ZEB is suppressed by the miR-200 family (Burk et al., 2008; Korpál et al., 2008). This plasmid contains eight miR-200 binding sites in ZEB-1 3' UTR region which is downstream of UbC and GFP. Thus, constitutive GFP expression indicates less binding of miR-200 in ZEB-1 3' UTR region, suggesting active ZEB-1 translation. In the present study, the increase in GFP

intensity in MDA-MB-231-Zcad cells suggested that less miR-200 was bound to ZEB-1 3' UTR region upon 4 weeks of Cd treatment. It is possible that Cd inhibits miR-200 that post-transcriptionally suppresses ZEB-1 translation, as speculated in Fig. 9. ZEB-1 is a transcription suppressor of E-cadherin (Gregory et al., 2008; Sanchez-Tillo et al., 2010). Therefore, prolonged Cd treatment appears to stabilize GFP (Fig. 4B), suggesting a potential mechanism of regulation of EMT in a post-transcriptional manner.

Beside the ZEB-GFP plasmid, the Ecad-RFP plasmid measures transcription activity at CDH1 promoter region. The decrease in RFP intensity indicated that Cd suppressed E-cadherin transcription (Fig. 4A). Snail represses CDH1 transcription through binding to the E-box in the promoter region of CDH1 (Batlle et al., 2000; Cano et al., 2000). The observation that Snail accumulated in the nucleus upon Cd treatment (Fig. 6) was consistent with the results showing reduced RFP in MCF10A-Zcad cells (Fig. 4A). It appears that Cd treatment affects the expression of transcription factors that regulate EMT.

The secondary key finding is that Cd increased the expression of Snail. Prolonged Cd treatment resulted in Snail accumulation in the nucleus, which explained the EMT upon 4 weeks of Cd treatment (Fig. 5 and Fig. 6). Also, treatment of cells with 1 μ M Cd for 4 - 8 h resulted in an increase in Snail expression (Fig. 7A), which was abolished by inhibition of transcription by ACD (Fig. 7B). These results suggest that transcriptional activity is crucial for Cd-induced Snail expression. Cd and nuclear export inhibitor LMB additively increased Snail expression (Fig. 8). LMB prevented Snail export from the nucleus and resulted in Snail accumulation. Cd-induced Snail

transcription and not inhibition of its export is likely the reason of additive effect upon co-treatment with LMB.

TGF β and receptor tyrosine kinase signaling pathways crosstalk and converge to regulate the transcription of Snail (Yook et al., 2005; Horiguchi et al., 2012). To maintain the homeostasis of Snail expression, GSK3 β phosphorylates Snail and prepares it for degradation by proteasomes (Zhou et al., 2004). Baroni et al. (2015) reported that treatment of bronchial epithelial cells with Cd affected TGF β pathway. Based on this report, it is possible that Cd upregulates Snail transcription through the TGF β pathway. Cd treatment activates Wnt/ β -catenin pathway and inhibits GSK3 β in metastasized breast cancer cells (unpublished data). Thus, it is possible that Cd could also decrease Snail degradation.

The progression of breast cancer to a more advanced stage involves the transition of hyperplastic epithelial tissue toward a metastatic carcinoma. EMT is a critical event in this process (Thiery, 2002). The biochemical changes that occur in EMT render the tumor cells more invasive phenotypes, which help the cells to migrate through the basement membrane into the circulation to distant tissues and organs (Trimboli et al., 2008). The study reported here is the first showing induction of EMT in normal and breast cancer cells upon Cd treatment. Besides the use of non-cancerous breast epithelial cell line MCF10A which is a well-accepted model to study EMT (Sarrio et al., 2008), the results in non-metastatic and metastatic cancer cell lines indicate that Cd not only induces, but also enhances EMT.

Although the detailed mechanism of Cd-induced Snail expression remains to be further elucidated, based on the results of this study, it is concluded that Cd treatment

upregulates Snail expression, substantially transforming epithelium-derived breast cancer cells to a more mesenchymal-like phenotype. The present study also suggests that Cd affects transcription of Snail and post-transcriptional activity of ZEB-1, which are two well-known biochemical mechanisms in the regulation of EMT.

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Figures

Fig. 1. Changes in morphology of MCF10A cells upon prolonged Cd treatment.

The cells were treated with 1 μ M Cd in MEGM for 4 weeks. Control cells were cultured in MEGM alone. Phase contrast images of the cultured cells were taken under 4X and 10X objective lenses.

Fig. 2. Expression of cadherins in MCF10A cells upon prolonged Cd treatment. The cells were treated with 1 μ M Cd in MEGM for 4 weeks. Control cells were cultured in MEGM. The cells were fixed and stained to visualize the abundance of E-cadherin and N-cadherin. Nuclei were stained with DAPI. The immuno-fluorescence images were taken under a 20X objective lens. Relative intensity of red fluorescence indicated cadherin levels.

MCF10A

MDA-MB-231

HCC 1937

HCC 38

Fig. 3. Expressions of EMT markers upon prolonged Cd treatment.

MCF10A cells were treated with 1 μ M Cd for 4 weeks in MEGM. MDA-MB-231,

HCC 1937, and HCC 38 cells were treated with 3 μ M Cd for 4 weeks in DMEM or

RPMI 1640 supplemented with 10 % FBS. Representative Western blots are shown.

Densities relative to the control group are plotted as mean $\pm$ SEM (n=3). *Significantly different from the respective control group ($p < 0.05$).

(A)

(B)

Fig. 4. Time-course of EMT upon prolonged Cd treatment.

