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CADMIUM PROMOTES PROLIFERATION OF TRIPLE-NEGATIVE BREAST CANCER CELLS THROUGH EGFR-MEDIATED CELL CYCLE REGULATION

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CADMIUM PROMOTES PROLIFERATION OF TRIPLE-NEGATIVE BREAST CANCER CELLS THROUGH EGFR-MEDIATED CELL CYCLE REGULATION

BY

ZHENGXI WEI

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OF

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2014

ABSTRACT

Cadmium (Cd) is a carcinogenic heavy metal which is implicated in breast cancer by epidemiological studies. In cell culture studies, it is reported to promote breast cancer cell growth through membrane estrogen receptors. Triple-negative breast cancer patients are non-responsive to endocrine and trastuzumab therapy and have the worst prognosis and lowest survival rate. The purpose of this study was to examine whether Cd can promote the growth of non-metastatic, triple-negative human breast cancer cells HCC 1937, which are positive for epidermal growth factor receptor (EGFR). It was found that Cd treatment (0.1-0.5 μM) promoted cell growth and accelerated cell cycle progression by increasing cyclins A, B, and E, and CDK 1, and 2 expressions. Further study using kinase inhibitors indicated that MAPK and PI3K activation was required for this process. The kinase activation in turn was mediated through EGFR activation. Based on these findings, it is concluded that sub-micromolar concentration of Cd induces proliferation of HCC 1937 cells through EGFR, MAPK and PI3K regulated cell cycle progression. The involvement of EGFR in Cd-stimulated early stage and triple-negative breast cancer cell growth implicates Cd's role in breast cancer progression.

Keywords: Cadmium, Breast Cancer, Cell Proliferation, Cell Cycle, EGFR

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He provides me enormous encouragement at all times. I am lucky to have a generous and thoughtful partner like him.

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PREFACE

This thesis was written in manuscript format.

The manuscript will be submitted to Toxicology and Applied Pharmacology with the following tentative title:

Cadmium promotes proliferation of triple-negative breast cancer cells through EGFR-mediated cell cycle regulation

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BACKGROUND

Cadmium exposure and health risk

Cadmium (Cd) is a toxic element and an environmental contaminant with growing health concerns. Occupational exposure to Cd is mainly through inhalation. General population is predominantly exposed to Cd via dietary intake, smoking and polluted air. Bioaccumulation of Cd in the body interferes with normal physiological functions. 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 (Cikrt et al., 1990; Cikrt et al., 1992).

Acute exposure to Cd at high concentration induces cellular stress, impairs mitochondrial function, DNA damage and cell death (Petanidis et al., 2013). Chronic exposure to low concentration of Cd (dietary intake level) causes deregulated cell growth because of endocrine disruption. Epidemiology studies showed that dietary Cd intake is associated with breast cancer incidence (Julin et al., 2012; Itoh et al., 2014). In ovariectomized rat model, single ip injection of 5 μg/kg Cd promoted mammary gland and uterus growth, which indicated that Cd behaved like a metalloestrogen at low concentration (Johnson et al., 2003; Hofer et al., 2009). Several in vitro studies reported that Cd could stimulate MCF7 and T47D cell proliferation (Garcia-Morales et al., 1994; Martin et al., 2003; Zang et al., 2009). However, Silva et al. (2006) failed to detect Cd's proliferative effect on the MCF7 cells. Still, Cd is accepted by many as an endocrine disruptor since some of its effects are similar to those of estrogen.

Cd as a xenoestrogen

Most researchers attribute Cd's estrogenic effect to the activation of estrogen receptor (ER). Garcia-Morales et al. (1994) first reported that low concentration of Cd stimulated MCF7 cell proliferation and induced transcription of ER regulated genes for cell growth. They concluded that Cd's estrogenic effect was ER dependent based on two observations: 1) Cd's estrogenicity was inhibited by ER inhibitor, ICI-164,384, 2) MDA-MB-231, an ER negative cell line, did not respond to Cd's estrogenic effect unless transfected with ER. To confirm that Cd interferes with the ER, Cd's binding behavior to ER was studied by several groups. In ERα binding assay, radiolabeled ^{109}Cd was shown to bind to ligand binding domain (LBD) of human recombinant ERα in a noncompetitive manner against estradiol (Stoica et al., 2000). Recently, RIfS (reflectometric interference spectroscopy), a label-free method, was utilized to study interaction between Cd and human ERα. It was reported that Cd was bound to cysteine residues in the LBD and changed ERα conformation without affecting estradiol binding to LBD of ERα (Fechner et al., 2011).

Cd's estrogenic effect is implemented by direct affecting ERα, and modulating ERα responsive signaling pathways. At the receptor level, Cd phosphorylates ERα and increases ERα translocation. Furthermore, it potentiates the interaction of ERα and c-jun which binds to other promoter regions and regulates c-myc and cyclin D1 leading to cell proliferation (Siewit et al., 2010; Ponce et al., 2013). At signaling level, Cd activates ERα-mediated pathways. It activates MAPK pathway and pERK reaches the nucleus, which recruits proto-oncongenes c-fos and c-jun to form transcription factors

AP-1, thereby increasing transcription of cell growth responsive genes. Constitutive transcription of these genes contributes to breast cancer progression (Choe et al., 2003; Liu et al., 2008; Zang et al., 2009).

