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Epithelial-mesenchymal transition in breast epithelial cells treated with cadmium and the role of Snail

Zhengxi Wei^{*}, Zhongguo Shan, and Zahir A. Shaikh[†]

Center for Molecular Toxicology, Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island, 02881, USA

Abstract

Epidemiological and experimental studies have implicated cadmium (Cd) with breast cancer. In breast epithelial MCF10A and MDA-MB-231 cells, Cd has been shown to promote cell growth. The present study examined whether Cd also causes epithelial-mesenchymal transition (EMT), a hallmark of cancer progression. Human breast epithelial cells 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. The MCF10A epithelial cells switched to a more mesenchymal-like morphology, which was accompanied by a decrease in the epithelial marker E-cadherin and an increase in the mesenchymal markers N-cadherin and vimentin. In both non-metastatic HCC 1937 and HCC 38 cells, treatment with Cd decreased the epithelial marker claudin-1. In addition, E-cadherin also decreased in the HCC 1937 cells. Even the mesenchymal-like MDA-MB-231 cells exhibited an increase in the mesenchymal marker vimentin. These changes indicated that prolonged treatment with Cd resulted in EMT in both normal and cancer-derived breast epithelial cells. Furthermore, both the 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 the mesenchymal marker ZEB-1. Treatment of cells with Cd significantly increased the level of Snail, a transcription factor involved in the regulation of EMT. However, the Cd-induced Snail expression was completely abolished by actinomycin D. Luciferase reporter assay indicated that the expression of Snail was regulated by Cd at the promoter level. Snail was essential for Cd-induced promotion of EMT in the MDA-MB-231 cells, as knockdown of Snail expression blocked Cd-induced cell migration. Together, these results indicate that Cd promotes EMT in breast epithelial cells and does so by modulating the transcription of Snail.

[†]Corresponding author: 7 Greenhouse Road, University of Rhode Island, Kingston, RI 02881, USA. Fax: (401) 874-2646. Telephone: (401) 874-5036, zshaikh@uri.edu.

^{*}Present address: 9800 Medical Center Drive, Division of Pre-Clinical Innovation, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, 20850, USA.

Conflict of Interest Statement

The authors have no conflict of interest to disclose.

Zhengxi Wei

Zhongguo Shan

Zahir A Shaikh

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Keywords

Cadmium; Breast cancer; Epithelial-mesenchymal transition; Snail; MDA-MB-231 cells; MCF10A cells

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; McElroy et al., 2006; Adams et al., 2012). The International Agency for Research on Cancer has categorized Cd as a group 1B carcinogen (IARC, 1993). Mechanistic studies implicating Cd in breast cancer initiation and promotion have been summarized by Waisberg et al. (2003). Breast cancer is the second most prevalent cancer in women and deaths occur due to tumor progression into advanced stages (Siegel et al., 2016). Investigation of environmental chemicals that potentially contribute to breast cancer progression could provide insight into cancer prevention. The study described herein aims to investigate the effect of Cd on breast cancer progression *in vitro*.

Progression of a tumor in situ to an invasive phenotype requires an increase in tumor cell plasticity (Park et al., 2010). Epithelial to mesenchymal transition (EMT) is a hallmark of increased cell plasticity and invasiveness (Fuchs et al., 2002). EMT is associated with a loss of epithelial markers such as E-cadherin and claudin-1, and a gain of mesenchymal markers such as N-cadherin, fibronectin and vimentin (Thiery, 2002; Willipinski-Stapelfeldt et al., 2005). Indeed, several studies, including one from our laboratory (Wei and Shaikh, 2017) have reported Cd-induced phenotypic changes in cell adhesion molecules and stress fibers, which are related to EMT. Expression of fibronectin and vimentin occurs upon treatment with Cd in kidneys of mice (Chakraborty et al., 2010) and in lung epithelial cells in vitro (Person et al., 2013). In human breast cancer-derived MCF7 cells, treatment with Cd for 6 months resulted in downregulation of E-cadherin (Ponce et al., 2015). Benbrahim-Tallaa et al. (2009) reported that even in normal breast epithelial MCF10A cells, treatment with Cd for 40 weeks caused transformation to a basal-like cancer phenotype. Thus, Cd seems capable of inducing EMT, however, the mechanism remains to be elucidated.