(A) E-cadherin expression in MCF10A-Zcad cells. The cells were seeded in MEGM and treated with MEGM containing 1 μ M Cd or 10 ng/ml TGF β for up to 4 weeks. Cells were harvested every week and analyzed by flow cytometry. Representative dot plots of the distribution of cells expressing RFP are shown. Cells expressing E-cadherin (RFP) were in the upper left (UL) quadrant. (B) ZEB-1 activity in MDA-MB-231-Zcad cells. The cells were seeded in DMEM supplemented with 10% FBS and treated with 3 μ M Cd or 10 ng/ml TGF β for up to 4 weeks. Cells were harvested every week and analyzed by flow cytometry. Representative dot plots of the distribution of cells expressing ZEB-1 (GFP) are shown. Cells expressing ZEB-1 are in the lower right (LR) quadrant. Data from three independent experiments are plotted as mean $\pm$ SEM (n=3). *Significantly different from the control group at the same time point ($p < 0.05$).

Fig. 5. Expression of Snail upon prolonged Cd treatment.

HCC 1937, HCC 38, and MDA-MB-231 cells were treated with 3 μ M for 4 weeks in RPMI 1640 or DMEM supplemented with 10 % FBS. Representative Western blots of the expression of Snail are shown. Densities relative to the control group are plotted as mean $\pm$ SEM (n=3). *Significantly higher than the respective control group (p < 0.05).

Fig. 6. Intracellular distribution of Snail upon prolonged Cd treatment.

HCC 1937 and HCC 38 cells were treated with 3 μ M Cd for 4 weeks in RPMI 1640 or DMEM supplemented with 10 % FBS. Representative Western blots of the expression of Snail in whole cell lysate, cytosolic, and nuclear fractions are shown. Densities relative to the control group are plotted as mean $\pm$ SEM (n=3). *Significantly higher than the respective control group (p < 0.05).

Fig. 7. Time-course of Snail expression.

(A) Time-course of Snail expression in HCC 1937 cells. The cells were treated with 1 μ M Cd in the RPMI 1640 for up to 8 h and cell lysates were analyzed for Snail expression. Representative Western blot of Snail expression is shown. Densities relative to the control group are plotted as mean $\pm$ SEM (n=3). (B) Inhibition of Snail transcription in HCC 1937 cells. The cells were treated with 1 μ M Cd or 10 ng/ml TGF β in the absence or presence of 10 nM ACD for 8 h. Representative Western blot of Snail expression is shown. Densities relative to the control group are plotted as mean $\pm$ SEM (n=3). *Significantly higher than the control group (p < 0.05).

[#]Significantly higher than the respective ACD treated group (p < 0.05).

Fig. 8 Inhibition of nuclear Snail transport by LMB.

HCC 1937 cells were treated with 1 μ M Cd in the presence or absence of 20 ng/ml LMB for 1, 4, or 8 h. Representative Western blots of Snail expression are shown. Densities relative to the respective control group at 1 h are plotted as mean $\pm$ SEM (n=3). *Significantly different from the control group (p < 0.05).

Fig. 9 Schematic diagram summarizing the effect of Cd treatment on the regulation of EMT.

(A) Cd treatment increases the expression of Snail, resulting in suppression of E-cadherin expression. Decrease in RFP indicates suppression of E-cadherin transcription, which is in concord with the upregulation of Snail.

(B) Increase in GFP indicates less miR-200 binding. Cd treatment may suppress miR-200 expression or inhibit miR-200 binding in ZEB-1 3'UTR region.

CHAPTER 3

CADMIUM ENHANCES METASTASIS-ASSOCIATED PHENOTYPES IN BREAST CANCER CELLS THROUGH INTEGRIN AND β -CATENIN SIGNALING

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Running Title: Cadmium and breast cancer

Abstract

Cadmium (Cd) is a carcinogenic heavy metal which is implicated in breast cancer development. While the mechanisms of Cd-induced breast cancer initiation and promotion have been well studied, but the molecular processes involved in breast cancer progression are not. The purpose of the present study was to evaluate the influence of Cd on metastasis-associated phenotypes, such as cell adhesion, migration, and invasion by using MDA-MB-231 cells. Treatment with 1 or 3 μM Cd for 1-6 h increased cell spreading and migration. This observation was found to be associated with integrin signaling since the activation of integrin $\beta 1$, FAK, Src, and Rac1. The treatment with 1 or 3 μM Cd also inhibited GSK3 β activity and induced T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription, indicating the involvement of β -catenin signaling. Furthermore, treatment with 3 μM Cd for 4 weeks increased the expression of β -catenin and enhanced TCF/LEF transcription. Also, the enhanced expressions of integrin $\alpha 5$ and $\beta 1$, paxillin, and vimentin indicated that prolonged Cd treatment reorganized the cytoskeleton, which aided malignancy, as evidenced by enhanced matrix metalloproteinase 2/9 (MMP2/9) secretion and cell invasion. In addition, prolonged Cd treatment caused an increase in cell growth rate. Together, these results indicate that Cd alters key signaling processes involved in the regulation of cytoskeleton to enhance cancer cell migration, invasion, adhesion, and proliferation.

Key words: Cadmium, metastasis, integrin, β -catenin

Highlights

- Cd treatment enhances cell adhesion and spreading in breast cancer cells through integrin activation.
- Inhibition of GSK3 β by Cd leads to activation of Wnt/ β -catenin pathway.
- Accumulation of β -catenin facilitates TCF/LEF transcription.
- Acquisitions of metastasis-associated phenotypes increases cell migration, invasion, and proliferation.