Activation of mitogenic pathway and cell cycle progression

Cell cycle is a process of cell division in which a parent cell divides into two daughter cells. Quiescent (non-dividing) cells are considered in G0 phase. When cell enters into the G1 phase, cell size increases and the cell prepares for DNA synthesis. Cells that enter the G1 phase but not pass restriction point go back to G0 phase. Cells that pass the restriction point move forward through G1/S/G2/M phases sequentially (Lim and Kaldis, 2013). G1/S and G2/M transitions are crucial checkpoints for cell cycle progression. The transition of cells from one phase to the next requires cyclins and CDKs working properly. Cell cycle regulation is a complicated system which is regulated by various kinase pathways. Mitogenic signaling control of cell cycle transition is well documented (Massague, 2004). When mitogen activates MAPK and PI3K, their downstream signals translocate and activate transcription factors such as myc and AP-1 which are responsible for enhancing cyclin/CDK complex formation (Shaulian and Karin, 2001). At G1/S transition, cyclin D/CDK 4, cyclin E/CDK 2 complexes phosphorylate retinoblastoma protein (Rb). Thereby E2F is released from Rb. Released E2F further promotes expression of cyclin A and CDK 2 which promote cells to enter S phase. At the end of S phase, accumulated cyclin A/CDK 2 complexes phosphorylate FoxM1, leading to the relief of its self-inhibition. Then FoxM1 recruits histone deacetylase p300/CREB binding protein (CBP) and transcribe cyclin B and

centromere protein F which are executor for entering mitosis (M phase) (Major et al., 2004; Laoukili et al., 2008; Park et al., 2008; Chen et al., 2009).

Role of EGFR in breast cancer development

Breast cancer has five subclasses based on presence of ERα/β, progesterone receptor (PR) and human epidermal growth factor 2 (HER2). Triple-negative cancer cells are negative for all of these. Approximately 20% of breast cancer patients are defined as triple-negative They have worse prognosis and survival rate because the breast cancer cells are highly malignant, more aggressive and unresponsive to endocrine treatment and trastuzumab (Herold and Anders, 2013). However, triple-negative breast cancer patients treated with dual EGFR and HER2 inhibitors have better overall survival rate (Chen and Russo, 2011). Therefore, EGFR is an important biomarker for breast cancer treatment and prognosis. The clinical therapy in triple-negative breast cancer patient prompts EGFR as the target to study the tumorigenesis potential of Cd in the absence of ER.

It is established that EGFR is the crucial receptor associated with cell proliferation and cell cycle progression. EGFR is a 170 kDa transmembrane glycoprotein expressed on many epithelial cells. It has an extracellular receptor binding domain and a cytoplasmic tyrosine kinase domain. When ligands bind to EGFR, the receptor dimerizes and results in EGFR and downstream kinase phosphorylation. The pEGFR translocates and phosphorylates transcriptional factors such as E2F1 and STAT3 which facilitate cell cycle progression and proliferation (Lo et al., 2005; Lo et al., 2006; Hanada et al., 2006;). The activated downstream pathways include MAPK,

PI3K and PLC. MAPK pathway promotes cell growth and PI3K/AKT pathway prevents apoptosis. Both downstream pathways lead to enhanced activity of transcription factors such as NFκB and c-myc which result in increasing DNA synthesis, cyclins and CDKs expression, and cell proliferation (Hoadley et al., 2007; Kundu et al., 2013). It is reported that Cd induces A549 human lung adenocarcinoma cell proliferation through EGFR (Kundu et al., 2011). So EGFR may be responsible for Cd's effect on cell proliferation and cell cycle progression.

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MANUSCRIPT

Cadmium promotes proliferation of triple-negative breast cancer cells through EGFR-mediated cell cycle regulation

by

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ABSTRACT

Cadmium (Cd) is a carcinogenic heavy metal which is implicated in breast cancer by epidemiological studies. In cell culture studies, it is reported to promote breast cancer cell growth through membrane estrogen receptors. Triple-negative breast cancer patients are non-responsive to endocrine and trastuzumab therapy and have the worst prognosis and lowest survival rate. The purpose of this study was to examine whether Cd can promote the growth of non-metastatic, triple-negative human breast cancer cells HCC 1937, which are positive for epidermal growth factor receptor (EGFR). It was found that Cd treatment (0.1-0.5 μM) promoted cell growth and accelerated cell cycle progression by increasing cyclins A, B, and E, and CDK 1, and 2 expressions. Further study using kinase inhibitors indicated that MAPK and PI3K activation was required for this process. The kinase activation in turn was mediated through EGFR activation. Based on these findings, it is concluded that sub-micromolar concentration of Cd induce proliferation of HCC 1937 cells through EGFR, MAPK and PI3K regulated cell cycle progression. The involvement of EGFR in Cd-stimulated early stage and triple-negative breast cancer cell growth implicates Cd's role in breast cancer progression.

Keywords: Cadmium, Breast Cancer, Cell Proliferation, Cell Cycle, EGFR

INTRODUCTION

Cadmium (Cd) is a toxic metal which is widely distributed in the environment. It is used in pigment, metal-plating, battery and plastics industries. The general population is exposed to Cd from fuel combustion, waste burning, and cigarette smoking, and through dietary intake from food and polluted water (Huang et al., 2013). Besides the acute toxicity to kidney and bone, Cd is an established Group 1 carcinogen because of its causative effect on lung cancer (Stayner et al., 1992). Retrospective and prospective epidemiology studies indicate that dietary Cd intake is associated with breast cancer incidence (Julin et al., 2012; Itoh et al., 2014). Also, bioaccumulation of Cd in breast tissue of breast cancer patients is higher than of normal subjects (Romanowicz-Makowska et al., 2011; Strumylaite et al., 2011). In studies with rats, Cd was found to be a highly potent endocrine disruptor because it promoted growth of mammary gland and uterus by a single i.p. injection of 5 μg/kg Cd (Johnson et al., 2003; Hofer et al., 2009).