EMT is a continuous process of epithelial cells transforming into motile mesenchymal cells with invasive properties (Mani et al., 2008). There are several transcription factors that regulate EMT. Snail, a zinc-finger transcription factor, 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). TGF β , Notch-1 and Wnt signaling pathways crosstalk and converge to regulate the transcription of Snail (Yook et al., 2005; Vincent et al., 2009; Horiguchi et al., 2012). Several studies reported that Cd activated these pathways in a highly context- and cell type-dependent manner (Baroni et al., 2015; Fujiki et al., 2014; Chakraborty et al., 2010). It is possible that Cd upregulates Snail through regulating different signaling pathways. Therefore, the present study assessed the effect of Cd on EMT in normal and breast cancer-derived cells and investigated the role of Snail in this process.

Materials and Methods

Materials

Mammary epithelial cell growth medium (MEGM), DMEM, RPMI 1640, and trypsin-versene were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA). Cd chloride, phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor were from Sigma-Aldrich (Dallas, TX). Bis-acrylamide powder, supersignal west femto maximum sensitivity substrate, and BCA kit were from Thermo-Fisher Scientific (Pittsburgh, PA). Phosphatase inhibitor cocktail tablets were from Roche (Indianapolis, IN). Laemmli buffer and blotting buffers were from Bio-Rad (Hercules, CA). Actinomycin D (ACD) was from Cayman Chemical (Ann Arbor, Michigan). The EMT Kit containing primary antibodies, lamin A/C, and HRP-conjugated secondary antibody were from Cell Signaling Technology (Danvers, MA). GAPDH primary antibody, transwell inserts, and slide chambers were from EMD Millipore (Billerica, MA). CF5.5 goat anti-rabbit IgG was from Santa Cruz Biotechnology (Dallas, TX). IRDye 680 goat anti-rabbit and anti-mouse IgG antibodies were from Licor (Lincoln, NE). Nuclear Extract Kit was from Active Motif (Carlsbad, CA). Snail siRNA (siGENOME Human SNAIL 6615 – SMARTpool, catalog # M-010847-00-0005), nonsense siRNA (catalog # D-001206-01-05), transfection reagent, and RNase were from GE Healthcare Dharmacon (Chicago, IL). Genjet DNA transfection reagent was from SignaGen Laboratories (Gaithersburg, MD). pGL3-enhancer vector, JM109 competent cells, and dual-luciferase assay kit were from Promega (Madison, WI). Snail_pGL2 plasmid was from Addgene (Cambridge, MA). Restriction enzymes and Q5 high-fidelity PCR kit were from New England BioLabs (Ipswich, MA). HiSpeed plasmid purification kit was from Qiagen (Germantown, MD).

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, penicillin (100 U/mL), and streptomycin (100 U/mL). HCC 1937 and HCC 38 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 U/mL). Cells undergoing prolonged Cd treatment were maintained in the corresponding medium for 4 weeks. All cells were maintained in a humidified incubator at 37°C with 5% CO₂. Cell morphology was observed under a Nikon phase contrast microscope.

Immunofluorescence 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, the cells were incubated with the primary antibody for E- or N-cadherin overnight. After washing with PBS for 5

min, three times, the cells were incubated with the 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 zinc-finger E-box binding homeobox-1 (ZEB-1) activity in Cd- or TGF β -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 blotting

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 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 three times in PBS containing 0.5% Tween 20, for 5 min 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 were scanned in a Licor Odyssey Imager. The optical densities of protein bands were quantified by the Odyssey Image Analysis Software.

Transwell assay

Transwell compartments (12 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 incubation for 48 h, 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 and the absorbance was measured at 450 nm in a Spectromax microplate reader. Migration of the Cd-treated cells was quantified as the absorbance relative to the control cells.

Preparation of Snail promoter plasmid

The promoter region of Snail (−1046 / −60) was amplified from Snail_pGL2 (Fujita et al, 2003) by using primers 5′-CTGAAAATCCTTCGGTGGCTCCCCA-3′ (sense) and 5′-GAGGGGCGGGGCCCTTATCTGCCAC-3′ (antisense). The amplifications were performed with Q5 high-fidelity PCR kit. The −1046 / −60 promoter fragment was cloned in the XhoI/

HindIII sites of pGL3-enhancer vector. The Snail_pGL3 plasmid was amplified in bacterial strain JM109 and purified by HiSpeed plasmid purification kit.