Introduction

Cadmium (Cd) is a toxic heavy metal and exposure to this metal has been associated with development in lung, kidney, prostate, and breast cancer (Stayner et al., 1992; Il'yasova and Schwartz, 2005; Sahmoun et al., 2005; Julin et al., 2012a; Julin et al., 2012b). Studies in mice and rats have confirmed that Cd treatment results in the development of lung, breast, and bladder cancer (Takenaka et al., 1983; Shiraishi et al., 1994; Johnson et al., 2003; Schrauzer, 2008; Davis et al., 2013). The International Agency for Research on Cancer has categorized Cd as a Group 1B carcinogen. Carcinogenesis is a continuous process comprised of initiation, promotion, and progression. Promotion of cell growth by Cd treatment is described as a xenoestrogenic effect (Siewit et al., 2010; Ponce et al., 2013; Song et al., 2015), involving membrane receptors (Yu et al., 2010; Wei et al., 2015).

A number of studies indicate that Cd initiates and promotes breast cancer development, with some suggesting that it also promotes breast cancer metastasis (Yang et al., 2004; Benbrahim-Tallaa et al., 2009; Siewit et al., 2010). Waalkes et al.

(2000) reported that chronic Cd exposure increased the metastatic potential of subcutaneously-injected sarcomas in rats. Metastasis is a multi-step process that includes dissemination, migration, intravasation, extravasation, and colonization to form secondary tumors. Specific mechanisms underlying these elements upon Cd treatment are poorly understood. Results reported by Ponce et al. (2015) suggested that Cd interfered with cell-cell interaction by disrupting E-cadherin, enhancing the ability of breast cancer cells to disseminate from the primary tumor.

After disseminating from primary tumors, metastatic breast cancer cells enter a prolonged period of latency before forming secondary tumors. The rate-limiting steps seem to be extravasation and cell proliferation (Luzzi et al., 1998). During extravasation, metastatic cancer cells migrate through endothelial barriers and settle in new microenvironments via cell adhesion molecules (Felding-Habermann et al., 2001). An important feature in metastatic cell movement is alteration of cellular microfilament dynamics and extracellular matrix (ECM) remodeling. Integrin adheres to ECM proteins and alters cytoskeletal dynamics, leading to cell adhesion and migration. FAK-Src complex transduces integrin signaling to downstream Rac/Rho, which initiates assembly of microfilaments (Mitra and Schlaepfer, 2006). Crosstalk with PI3K and MAPK promotes cell survival and proliferation (Giancotti and Ruoslahti, 1999). In lung epithelial cells, 0.5 and 1 μ M Cd stimulated actin filament formation (Go et al., 2013). Baroni et al. (2015) reported that 10 μ M Cd increased integrin β 1 and β 5 gene expression and directly affected the production of ECM proteins in human bronchial epithelial cells. Accordingly, it seems reasonable to

hypothesize that Cd affects metastasized breast cancer cell adhesion through alterations in integrin activation.

Colonization at metastasized sites requires cells to proliferate to counterbalance cell death. Activation of the Wnt/ β -catenin pathway, that normally regulates cell proliferation during embryonic development, has been implicated in tumorigenesis in colon cancer, leukemia, and breast cancer (Reya and Clevers, 2005). The Wnt pathway is initiated after Wnt ligands bind to Frizzled and Lrp 5/6 receptors. Receptor occupancy leads to accumulation of an important downstream signaling protein, β -catenin. As a consequence of GSK3 β -mediated phosphorylation, β -catenin translocates into the nucleus and forms co-transcriptional activators with TCF/LEF, driving transcription of many oncogenes (Eastman and Grosschedl, 1999). Mice exposed to Cd increased release of Wnt ligand and transcription of Wnt receptors (Chakraborty et al., 2010b). The same group (Chakraborty et al., 2010a) reported that Cd enhanced nuclear translocation of β -catenin, which triggered TCF/LEF-mediated transcription in human kidney tubular cells. In addition, Cd increased free β -catenin accumulation through disruption of cadherins (Edwards et al., 2013). Based on these studies conducted in mouse or human kidney cells, Cd may stimulate secondary tumor growth through activation of Wnt/ β -catenin-dependent pathways.

In summary, cellular migration and metastasis involve increased ECM interaction, activated adhesion-related signaling, crosstalk with β -catenin and mitogenic signaling. Thus, the present study focuses on these processes to test the hypothesis that Cd exerts carcinogenic effects on breast cancer progression.

Materials and Methods

Materials

DMEM and trypsin-versene were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). CdCl₂, PMSF and protease inhibitor were purchased from Sigma-Aldrich (Dallas, TX). Bis-acrylamide powder, BCA kit and extracellular matrix components were purchased from Thermo-Fisher Scientific (Pittsburgh, PA). Phosphatase inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Gelatin-zymogram gel, Laemmli buffer and blotting buffers were obtained from Bio-Rad (Hercules, CA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Cayman Chemical (Ann Arbor, Michigan). Active-integrin β 1, GAPDH antibodies, Amicon centrifugal filter units (NMWL 10), slide chambers and Transwell inserts were purchased from EMD Millipore (Billerica, MA). Phosphor Rac1, α -actinin, phosphor-ERK 1/2 (Thr 202/Tyr 204), ERK, phosphor-Src (Tyr 416), Src, phosphor-FAK (Tyr 397) and FAK primary antibodies were from Cell Signaling Technology (Danvers, MA). CF5.5 goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Dallas, TX). IRDye 680 goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Licor (Lincoln, NE). TOPFlash and pRL-TK plasmids were purchased from Addgene (Cambridge, MA). Nuclear Extract Kit was purchased from Active Motif (Carlsbad, CA). Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Rockville, MD). Cultrex 3-D spheroid cell invasion assay kit was obtained from Trevigen (Gaithersburg, MD). E-PlateView 16-well plates were purchased from

ACEA Biosciences (San Diego, CA). Dual-luciferase assay kit was from Promega (Madison, WI).