The mechanism of breast cancer cell growth promotion by Cd has been explored by a number of investigators. Garcia-Morales et al. (1994) found that Cd stimulated the growth of MCF-7 cells by activating estrogen receptor alpha (ERα) and inducing the expression of ERα targeted genes which were involved in cell growth. Cd has also been shown to bind to the ligand-binding domain of ERα in a noncompetitive manner against estradiol (Stoica et al., 2000). Several other studies have confirmed that Cd stimulates the proliferation of ER-positive MCF7 and T47D cells (Martin et al., 2003; Zang et al., 2009). However, Silva et al. (2006) were unable to observe the

estrogenicity of Cd in MCF7 cells by E-Screen assay. Yu et al. (2010) reported that Cd stimulated cell growth via G protein coupled receptor 30 (GPR30) in ERα negative breast cancer cell line SKBR3. Furthermore, in a transgenic estrogen response element (ERE)-luciferase reporter mouse model, Cd did not increase the ERE-luciferase activities in any reproductive or non-reproductive organ (Ali et al., 2010; Ali et al., 2012). Still, most researchers accept Cd as an endocrine disruptor since a number of its effects are similar to those of estrogen.

Although currently there is no consensus on whether Cd's estrogenic effect is ER mediated, there is a general agreement that Cd activates the mitogen signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) in breast cancer cells (Choe et al., 2003; Liu et al., 2008; Zang et al., 2009). These pathways converge signaling from various membrane receptors, including ERs, G-protein coupled receptors (GPCRs), and receptor tyrosine kinases (RTKs), and cause the activation of genes involved in cell cycle regulation, proliferation, and survival (Martin et al., 2000). Epidermal growth factor receptor (EGFR) is one of the RTKs which plays a pivotal role in integrating hormone-mediated growth factor stimulation and subsequent activation of MAPK and PI3K pathways (Yarden and Sliwkowski, 2001; Filardo et al., 2002; Navolanic et al., 2003).

We hypothesized that, in addition to ERs, EGFR might be involved in promoting the proliferation of breast cancer cells. To test this hypothesis, we utilized an early stage human ductal breast cancer cell line, HCC 1937. These cells are triple-negative with respect to ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), but are positive for EGFR. This early stage triple-negative breast

cancer cell line renders a good model to study the tumorigenic potential of Cd in the absence of ER. Based on a series of experiments with the HCC 1937 cells, we present here four observations regarding Cd-induced human breast cancer cell proliferation:

1) ER is not essential for Cd's action.

2) The cell cycle signal is initiated at the cell membrane through EGFR.

3) Intracellular signal transduction involves activation of MAPK and PI3K.

4) In the nucleus, cyclins and CDKs facilitate cell cycle progression.

MATERIALS AND METHODS

Materials RPMI 1640 and Trypsin-Versene were purchased from Lonza (Walkersville, MD). Fetal bovine serum was purchased from Atlanta Biologicals (Flowery Branch, GA). Phenol red-free RPMI 1640 medium was purchased from Life Technologies (Grand Island, NY). MTT, BSA, DMSO, PMSF and protease inhibitor were purchased from Sigma-Aldrich (Dallas, TX). Acrylamide-Bis acrylamide powder and BCA kit were purchased from Fisher Scientific (Pittsburgh, PA). Phosphatase inhibitor cocktail tablet was purchased from Roche (Indianapolis, IN). Laemmli buffer and blotting buffers were obtained from Bio-Rad (Hercules, CA). PD 184161, wortmannin and AG 1478 were purchased from Cayman Chemical (Ann Arbor, Michigan). Propidium iodide staining solution was part of Apoptosis Detection Kit from BD Bioscience (San Jose, CA). Phospho-p44/42, p44/42, phosphor-AKT and AKT primary antibodies were from Cell Signaling Technology (Danvers, MA). Cyclin A (H-432), cyclin B1 (M-20), cyclin E (M-20), CDK 1 (p34) and CDK 2 (H-298) were obtained from Santa Cruz Biotechnology (Dallas, TX). EGFR siRNA and transfection reagent were obtained from Thermo Scientific (Pittsburgh PA).

Cell Culture HCC 1937 cells were obtain from ATCC and cultured in RPMI 1640 medium in the presence of 5% CO_2 at 37℃. The medium was supplemented with 10% FBS, 100 unit/ml penicillin, 100 μg/ml streptomycin and non-essential amino acids.

Cell proliferation assay The cells were seeded into 24-well plates at a density of 8×10^3 cells/well in phenol red-free RPMI 1640 medium. After 24 h, the cells were

serum-starved and cultured in 0.2% BSA-supplemented phenol red-free RPMI 1640 medium for 48 h. Following this, the cells were treated with 0.05, 0.1 or 0.5 μM $CdCl_2$ in phenol-red free RPMI 1640 for 4 days. The medium was changed every 2 days. At the end of Cd treatment, the cells were incubated with 10% MTT solution (5 mg/ml) for 3 h. The MTT solution was aspirated and formazan was dissolved by DMSO. Cell viability was determined by measuring absorbance at 570 nm minus the background absorbance at 690 nm.

Flow cytometry HCC 1937 cells were seeded in 6-well plates at a density of 4×10^4 cells/well and serum-starved as described above. The cells were treated with 0.05, 0.1, 0.5 or 1 μM $CdCl_2$ in phenol red-free RPMI 1640. At the end of Cd treatment, cells were harvested and fixed overnight in 70% ethanol. For measuring DNA content by flow cytometry, the cells were washed with PBS before staining with a solution of 18 μg/mL propidium iodide and 40 μg/mL RNase A for 40 min.

siRNA transfection HCC 1937 cells were seeded in 12-well plates at a density of 2×10^4 cells/well in RPMI 1640 medium containing 10% FBS. Cell culture medium was replaced by fresh complete medium before siRNA transfection. Each well was transfected with 2 μl EGFR siRNA (20 μM) and 2 μl transfection reagent for 48 h. The cells were serum-starved in 0.2% BSA-supplemented phenol red-free RPMI 1640 medium for 12 h before treatment with Cd.