Luciferase reporter assay

MDA-MB-231 cells were seeded in 48-well plates and cultured in DMEM with 10% FBS. After 24 h, the cells were transfected with pRL-TK (2 ng/well) and Snail_pGL3 (200 ng/well) plasmids for 48 h. The cells were serum starved by incubating in blank medium overnight, prior to treatment with Cd for 6 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.

Data analysis

Data from at least three experiments was analyzed by one-way ANOVA followed by Tukey's post hoc test using Prism. Statistical significance was set at $p < 0.05$.

Results

Effect of Cd on cell morphology and cadherins in normal cells

Treatment of MCF10A cells with 1 μM Cd for 4 weeks appeared to transform the morphology of normal breast epithelial cells. The untreated cells displayed epithelial sheet-like structure, indicating tight cell-cell adhesion. In contrast, cells treated with Cd were spindle-like and appeared to have reduced intercellular adhesion (Fig. 1). The apparent loss of epithelial characteristic was further investigated using biochemical markers of EMT.

Intercellular adhesion is mediated through cadherins. Thus, the MCF10A cells were stained for E- and N-cadherin and the immunofluorescence pattern indicating the relative expression and location of cadherins was investigated. As shown in Fig. 2, the control cells displayed strong fluorescence for E-cadherin, but only faint and diffused fluorescence for N-cadherin. In contrast, treatment with 1 μM Cd for 4 weeks markedly reversed the fluorescence pattern of cadherins, while the epithelial marker E-cadherin was faint and diffused. The mesenchymal marker N-cadherin was clearly visible at well-defined cell borders. Together, these morphological and phenotypical changes indicated that prolonged Cd treatment transformed the normal breast epithelial cells to a more mesenchymal-like phenotype.

Effect of Cd on the EMT markers in cancer-derived cells

To determine the effect of Cd on EMT markers in cancer cells, three human breast epithelial cell lines consisting of, non-metastatic HCC 38 and HCC 1937, and metastatic MDA-MB-231 were examined. The cells were treated with 3 μM Cd for 4 weeks and the expression of epithelial and mesenchymal markers was evaluated. The normal breast epithelial MCF10A cells treated with 1 μM Cd for 4 weeks were also included for comparison. As shown in Fig. 3, in the MCF10A cells, treatment with Cd reduced the level of E-cadherin to 16% and increased the expression of N-cadherin and vimentin by 1.6- and 2.6-fold, as compared to the respective controls. The MDA-MB-231 cells are known to express very low level of both E- and N-cadherin (Blick et al., 2008). However, in these cells

the level of a mesenchymal marker, vimentin, was upregulated 5.1-fold. In the HCC 1937 cells, Cd decreased the two epithelial markers, E-cadherin and claudin-1, by 55 and 63%, respectively. Again, the N-cadherin levels were too low to provide a distinction between the Cd-treated and control cells. The HCC 38 cells expressed high basal levels of both E- and N-cadherins. In these cells, although the Cd-induced reduction in E-cadherin was not statistically significant, the level of claudin-1, was reduced 59%, and the mesenchymal marker N-cadherin was elevated 2.7-fold over the control. Thus, not only in normal, but also cancer-derived breast epithelial cells, prolonged Cd treatment caused a decrease in epithelial markers and increase in mesenchymal markers confirming that prolonged treatment with Cd caused EMT in the breast epithelial cells.

Further confirmation of EMT by the Zcad sensor

Transition to mesenchymal-like phenotype is a gradual process. Thus, to track the changes in E-cadherin and its transcription suppressor ZEB-1, the Zcad sensor was utilized. This sensor is comprised of an Ecad-RFP plasmid containing RFP driven by the CDH1 promoter and a ZEB-GFP plasmid containing destabilized GFP serving as an indicator of ZEB-1 translation (Toneff et al., 2016). So, in this dual fluorescent system, RFP intensity measures E-cadherin expression, and GFP intensity indicates ZEB-1 expression. MCF10A cells express more E-cadherin, which shows strong RFP intensity when transfected with Zcad plasmid. Stably transfected MCF10A cells were treated with either 1 μ M Cd or TGF β (positive control) for up to 4 weeks. As expected, the MCF10A-Zcad cells treated with TGF β expressed significantly less E-cadherin, indicated by a marked reduction (45%) in RFP intensity in the UL quadrant compared to the control (Fig. 4). Similar, but less pronounced effect (13% decrease in RFP intensity compared to the control) was observed in cells treated with 1 μ M Cd for 4 weeks..