Cell lines and cell culture

Breast cancer derived MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 U/mL). Cells were maintained in a humidified incubator (37°C) with 5% CO₂.

Wound healing assay

The wound healing assay was performed in a 12-well plate. MDA-MB-231 cells were seeded in three replicates and cultured in DMEM with 10 % FBS. Wounds were generated by scratching the 90% confluent cell monolayer with a 1-20 µl pipet tip. The unattached cells were washed away with PBS and attached cells were treated with 0.5-1 µM Cd in 10 % FBS-supplemented DMEM. Images of the wounds were acquired every 24 h under phase contrast microscopy.

Electrical impedance assay

MDA-MB-231 cells were seeded at a density of 8,000 cells/well in E-PlateView 16 plates and cultured in DMEM with 10% FBS. The bottom of the plate was covered with gold microelectrode sensors that generated an electric field when a low voltage (less than 20 mV) was applied between electrodes. Cells in the logarithmic growth phase were treated with DMEM containing 1 or 3 µM Cd and adhesion was monitored

by using xCELLigence RTCA system at a sweeping interval of 15 s. Results were profiled and plotted by using the RTCA software.

Immuno-fluoresce staining

MDA-MB-231 cells were seeded in slide chambers and grown in DMEM with 10 % FBS. After 24 h, the cells were treated with 1 or 3 μ M Cd in DMEM for 3 h. For specimen preparation, cells were fixed by treatment with 4% paraformaldehyde in PBS for 15 min followed by permeation with 0.3% Triton X-100 in 1% BSA-supplemented PBS. Cells were then blocked in 5% BSA-supplemented PBS. Finally, the cells were incubated with primary antibody for α -actinin overnight. After washing with PBS for 5 min, three times, the cells were stained using secondary antibody, goat anti-rabbit IgG. Nuclei were stained with DAPI. Images of the stained cells were captured with a Nikon NE2000 fluorescence microscope.

Western blot analysis

Whole cell lysates were prepared in RIPA buffer. Nuclear and cytoplasmic fraction samples were prepared using an Active Motif nuclear extraction kit. Protein concentration in lysates was determined using the BCA kit. Proteins were denatured by heating the lysate to 95°C in Laemmli buffer containing 5% β -mercaptoethanol. Samples requiring non-reduced proteins were prepared in Laemmli buffer without β -mercaptoethanol. Proteins in the samples were then separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with 5% non-fat milk for 1 h followed by incubation with the primary

antibody overnight. The membranes were rinsed for 5 min three times in PBS containing 0.5% Tween 20, and incubated with secondary antibody for 1 h, followed by three additional washings in PBS containing 0.5% Tween 20, before imaging. The membranes were scanned by an Odyssey Infrared Imager. The optical density of the bands was quantified by the Odyssey image analysis software.

Luciferase reporter assay

MDA-MB-231 cells were seeded in 96-well plates and cultured in DMEM with 10% FBS. After 24 h, the cells were transfected with pRL-TK (2 ng/well) and TOPFlash (50 ng/well) plasmids for 48 h. The cells were serum starved by incubating in blank medium overnight, prior to treatment with Cd for 8 h. Firefly and renilla luciferase activities were generated by using Promega Dual-Luciferase assay and measured in a Glomax luminometer. Relative luciferase activity was quantified as the ratio of firefly to renilla luciferase activities.

Transwell assay

Transwell compartments (8 μ m pore size) were prepared and inserted in a 24-well plate. The membranes were coated with 2 μ g/ml collagen, 2 μ g/ml fibronectin, 8 μ g/ml vitronectin, and 8 μ g/ml laminin. Cells were seeded into upper chambers in DMEM supplemented with 10% FBS. After a 48 h incubation, cells in the upper chamber were carefully removed with a cotton swab. The cells remaining on the underside of the membranes were fixed in 70% ethanol for 10 min and then stained with 0.5% crystal violet solution. Membranes with stained cells were imaged using an

EVOS microscope at scan mode. To quantify the number of cells that migrated to the lower chamber, crystal violet stained cells were de-stained in 20% methanol. Migrated cell numbers in control and Cd-treated groups were determined by the absorbance of de-staining solutions at 450 nm in a Spectromax microplate reader. The relative migration tendency of Cd-treated cells was quantified as the ratio of absorbance reading by the treated cells over that of the control cells.

Gel zymography analysis

Precast gelatin-zymogram SDS-polyacrylamide gels were co-polymerized with gelatin. Media from cultured cells were concentrated 10 fold by centrifuging in Amicon filter units at 4,000 g for 30 min before adding to non-denaturing loading buffer solution. Protein concentration of all samples was determined and normalized to the same level before loading the samples onto gels. Electrophoresis of gelatin-zymogram gels was performed at 100V for 1 h. Gels were washed and incubated in renaturing buffer for 40 min and incubated in developing buffer overnight at 37°C. Then gels were stained with 0.1% coomassie R-250 brilliant blue for 1 h and washed for 30 min until clear bands developed. Gelatinolytic activity (relative intensity of clear bands) of secreted MMP 9 and MMP 2 was quantified by densitometry analysis using the Odyssey image analysis software.