Immunoblotting HCC 1937 cells were seeded in 6-well plates and serum-starved as described above. To detect cyclin and CDK expression, the cells were treated with $CdCl_2$ for 48 h in 0.2 % BSA-supplemented phenol red-free RPMI 1640 medium. To observe ERK and AKT phosphorylation, the cells were treated with $CdCl_2$ for up to 6

h in serum-free RPMI 1640 medium. The cells were washed with PBS before harvest and lysed in RIPA Buffer supplemented with protease inhibitor (1:1000 dilution), 2 mM PMSF and phosphatase inhibitor (10 ml/tablet). The lysate was centrifuged at 12,000 rpm for 10 min at 4℃. The supernatant was heated to 95℃ with laemmli buffer containing 5% β-mercaptoethanol. Protein concentration was measured by BCA kit and the same amount of protein was applied to each well before electrophoresis. SD Semi-dry Transfer Cell was used to transfer protein to nitrocellulose membrane at 24V for 1 h. The membrane was blocked in 5% non-fat milk for 1 h and incubated with corresponding primary antibody at 4℃ overnight. The membrane was washed 3×10 min with 0.1% PBST before incubation with the secondary antibody at 1:10000 dilution. The protein bands on the membrane were scanned by using Odyssey Infrared Imager after PBST washing for 3×10 min.

Data analysis All experiments were repeated at least three times. Flow cytometry data were analyzed by ModFit LT Software. Western blot data were analyzed and quantified by Odyssey Infrared Imaging Software. One-way ANOVA was performed by SPSS followed by Tukey's post hoc test at $p<0.05$.

RESULTS

Effect of Cd on cell growth

In order to establish that Cd promoted early stage cancer cell growth, HCC 1937 cells, a triple-negative human breast cancer cell line, was used in this study. The cells were serum-starved prior to treatment with Cd and proliferation was measured by the MTT assay. As shown in Fig. 1, there was a markedly increased the cell density of HCC 1937 cells after four days of culture in the presence of Cd. The MTT assay revealed 23 and 45% increase in cell density at 0.1 and 0.5 μM Cd, respectively, when compared to the BSA control group.

Effect of Cd on cell cycle progression

To test whether Cd induced HCC 1937 cell growth by cell cycle progression, the cells were synchronized by 48 h serum starvation and treated with various concentrations of Cd for 24 h. Cells grown in the presence of 10% FBS were used as positive control. The distribution of different cell cycle phases was determined by using flow cytometry. The results were shown in Fig. 2, treatment with 0.05-0.5 μM Cd significantly increased the proportion of cell population in the G2/M phase. At the treatment with 0.5 μM Cd, there was a 57% increase over the BSA control group. Higher concentration of Cd (1 μM) was toxic in these cells.

To determine the time-course of cell cycle progression, serum-starved cells were treated with 0.5 μM $CdCl_2$ for 12-48 h. Cells grown in the presence of 10% FBS were used as positive control. As shown in Fig. 3, between 24 to 48 h treatment with 0.5

μM Cd, there was a 49-58% increase in proportion of cell population in the G2/M phase when compared to the BSA control. The increase of cell population in G2/M phase indicated Cd accelerated cell cycle progression.

Effect of Cd on cyclins and CDKs

Cell cycle progression is precisely regulated by periodic expression of phase-specific heterodimeric protein kinases-cyclins (regulatory subunit) and cyclin-dependent kinases (CDKs) (catalytic subunit). Cyclin D/CDK4 and 6 prompt cells entering cell cycle and drive the cells going through the G1 phase. Cyclin E/CDK 2 complex is responsible for the G1/S transition. Cyclin A/CDK 2 and Cyclin B/CDK 1 drive cell through the S and G2 phases, respectively. The effect of Cd on cyclins and CDKs expression was determined in serum-starved cells treated with 0.05-1 μM Cd for 48 h. The results were shown in Fig. 4, Cd treatment significantly increased cyclins A, B, and E, and CDK 1 and 2, however, not in a concentration-dependent manner, except cyclin E. It had no effect on the cyclin D level. The cyclin and CDK data indicated that Cd accelerated the progression of HCC 1937 cells into the G2/M phase.

Effect of Cd on MAPK and PI3K activation

MAPK and PI3K signaling pathways regulate cell growth and cell cycle progression. To test whether Cd activated these kinases, the time course and concentration response of ERK 1/2 and AKT phosphorylation were determined by western blot analysis. As shown in Fig. 5A, maximal ERK 1/2 phosphorylation by 0.5 μM Cd was observed at 15 min, followed by a gradual decrease over the 2 h period. In

comparison, AKT phosphorylation peaked at 6 h after Cd treatment. As shown in Fig. 5B, treatment with 0.1-1 μM Cd resulted in up to 2.5-fold increase in ERK 1/2 phosphorylation at 15 min and up to 3-fold increase in AKT phosphorylation in 6 h.

Role of ERK and AKT in the expression of cyclins and CDKs

It is well known that MAPK and PI3K activations regulate cell cycle progression. To ascertain that Cd-induced cell cycle progression was also dependent on ERK and AKT phosphorylation, inhibitors of MEK (PD184161) and PI3K (Wortmannin) were utilized to check the effect on expression of cyclins and CDKs after Cd treatment. EGF was used as a positive control. As shown in Fig. 6, the inhibitors abrogated both Cd and EGF's effects on cyclin and CDK expressions. These results confirmed that the activation of MAPK and PI3K pathways was required for Cd-induced increases in cyclins and CDKs.

Role of EGFR on Cd-induced MAPK and PI3K activation

EGFR is a key receptor that mediates MAPK and PI3K pathways. In ER negative cells, Cd-induced kinase activation may be mediated through other membrane receptors such as EGFR. To identify the contribution of EGFR in Cd-induced cell cycle progression, an EGFR inhibitor (AG 1478) was utilized. The serum-starved cells were treated with 0.5 μM Cd or 10 ng/ml EGF in the presence or absence of 2 μM AG 1478 for 15 min or 6 h. Both Cd- and EGF-induced ERK and AKT phosphorylation were completely eliminated by AG 1478 (Fig. 7).