The MDA-MB-231-Zcad cells which had relatively high basal level of ZEB-1 activity showed strong GFP intensity in the LL quadrant (Fig. 5). Treatment with the positive control, TGF β , caused a 6-fold increase in ZEB-1 expression (LR quadrant) after 4 weeks. In comparison, treatment with 3 μ M Cd for 4 weeks caused a 1.7-fold increase in GFP intensity (LR quadrant). The increase in GFP expression in Zcad cells indicated enhanced translation of ZEB-1 which is a direct suppressor of E-cadherin transcription (Postigo et al., 2003; Sanchez-Tillo et al., 2010). Enhanced ZEB-1 activity in cells following treatment with Cd suggested a more mesenchymal-like property and suppression of E cadherin transcription.

Effect of Cd on Snail levels

Snail is a transcription factor which primarily functions as an EMT regulator (Peinado et al., 2007). Therefore, the effect of prolonged treatment with Cd on Snail was investigated in HCC 1937, HCC 38, and MDA-MB-231 cells treated with 3 μ M Cd for 4 weeks. Fig. 6 shows that the Snail levels in these cells increased 3.5-, 3.4-, and 2.1-fold, respectively.

Snail is formed in the cytosol and translocated to the nucleus to exert its function. The intracellular distribution of Snail following Cd treatment was analyzed in the HCC 1937 and HCC 38 cells treated with 3 μ M Cd for 4 weeks. As shown in Fig. 7, Snail was concentrated

primarily in the nuclear fraction and its level was 2.5- and 3.4-fold higher than the respective controls. The accumulation of Snail in the nucleus following treatment with Cd suggested that the Cd-induced EMT could possibly be mediated through Snail.

Time-course and mechanism of Snail expression

The HCC 1937 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. 8, the expression of Snail increased almost linearly (2.8-fold) during the 8 h following treatment with Cd. To evaluate whether this was due to increased transcription of Snail, the cells were co-treated with 1 μ M Cd and ACD for 8 h. TGF β served as a positive control. Cd and TGF β caused a 2.1- and 2.0-fold increase in Snail expression, respectively (Fig. 9). Co-treatment with ACD completely abolished this effect, indicating that elevation of Snail levels was due to increased transcription.

Snail transcription is dependent on the activation of its promoter region (Barbera et al., 2004). To establish that Cd affected Snail transcription through its promoter, the MDA-MB-231 cells were transfected with Snail_pGL3 plasmid which contained Snail promoter region (−1046 / −60). As shown in Fig. 10, treatment of cells with 1 or 3 μ M Cd for 6 h increased luciferase activity 1.5-fold, confirming that Cd enhanced Snail transcription by elevating the activity of its promoter.

Role of Snail in Cd-induced EMT

To investigate the importance of elevated expression of Snail in Cd-induced EMT, the MDA-MB-231 cells were treated with 3 μ M Cd for 4 weeks before being seeded on the transwell inserts for the cell mobility assay. Photographs of the underside of transwell inserts are shown in Fig. 11 (top). Treatment with Cd resulted in migration of 1.4-fold more cells through the membrane toward the lower chamber than in the control. Next, Snail expression was knockdown with siRNA prior to seeding the cells on the transwell inserts. Successful knockdown of Snail expression was confirmed by Western blot analysis (Fig. 11, bottom). Knockdown of Snail expression suppressed cell migration by 24% in the control cells and 46% in cells treated with Cd. These results indicated that Snail was essential for Cd-induced EMT.

Discussion

Prozialeck's group first reported that Cd reduces E-cadherin expression at cell membrane in kidney tubular cells, causing cell-cell dissemination (Prozialeck and Niewenhuis, 1991; 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 both normal and cancer-derived breast epithelial cells. Such alteration was attributed to EMT based on the following observations.

The primary key finding in the present study was that prolonged treatment of cells with Cd changes the expression of epithelial and mesenchymal markers E- and N-cadherins, vimentin, and claudin-1. Change in the expression of these markers is a hallmark of EMT

(De Craene and Berx, 2013). The initiation or progression of EMT by non-cytotoxic concentrations of Cd (1 or 3 μ M) was not restricted to normal or cancer-derived breast epithelial cells. It 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, where RFP and GFP intensities represented relative expression of E-cadherin and ZEB-1, respectively, Cd-treated MCF10A-Zcad and MDA-MB-231-Zcad cells exhibited a decrease in RFP and an increase in GFP intensity, indicating suppression of E-cadherin transcription and active ZEB-1 translation (Fig. 4 and 5).