Cell proliferation assay

Untreated and 4-week Cd-treated MDA-MB-231 cells were harvested and seeded in a 96-well plate at a density of 10,000 cells/well in DMEM supplemented with 10 %

FBS. After 4 days, cell growth was measured by Cell-Counting Kit 8. Absorbance was read at 450 nm and relative cell growth of Cd-treated cells was compared to untreated control cells.

3D cell culture

A spheroid invasion assay was used to estimate cell invasiveness. The experiment was performed by using a Cultrex 3-D spheroid cell invasion assay kit. Control and 4-week Cd-treated cells were harvested and seeded at a density of 5,000 cells/well in low-adhesive round-bottom 96-well plates. The cells were cultured in DMEM supplemented with 10 % FBS and basal membrane extract. After 4 days, the cells assembled into a compact spheroid. At that point, matrigel was added to each well to provide a solidified invasion matrix. The growth of spheroids was observed microscopically and imaged daily.

Data analysis

All experiments were repeated at least three times. Western blot and zymography data were analyzed and quantified by Odyssey imaging analysis software. One-way ANOVA was performed by Prism with Tukey's post hoc test on all data and $p < 0.05$ was considered statistically significant.

Results

Effects of Cd treatment on cytoskeletal dynamics

In the wound healing assay, the scratches in 0.5 and 1 μM Cd treatment groups were healed by 48 h, while scratches in control group remained. These results

indicated that Cd induced MDA-MB-231 cell migration without producing visible cytotoxic effects (Fig. 1A).

A real-time electrical impedance assay was used to track cell spreading. Adding 1 or 3 μ M Cd to attached cells caused an increase in the cell spreading index at both Cd concentrations and peaked around 4 h. (Fig. 1B). Cell migration and spreading was also assessed by using a dynamic cytoskeletal construction indicator, α -actinin, which cross-links actin filaments (Otey et al., 1990). Cells treated with Cd exhibited longitudinal filamentous α -actinin staining in 3 h (Fig. 1C). Its spatial distribution in filopodia indicated that Cd stimulated the buildup of cytoskeletal proteins for cell spreading and subsequently cell migration.

Effects of Cd treatment on activation of integrin and downstream signaling

Upon stimulation, integrin transduces adhesion signaling to focal adhesion kinases, which is essential in assembly of actin filaments (Mitra and Schlaepfer, 2006). Effects of Cd treatment on integrin activation and downstream kinases were examined by Western blot. As shown in Fig. 2, treatment with 1 or 3 μ M Cd or 100 nM PMA (positive control) significantly increased active integrin β 1 expression 2-fold in MDA-MB-231 cells. Cd-treated cells displayed a 1.4 - 2.3-fold increase in phosphorylation of FAK (Tyr 397) and Src (Tyr 416), which indicated activation of kinases at focal adhesion sites. Rac1 is a downstream target of focal adhesion kinase, and is involved with regulation of actin polymerization (Vial et al., 2003). Here, 1 or 3 μ M Cd elevated Rac1 phosphorylation 2.3-fold, which was associated with the appearance of filamentous α -actinin (Fig. 1C). Additionally, ERK 1/2 signaling was

activated by Cd treatment, presumably because of integrin pathway crosstalk with MAPK signaling (Vial et al., 2003). The activation of integrin itself and downstream signal transduction indicated that integrin was involved in Cd-induced cell adhesion.

Effects of Cd treatment on β -catenin signaling and expression

To examine activation of the Wnt/ β -catenin pathway in Cd-treated breast cancer cells, the MDA-MB-231 cells were transfected with TOPflash plasmids which contained repeated TCF/LEF sites upstream of the luciferase reporter. As shown in Fig. 3A, treatment with 3 μ M Cd increased luciferase activity in transfected cells 1.8-fold, suggesting increased TCF/LEF transcription. This was further confirmed by the observation that Cd treatment enhanced phosphorylation of GSK3 β (Ser 9) 1.5-fold, which led to GSK3 β inhibition (Fig. 3B). Thus, the Wnt/ β -catenin pathway was activated by Cd treatment.

The distribution of β -catenin in cellular fractions was determined in MDA-MB-231 cells treated with 3 μ M Cd for 4 week. Western blot analysis showed that prolonged Cd treatment led to a 2.9-fold increase in β -catenin level in the whole lysate, a 1.9-fold increase in the cytosolic fraction, and a 1.9-fold increase in the nuclear fraction. Increasing β -catenin was accumulated in both the cytosol and the nucleus (Fig. 4A). Accumulation of β -catenin generated a larger pool of co-activator for TCF/LEF transcription. As shown in Fig. 4B, the luciferase activity in 4-week Cd-treated cells was higher than 4-week untreated cells, indicating basal TCF/LEF transcription activity was elevated after the 4-week Cd treatment. Under the same treatment with 1 μ M Cd, 4-week Cd-treated cells further increased TCF/LEF

transcription activity 2.8-fold over 4-week untreated cells. Therefore, the additional activation of TCF/LEF transcription in the 4-week Cd-treated cells seems to follow the increase in β -catenin expression.