To further identify EGFR's role in Cd-induced ERK phosphorylation, EGFR in HCC 1937 cells was knocked down by siRNA transfection. The transfected cells were serum-starved for 12 h and then treated with 0.5 μM Cd or 1 ng/ml EGF. Mock transfected cells were used as control. As shown in Fig. 8, EGFR siRNA and AG 1478 both eliminated Cd-induced ERK phosphorylation. The blockage of EGFR-mediated MAKP and PI3K signaling will affect cell cycle progression. As shown in Fig. 9, the EGFR inhibitor decreased the Cd- and EGF-induced expressions of cyclins and CDKs. This indicated that EGFR was essential for Cd-induced cell cycle progression.

DISCUSSION

At high concentration, Cd is toxic and causes cell cycle arrest (Yang et al., 2004; Xie and Shaikh, 2006). More recent studies have reported that at micromolar concentration Cd increases cell cycle progression in prostate epithelial cell, mesangial cells, and lung epithelial cells (Bakshi et al., 2008; Xiao et al., 2009; Kundu et al., 2011; Kundu et al., 2013). The results reported in this study in breast cancer cells are similar to those reported in other types of cells.

The present study showed that Cd prompted cells to enter G2/M phase without affecting G0/G1 phase, which is similar to what was observed in the presence of FBS. The mechanism of Cd-induced cell cycle progression appears to be through regulation of cyclins A, B, and E and CDK 1 and 2. Cyclin E/CDK 2 complex is responsible for G1/S transition and Cyclin A/CDK 2 and Cyclin B/CDK 1 drive cells through S phase and G2 phase, respectively (Lim and Kaldis, 2013). Therefore, increased expression of cyclins and CDKs accelerated cell progressing to G2/M phase. In the present study, Cd did not increase cyclin D expression, which supports the cell cycle results. No significant change in the proportion of cell population in G0/G1 phase was detected because HCC 1937 cells were transformed cells which had deregulated control of entering cell cycle (Klein and Assoian, 2008). So extracellular stimuli neither significantly affected the cells progression into G1 phase, nor increased the corresponding cyclin D expression.

The cell cycle results indicated that Cd prompted cells to enter G2/M phase by activating mitogenic signals. Inhibition of MAPK and PI3K by PD 184161 and

Wortmannin resulted in suppressing the expression of cyclins and CDKs. Extensive studies report that progression into G2/M phase requires precise control of cell cycle machinery, which is regulated by kinases involved signal transduction through a series of steps. Activation of MAPK pathway leads to enhanced activity of transcription factors such as Myc, AP-1, E2F1 and STAT3 (Lo et al., 2005; Hanada et al., 2006; Lo et al., 2006). These transcription factors promote expression of cyclins and CDKs (Biswas et al., 2000; Major et al., 2004; Shelton et al., 2004; Laoukili et al., 2008; Park et al., 2008; Chen et al., 2009). Cyclin A/CDK 2 and Cyclin B/CDK 1 complexes sequentially phosphorylate FoxM1 to translocate into the nucleus and to transcribe executor of mitosis, such as cyclin B, and centromere protein F (Major et al., 2004; Laoukili et al., 2008; Park et al., 2008; Chen et al., 2009). Meanwhile, activation of the PI3K/AKT pathway induces the cell to progress to late G2 phase, but the mechanism is not fully understood (Liang and Slingerland, 2003; Ornelas et al., 2013). Although these details of mitogen-activated pathways were not investigated in the present study, the major cell cycle regulatory pathways are known to be well conserved (Shelton et al., 2004; Ma et al., 2010).

Beside MAPK activation which has been described above, the activation of PI3K is commonly regarded as an anti-apoptotic signal because AKT phosphorylation induces anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and survivin expression (Siddiqa et al., 2008). The sustained AKT activation contributes to pro-survival by directly inactivating caspase family (Longo et al., 2008). In this study, the relative long duration of AKT phosphorylation by Cd treatment explained Cd's pro-proliferative effect in HCC 1937 cells, which supported pro-survival effect of Cd in

MCF7 (Brama et al., 2007). Also, similar phosphorylation pattern of AKT was observed in cobalt promoted cell cycle progression (Ryu et al., 2010).

Cd-induced MAPK and PI3K activation was previously studied in ER-positive cell lines such as MCF7 and T47D, which was attributed to Cd's estrogenic effect on ER (Brama et al., 2007; Ponce et al., 2013). In ER-negative cells, Yu et al. (2010) reported that GPR30 mediated Cd's estrogenic signaling. GPR30 functions by activating EGFR which causes rapid signaling of downstream cascade such as MAPK and PI3K (Filardo et al., 2002; Navolanic et al., 2003). The present study showed that Cd's mitogenic effect in the triple-negative breast cancer cells required EGFR-mediated MAPK and PI3K activation and subsequent expression of cyclins and CDKs.

In clinical therapy, EGFR is an important biomarker for triple negative breast cancer patients who are only responsive to EGFR/HER2 inhibitors (Chen and Russo, 2011). It is reported that early stage ductal carcinoma with triple-negative receptor phenotype tends to proceed to invasive breast cancer (Bryan et al., 2006). Therefore, HCC1937 cell model renders a rational approach to study association between triple-MAPK and PI3K signaling pathways negative breast cancer and Cd exposure. Recent reports in lung epithelial cells and rat mesangial cells supported that Cd-induced cell proliferation was mediated via EGFR. The studies on lung epithelial cells showed that Cd activated EGFR and promoted an initial inflammatory response. With activated EGFR, NF κ B upregulated the expression of pAKT, pSTAT3 and cyclin D1 which contributed to cell proliferation and cell cycle progression (Kundu et al., 2011; Kundu et al., 2013). The study on rat mesangial cells showed that Cd influences

Ca^{2+}/calmodulin-dependent kinases which resulted in activation of EGFR and downstream Src, ERK and AKT phosphorylation (Xiao et al., 2009).