Reported molecular mechanisms of EMT vary from disruption of developmental signaling to activation of inflammatory pathways (Xu et al., 2009; Luo et al., 2013). Chakraborty et al. (2010) reported that Cd increased transcriptional activation of Wnt pathway and upregulation of EMT markers. Baroni et al. (2015) reported that treatment of bronchial epithelial cells with Cd affected TGF β pathway. According to Fujiki et al. (2017), prolonged treatment with Cd increased Notch-1 intracellular domain and its target gene Snail in A549 lung cancer cells. Also, knockdown of Notch1 partially suppressed Cd-induced alterations of E-cadherin and Snail levels. In the present study, the use of Zcad cells revealed related mechanism in Cd-induced EMT in breast epithelial cells. According to the construct sequence reported in Toneff et al. (2016), the Ecad-RFP plasmid measures transcriptional activity at CDH1 promoter region. The decrease in RFP intensity in the present study indicated that Cd suppressed E-cadherin transcription (Fig. 4).

Snail represses CDH1 transcription through binding to E-box in the promoter region of CDH1 (Batlle et al., 2000; Cano et al., 2000). The secondary key finding of this study is that treatment with Cd increased the expression of Snail which was required for Cd-induced EMT. The observation that Snail accumulated in the nucleus upon treatment with Cd (Fig. 7) was consistent with the results showing reduced RFP in MCF10A-Zcad cells (Fig. 4). Elevated Snail expression in epithelial cancer cells promotes mesenchymal properties and subsequently facilitates tumor migration (Come et al., 2006; Mezencev et al., 2016). Chang and Yu (2013) observed that prolonged nicotine exposure enhanced EMT and knockdown of Snail reversed this process. Results of the transwell assay revealed that indeed cells treated with Cd, expressing higher level of Snail, were more migratory than the control cells (Fig. 11). As a key transcription factor that drives EMT, knockdown of Snail blocked the elevated cell migration in Cd-treated cells (Fig. 11). Thus, it is apparent that Snail was essential for Cd-induced EMT and elevated migratory activity.

Treatment of cells with Cd resulted in an increase in Snail expression, which was abolished by inhibition of transcription by ACD (Fig. 9). Regulation of Snail transcription is dependent on the activation of its promoter region (Barbera et al., 2004). To evaluate whether Cd affects the activity of Snail promoter, the MDA-MB-231 cells were transfected with Snail_pGL3 plasmid which contained Snail promoter region (−1046 / −60). As shown in Fig. 10, treatment with 1 or 3 μ M Cd increased luciferase activity 1.5-fold, suggesting that the treatment of Cd enhanced Snail promoter activity. These results suggest that transcriptional activity is crucial for Cd-induced Snail expression.

ZEB-1 is a transcription suppressor of E-cadherin (Gregory et al., 2008; Sanchez-Tillo et al., 2010). Zcad is a dual fluorescence sensor which consists of Ecad-RFP and ZEB-GFP plasmids. Beside the Ecad-RFP plasmid, ZEB-GFP plasmid is designed based on the knowledge that ZEB is suppressed by the miR-200 family (Burk et al., 2008; Korpai 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 treatment with Cd. It is possible that Cd inhibits miR-200 that post-transcriptionally suppresses ZEB-1 translation. Therefore, prolonged treatment with Cd appears to stabilize GFP (Fig. 5), suggesting a potential mechanism of regulation of EMT in a post-transcriptional manner.

Although the detailed mechanism of Cd-induced Snail expression remains to be further elucidated, the present study demonstrates 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. Based on the results of this study, it is concluded that Cd upregulates Snail expression, transforming epithelium-derived breast cancer cells to a more mesenchymal-like phenotype which is conducive to greater mobility. These findings may be relevant in further exploring the role of Cd in the progression of breast cancer metastasis.

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Highlights

- Cd promotes EMT in breast epithelial cells.
- The EMT is associated with accumulation of Snail in the nucleus.
- Snail accumulation occurs through transcriptional regulation.
- Snail is essential for Cd-induced EMT

Fig. 1. Changes in morphology of MCF10A cells upon prolonged treatment with Cd
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 20X objective lenses.