Effects of prolonged Cd treatment on cytoskeleton reorganization

To study how prolonged Cd treatment affects the cytoskeleton, MDA-MB-231 cells were treated with 3 μ M Cd for 4 weeks. The Cd-treated cells stained with vimentin exhibited different spatial distribution of intermediate filaments when compared to control cells (Fig. 5A). The expression of not only vimentin but also integrins $\alpha 5$ and $\beta 1$, and paxillin were increased by 5.3-, 2.1-, 6.3-, and 3-fold, respectively (Fig. 5B). The increases in intermediate filaments, integrins, and scaffold proteins indicated that prolonged Cd treatment re-organized the cytoskeleton.

Effects of prolonged Cd treatment on metastatic potential

Transwell assay and MMP secretion were used to assess cell migration and invasion. To evaluate the effect of Cd on cell proliferation, cell growth was compared between Cd-treated cells and control cells. Furthermore, the spheroid invasion assay was used to comprehensively examine invasion and proliferation.

The microscopic pictures in Fig. 6A show that a greater number of Cd-treated cells than untreated cells migrated to the lower chamber and attached to the underside of the membrane in Transwell inserts. Quantified result indicated that 4 weeks of Cd treatment increased cell migration by 1.6-fold (Fig. 6A). Activities of MMP 2 and 9 in cell culture medium were detected by in-gel zymography. As shown in Fig. 6B, the

clear bands in the gel image indicated gelatinolytic activity of MMPs. Determined by molecular weight, the intensities of clear bands at 58 and 82 KDa represented activities of MMP 2 and 9, respectively. Together, the secretion of MMP 2 and 9 in Cd-treated cells was 3.4-fold higher than in the control cells.

To investigate whether 4 weeks of treatment with 3 μ M Cd altered growth rate of MDA-MB-231 cells, the cells were harvested and cultured in the absence of Cd for 4 days. As shown in Fig. 7, the pretreated cells exhibited 30% higher growth than the control cells.

Invasiveness of prolonged Cd-treated cells was evaluated in a 3D spheroid invasion assay. The Cd-treated cells exhibited a shady outer ring around the spheroid core (arrow pointed), which indicated cell invasion into the surrounding matrigel (Fig. 8). In control cells, this ring was evident on day 12. In comparison, the ring in 4-week Cd-treated cells began as early as on day 6. The Cd-treated cells thus migrated and invaded more aggressively than the control cells.

Discussion

Metastasis is a consecutive process which includes invasion into surrounding tissue, intravasation, extravasation, and colonization into secondary tumors (Nguyen et al., 2009). Cell movement during this process is conducted through sequential activation of cell adhesion receptors which dynamically regulate cytoskeletal construction, leading to cell spreading and migration (Felding-Habermann et al., 2001).

The results presented here suggest that Cd enhances metastasized MDA-MB-231 breast cancer cell attachment through integrin activation. Cells receiving prolonged Cd treatment aid malignancy, as evidenced by cytoskeleton reorganization and enhanced cell migration and invasion. Enhanced cell proliferation further facilitates metastasized cell growth. All of these changes are key indicators of metastasized cells migration to a new environment and growth into a secondary tumor. Moreover, cellular responses shortly after Cd treatment correlated with alterations in cells treated with Cd for 4 weeks. First, short-term Cd treatment led to integrin $\beta 1$ activation, which preceded cell adhesion and cell migration. Thus, prolonged Cd treatment elevated integrin $\alpha 5$ and $\beta 1$ expression and displayed markedly higher migration and invasion characteristics. Second, short-term Cd treatment activated β -catenin signaling. Prolonged Cd treatment elevated expression of β -catenin which rendered Cd-treated cells more responsive to the activation of Wnt/ β -catenin signaling which contributed to cancer progression.

Cd-induced cytoskeletal perturbation has been well studied in animal cells. Exposure to Cd leads to disassembly of microtubules in mouse 3T3 cells (Perrino and Chou, 1986). In Cd-treated mouse mesangial cells, actin filaments become irregular and are lost because of disturbances in focal adhesion (Choong et al., 2013). In contrast to these earlier findings of cytoskeleton disruption upon Cd treatment, the results of present study show that Cd increased α -actinin, a binder of actin filaments polymerization, indicating active cytoskeleton buildup in the MDA-MB-231 cells (Fig. 1C). Real-time tracking of cell attachment indicated that Cd treatment stimulated cell spreading. These results led to examination of the effects of Cd on receptors that

regulate cell adhesion (Huttenlocher et al., 1996). The present study is the first to report that low level Cd treatment induces cell adhesion through integrin activation (Fig. 2). In this study, it was found that Cd, instead of disrupting focal adhesion (Choong et al., 2013), induced phosphorylation of focal adhesion kinases in breast cancer cells. This is a significant finding, because metastatic colonization at distant sites requires activation of integrin-mediated signaling (Reticker-Flynn et al., 2012). Increased expressions of intermediate filament-vimentin and scaffold protein-paxillin upon Cd treatment for 4 weeks (Fig. 5) provided evidence of cytoskeleton reorganization (Fife et al., 2014). The elevation of vimentin increases focal contact size, which is critical for cancer cell movement (Tsuruta and Jones, 2003).