EGFR and β1 integrin are reported to physically interact and are co-regulated and share most signaling pathways, such as PI3K/AKT and MAPK/ERK, Rho/Rac GTPase (Jeanes et al., 2012). It has been reported that Cd has high binding affinity with cysteine containing motifs in EGFR and integrin (Ahmadibeni et al., 2007). It is possible that Cd may cause cell proliferation through promoting the interaction between EGFR and integrin.

In conclusion, the present study showed that submicromolar concentrations of Cd cause breast cancer cell proliferation by regulating cell cycle progression. By using kinase inhibitors, it was observed that Cd induces the expression of cyclins and CDKs by activation of MAPK and PI3K pathways. Furthermore, these mitogenic effects of Cd were mediated through its upstream effect on EGFR.

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FIGURES

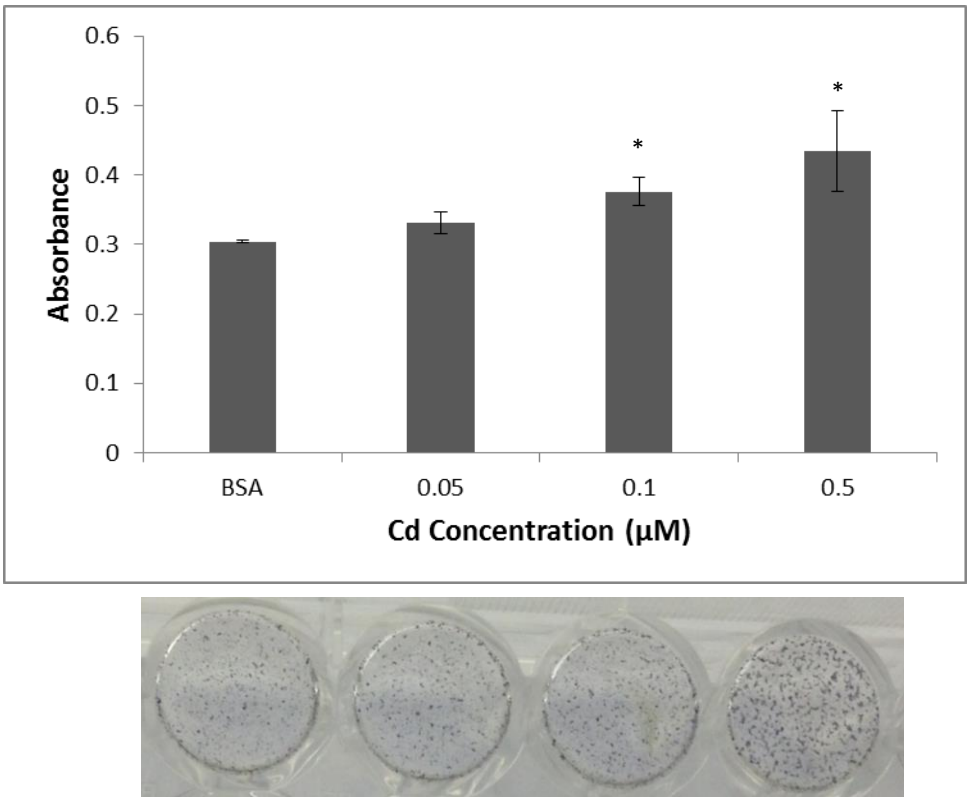


Fig. 1. Effect of Cd treatment on growth of HCC 1937 cells. The cells were seeded in RPMI 1640 containing 10% FBS medium and starved in 0.2% BSA for 48 h. Then the cells were treated with Cd for 4 days. Cell growth was measured by the MTT assay. Data from three independent experiments were plotted as mean ± SE. The image under the graph shows one well of MTT stained cells/treatment. *Significantly different from the BSA control group ($p<0.05$, $n=3$).