Fig. 2. Expression of cadherins in MCF10A cells upon prolonged treatment with Cd

The cells were treated with 1 μ M Cd in MEGM for 4 weeks. Control cells were cultured in MEGM alone. The cells were fixed and stained to visualize the abundance of E-cadherin and N-cadherin. Immuno-fluorescence images were taken under a 20X objective lens. Relative cadherin levels are indicated by the intensities of red fluorescence.

Fig. 3. Expressions of EMT markers in breast epithelial cells upon prolonged treatment with Cd The MCF10A cells were treated for 4 weeks with 1 μ M Cd in MEGM. The MDA-MB-231, HCC 1937, and HCC 38 cells were treated with 3 μ M Cd for 4 weeks in DMEM and RPMI 1640, respectively, supplemented with 10% FBS. Representative Western blots of the EMT markers from three independent experiments are shown. Densities relative to the respective controls are plotted as mean $\pm$ SEM. *Significantly different from the control ($p < 0.05$).

Fig. 4. Effect of prolonged treatment with Cd on E-cadherin

The MCF10A Zcad cells were treated for up to 4 weeks with 1 μ M Cd or 10 ng/ml TGF β in MEGM. The cells were harvested at the end of each week and analyzed by flow cytometry. Representative dot plots of the distribution of cells expressing E-cadherin (RFP) are shown. Cells expressing E-cadherin are in the upper left (UL) quadrant. Data from three independent experiments are plotted as mean $\pm$ SEM. *Significantly lower than the control at the same time point ($p < 0.05$).

Fig. 5. Effect of prolonged treatment with Cd on ZEB-1 activity

The MDA-MB-231-Zcad cells were treated for up to 4 weeks with 3 μ M Cd or 10 ng/ml TGF β in DMEM supplemented with 10% FBS. The cells were harvested at the end of each 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.

*Significantly higher than the control at the same time point ($p < 0.05$).

Fig. 6. Effect of prolonged treatment with Cd on Snail levels

The 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. A representative Western blot of the Snail levels is shown. Densities relative to the control from three independent experiments are plotted as mean $\pm$ SEM. *Significantly higher than the respective control ($p < 0.05$).

Fig. 7. Intracellular distribution of Snail in cells treated with Cd

The 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 Snail in whole cell lysate, cytosolic, and nuclear fractions are shown. Densities relative to the control from three independent experiments are plotted as mean $\pm$ SEM. *Significantly higher than the respective control group ($p < 0.05$).

Fig. 8. Time-course of Snail accumulation following treatment with Cd

The HCC1937 cells were treated for up to 8 h with 1 μ M Cd in RPMI 1640. A representative Western blot of the Snail levels is shown. Densities relative to the control at time zero from three independent experiments are plotted as mean $\pm$ SEM. *Significantly higher than the control at time zero ($p < 0.05$).

Fig. 9. Transcriptional control of Snail expression in response to Cd

The HCC 1937 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. A representative Western blot of the Snail levels is shown. Densities relative to the untreated control from three independent experiments are plotted as mean $\pm$ SEM. *Significantly higher than the control ($p < 0.05$). #Significantly higher than the cells treated with ACD ($p < 0.05$).

Fig. 10. Effect of Cd on the activity of Snail promoter

The MDA-MB-231 cells were transfected with Snail_pGL3 and pRL-TK plasmids and then serum-starved overnight, followed by treatment with 1 or 3 μM Cd for 6 h. Firefly and renilla luciferase activities were measured and the ratio of the activities was normalized to the untreated control. Data from three independent experiments are plotted as mean±SEM.

*Significantly higher than the control ($p < 0.05$).

Fig. 11. Effect of Snail expression on cell migration upon prolonged treatment with Cd
 The MDA-MB-231 cells were cultured in DMEM containing 3 μ M Cd for 4 weeks. Control cells were cultured in medium alone. Both the control and Cd-treated cells were incubated with either the control or Snail siRNA for two days. Knockdown of Snail expression was confirmed by Western blot analysis. To assess cell mobility, the cells were seeded in transwell inserts at equal density. After 48 h, cells that migrated to the other side of the insert were stained by crystal violet and photographed for qualitative comparison. Quantitative analysis was performed by destaining the cells and measuring absorbance of the de-staining solution. Data from three independent experiments are plotted as mean $\pm$ SEM. *Significantly higher than the respective control. #Significantly higher than the cells treated with Snail siRNA ($p < 0.05$).