Integrin plays an important role in tumor cell invasion and migration (Hood and Cheresch, 2002). Disseminated tumor cells use integrin and protease to develop a mesenchymal-type migration pattern (Friedl and Wolf, 2003). Several studies reported that integrin-associated MMPs became localized to cell-ECM sites when integrin was bound to the ECM (Mueller et al., 1999; Dumin et al., 2001). The present study observed an increase in total cell migration in cells treated with Cd for 4 weeks in the Transwell assay (Fig. 6A). Apparently, migrated cells had to degrade ECM on the membrane to enter the lower chamber because ECM blocked the membrane pores. The enhancement of MMP2/9 secretion (Fig. 6B) was parallel to integrin expression in Cd-treated breast cancer cells (Fig. 5B). Increases in integrin and MMP levels are important for local tumor invasion because their interactions likely facilitate ECM degradation. Moreover, enhanced MMP secretion also promotes the release of growth factors including transforming growth factor β (TGF β) and vascular endothelial

growth factor (VEGF) (Bergers et al., 2000; Yu and Stamenkovic, 2000; Mu et al., 2002). The released growth factors bind to membrane receptors which further stimulate cell proliferation and migration (Schenk et al., 2003). Therefore, the increased ERK1/2 phosphorylation in the present study (Fig. 2) might be a consequence of the release of growth factors.

Canonical Wnt/ β -catenin activation may facilitate carcinogenesis (Reya and Clevers, 2005) since it is marked by translocation of β -catenin to the nucleus with subsequent triggering of TCF/LEF transcriptional machinery. In this study, Cd activated TCF/LEF luciferase reporter in breast cancer cells (Fig. 3A), which was similar to observations reported previously in Cd-treated human kidney cells (Chakraborty et al., 2010a; Edwards et al., 2013). However, the related molecular events summarized in Fig. 9 were at variance with their previously reported mechanisms related to disruption of E-cadherin. For example, β -catenin, an important co-activator of TCF/LEF transcription, was caged by GSK3 β for degradation. The result in Fig. 3B showed that Cd inhibited GSK3 β , resulting in β -catenin's escape from degradation. The free β -catenin translocated into the nucleus and increased TCF/LEF transcription (Fig. 3A). Furthermore, prolonged Cd treatment elevated the expression of β -catenin (Fig. 4A). Although the mechanism responsible for β -catenin accumulation remains unclear, some insight might be provided by the observation that upregulation of β -catenin rendered cells more responsive to Wnt/ β -catenin pathway stimulation (Fig. 4B).

Integrin signaling converges with the Wnt/ β -catenin pathway at the GSK3 β node (Burkhalter et al., 2011). The present study also examined whether Wnt/ β -catenin

could be activated without engaging integrins. It was found that TCF/LEF transcription was diminished if cells were treated with Cd in low-adhesive plates (data not shown). This suggested that the involvement of integrin was important in Wnt/ β -catenin activation. Cd probably activated integrin-linked kinase (ILK), which in-turn inhibited GSK3 β , resulting in β -catenin-initiated TCF/LEF transcription. In partial support of this hypothesis, it was found that prolonged Cd treatment increased both integrins and β -catenin expression. The observed increase in cell proliferation in these cells (Fig. 7) could be a combined effect of more active proliferative signaling by integrins and β -catenin.

Breast tumor cells that migrate to distant organs frequently enter a long latency period before developing into life-threatening metastasized breast cancer. During this process, environmental factors may affect tumor malignancy. Therefore, it is important to understand how environmental risk contributes to breast cancer progression. Results reported here may provide some insight into this question. This study reports for the first time that a possible integrin-related mechanism is involved in the enhancement of breast cancer cell migration by the environmental contaminant Cd. Furthermore, it was shown that prolonged Cd treatment results in β -catenin accumulation, which renders breast cancer cells more sensitive to activate Wnt pathway. Extrapolating the in vitro observation of Cd-induced integrin activation, metastasized cancer cells might be more likely to lodge at distant organs after Cd exposure in vivo. There is growing evidence that tumor spheroids represent tumor conditions in vivo. Spheroids exhibit several traits which 2D monolayer cultures lack, such as characteristic tumor morphology, cell-cell contact, and hypoxic centers. The

results from this in vitro assay indicated that prolonged Cd treatment increases the tendency to form secondary tumors, which may shorten the latency of breast cancer relapse.

In summary, it is concluded that Cd treatment activates integrin in breast cancer cells, substantially affecting cell adhesion and migration. Activation of TCF/LEF transcription upon Cd treatment can be explained as the result of Cd-induced GSK3 β inhibition, which in turn could be the combined effects of integrin and β -catenin signaling. Activation of both pathways resulted in Cd-increased cell proliferation. Overall, it appears that Cd treatment increases the possibility of secondary tumor formation in metastatic sites. The results of this study contribute to a better understanding of Cd-induced breast cancer progression and may be of value in designing better strategies for cancer interventions in the future.

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Figures

Figure 1. Cell spreading and adhesion in Cd-treated cells.

(A) Wound healing assay. Before treatment with Cd, cross-scratches were made evenly on MDA-MB-231 cell monolayers. The cells were then treated with 0.5 or 1 μM Cd for 2 days. (B) Real-time electrical impedance assay. MDA-MB-231 cells in the logarithmic growth phase were treated with 1 or 3 μM Cd. Electrical impedance was measured every 15 s. Increased attachment between cell and electrode coated-plates was quantified as elevations in cell index. (C) Immuno-fluorescence staining for α -actinin. Attached MDA-MB-231 cells were treated with 1 or 3 μM Cd for 3 h. Then cells were fixed and stained with α -actinin antibody. DAPI was added to the slide before microscopic imaging. Upper images were under 10 X objective lens. Lower images were taken under oil lens at 100 X.

Figure 2. Activation of integrin $\beta 1$ and downstream signaling pathway upon Cd treatment.