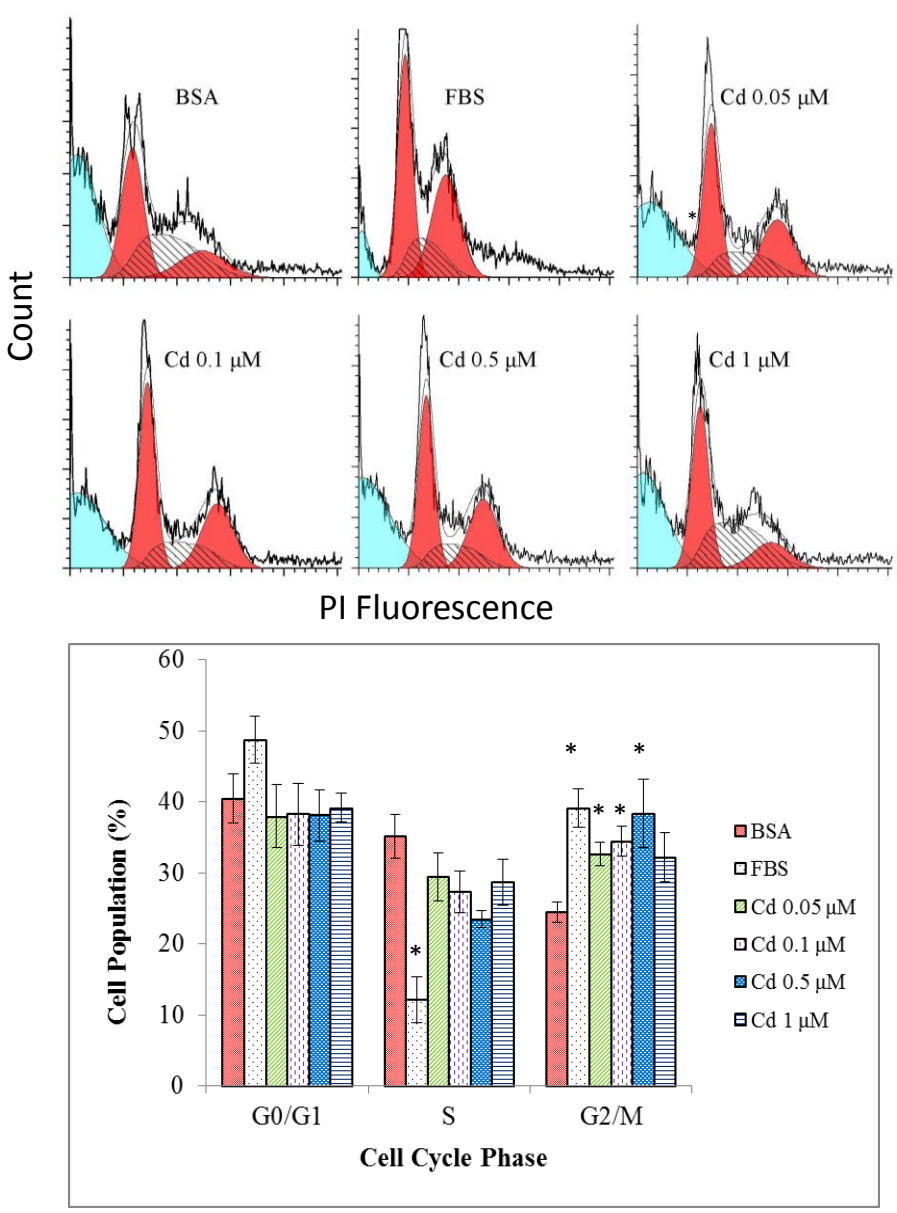
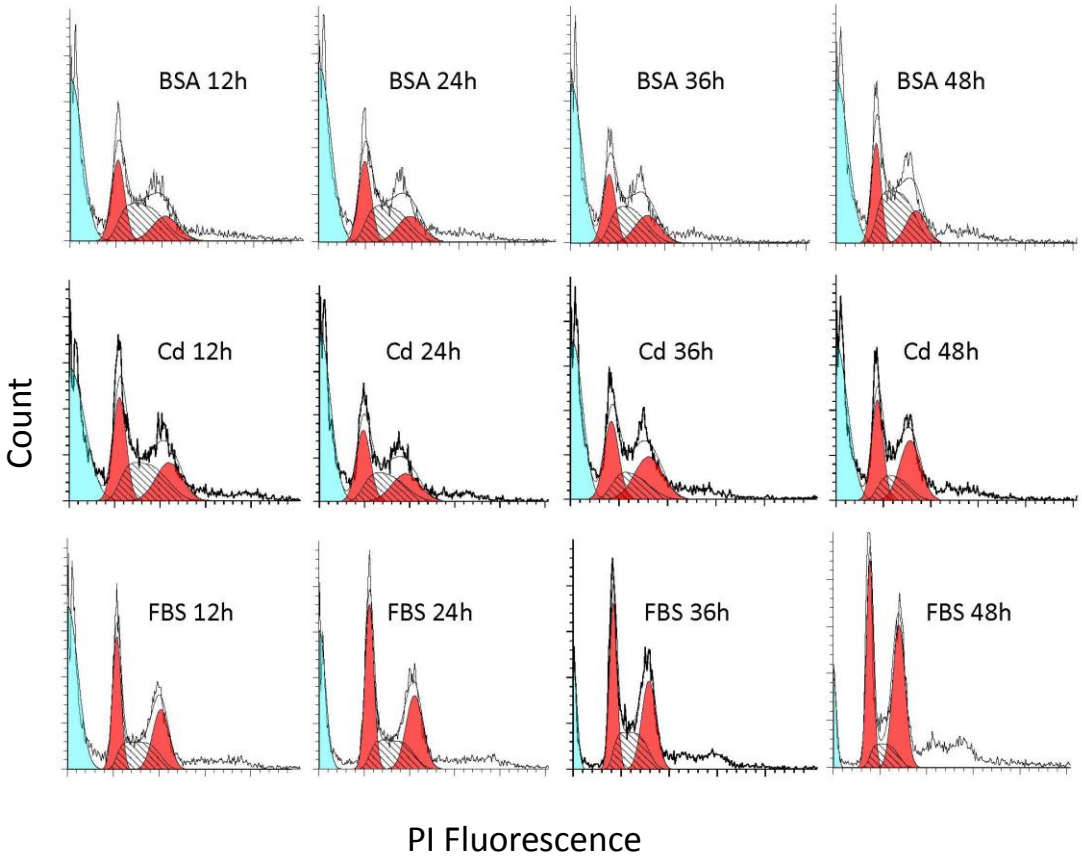


Fig. 2. Effect of Cd treatment on cell cycle progression. HCC 1937 cells were seeded in 10% FBS for 24 h and serum-starved for 48 h. Then cells were treated with 0.05, 0.1, 0.5 or 1 µM $CdCl_2$ for 24 h. Cells were harvested and fixed at the end of treatment. DNA was stained with PI. Proportion of cells in various phases of cell cycle was plotted as PI staining (y axis) versus DNA content (x axis). The mean ± SE of cell phase fraction in G0/G1, S and G2/M phase was plotted as bar graph. *Significantly different from the BSA control group ($p<0.05$, n=3).