The cells were treated with 1 or 3 μM Cd for 15 min or 1 h. Cells treated with 100 nM PMA for 1 h were positive controls. Representative Western blot images of active integrin $\beta 1$, pSrc (Tyr 416), pFAK (Tyr 397), pERK1/2 (Thr 202/Tyr 204), and pRac1 are shown. Densities relative to the untreated control cells are plotted as mean $\pm$ SEM (n =3). *Significantly higher than the untreated control group (p < 0.05).

(A)

(B)

Figure 3. Effects of Cd treatment on the Wnt/ β -catenin pathway.

(A) Luciferase reporter assay. The cells were transfected with TOPflash and pRL-TK plasmids for 2 days. Transfected cells were serum-starved overnight and treated with 1 or 3 μ M Cd for 8 h. Firefly and renilla luciferase activities were then measured. The ratio of firefly to renilla luciferase activities was normalized to control. The relative luciferase activity is plotted as mean $\pm$ SEM. (B) Inhibition of GSK3 β . Serum-starved cells were treated with 1 and 3 μ M Cd for 15 min or 1 h. Representative Western blot images of phosphorylated-GSK 3 β (Ser 9) and GSK 3 β are shown. Densities relative to the untreated control group are plotted as means $\pm$ SEM (n =3). *Significantly different from the untreated control group (p < 0.05).

(A)

(B)

Figure 4. Effect of prolonged Cd treatment on β -catenin levels.

The cells were cultured in DMEM supplemented with 3 μ M Cd for 4 weeks. Control cells were cultured in medium alone. (A) Expression of β -catenin in cellular fractions. Representative western blot images of β -catenin expression are shown. Lamin A/C and GAPDH were loading controls for the nuclear and cytosol fractions, respectively. Densities relative to the respective fraction of 4-week untreated group are plotted as mean $\pm$ SEM (n=3). (B) Luciferase reporter assay. Untreated and 4-week Cd-treated cells were transfected with TOPflash and pRL-TK plasmids and then serum-starved overnight, followed by treatment with 1 μ M Cd for 8 h. Firefly and renilla luciferase activities were measured. The ratio of firefly and renilla luciferase activities was normalized to untreated control cells and plotted as mean $\pm$ SEM. *Significantly higher than the 4-week untreated control group. #Significantly higher than 4-week untreated group that was treated with 1 μ M Cd ($p < 0.05$).

Figure 5. Effects of prolonged Cd treatment on the cytoskeleton.

The cells were cultured in DMEM containing 3 μ M Cd for 4 weeks. Control cells were cultured in medium alone. (A) Immuno-fluorescence staining with vimentin. Cells were fixed and stained with vimentin antibody. DAPI was added to the slide before microscopic imaging. (B) Western blot of integrin α 5 and β 1, paxillin, and vimentin. Control and Cd-treated cells were seeded in 6-well plates. After 48 h, attached cells were harvested and analyzed for expression of integrin α 5 and β 1, paxillin, and vimentin and representative images are shown. Densities relative to the control cells are plotted as mean $\pm$ SEM (n =3). *Significantly higher than the control cells (p < 0.05).

(A)

(B)

Figure 6. Effects of prolonged Cd treatment on cell migration.

The cells were cultured in DMEM containing 3 μ M Cd for 4 weeks. The control cells were cultured in medium alone. (A) Transwell assay. Control and Cd-treated cells were seeded in Transwell inserts. After 48 h, migrated cells were stained by crystal violet. Quantification of migrated cells was conducted by measuring the absorbance of de-staining solution. (B) Gelatin zymograph. Equal number of control and Cd-treated cells were cultured in serum-free medium for 2 days. The supernatant was concentrated 10-fold and applied to gelatin-zymogram gels. MMP2/9 secretion levels were visualized as the intensity of clear bands at 58 KDa and 82 KDa. Representative gel images are shown above the graph. Densities relative to those of the control cell group are plotted as mean $\pm$ SEM (n=3). *Significantly different from control cells (p < 0.05).

Figure 7. Effects of prolonged Cd treatment on cell proliferation.

The cells were cultured in 10% FBS-supplemented DMEM containing either no Cd or 3 μ M Cd. After 4 weeks, the cells were harvested and seeded at equal densities in DMEM supplemented with 10% FBS, but no Cd for 4 days. Cell proliferation was quantified using Cell Counting Kit-8. Absorbance at 450 nM relative to the control cells was plotted as mean $\pm$ SEM (n=3). *Significantly higher than the control cells ($p < 0.05$).

Figure 8. Effect of prolonged Cd treatment on cell invasion.

The cells were cultured in DMEM supplemented with 3 μ M Cd for 4 weeks. Control cells were cultured in medium alone. The cells were harvested and seeded in a round bottom 96-well plate at an equal density and grown in DMEM supplemented with basal membrane extract. Matrigel was added into each well after cells aggregated into spheroids. Cellular invasion of the spheroid was assessed by the appearance of a shady outer ring which was composed of cells invading into the matrigel.

Figure 9. Schematic diagram summarizing the effects of Cd on integrin and β -catenin signaling.

(A) Cd activates integrins on the cell membrane. Focal adhesion kinase (FAK) then transduces integrin signaling to downstream Rac1 to regulate actin assembly. FAK-initiated signaling also activates ERK 1/2 through crosstalk with MAPK, leading to cell proliferation. (B) At the same time, the active adhesion signaling is transduced to integrin-linked kinase (ILK) which inhibits GSK3 β , resulting in β -catenin's escaping from degradation. The free β -catenin translocates into the nucleus and stimulates TCF/LEF-mediated oncogene transcription, which contributes to carcinogenesis.