A


BSA 12h
BSA 24h
BSA 36h
BSA 48h
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FBS 12h
FBS 24h
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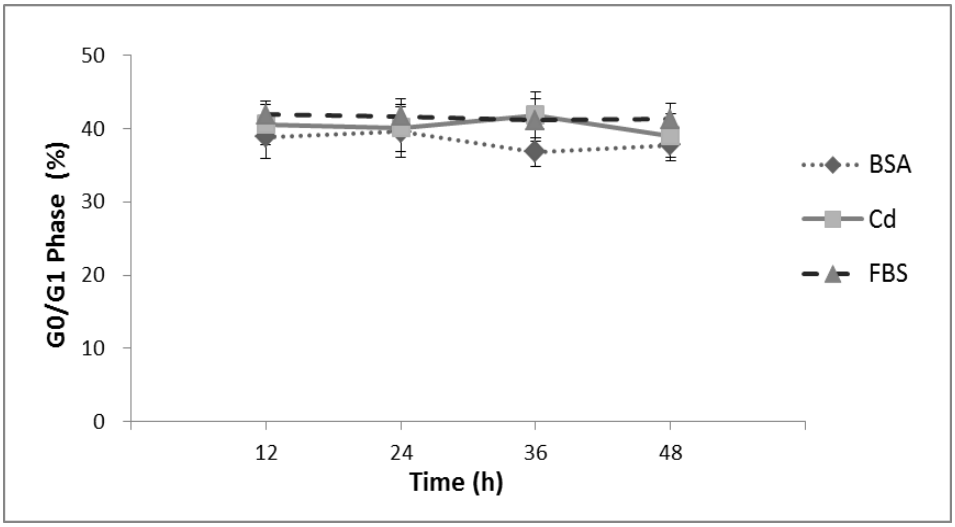
FBS 48h
Count
PI Fluorescence

B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G0/G1 Phase (%)
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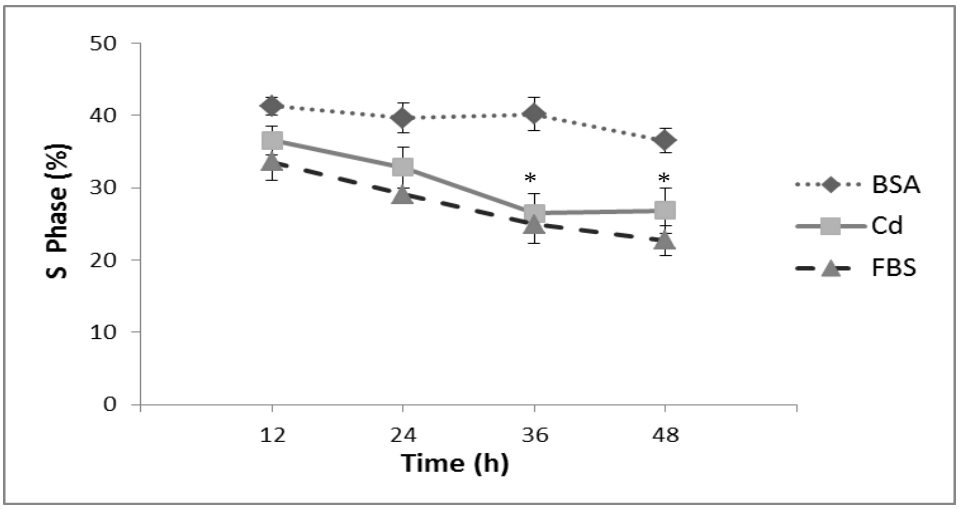
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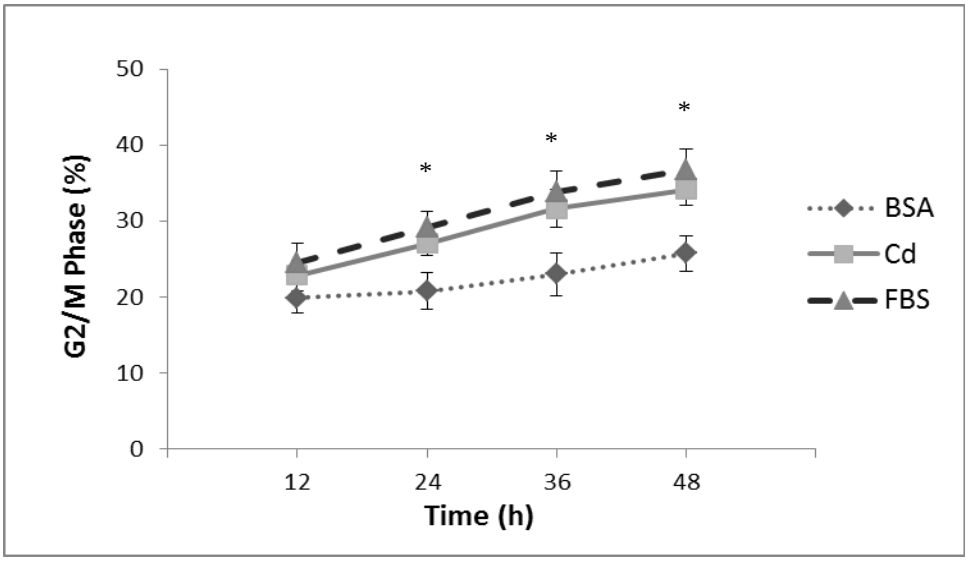
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G2/M Phase (%)
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Time (h)
BSA
Cd
FBS

Fig. 3. Time-course of cell cycle progression in response to Cd treatment. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.5 μM $CdCl_2$ for 12, 24, 36 or 48 h. BSA and FBS were negative and positive controls, respectively. Cells were harvested and fixed at the end of treatment. DNA was stained with PI. (A) Proportion of cells in various phases of cell cycle was plotted as PI staining (y axis) versus DNA content (x axis). (B) The mean ± SE of cell phase fraction in G0/G1, S and G2/M phase was plotted as line graph. *Significantly different from the BSA control group ($p<0.05$, $n=6$).

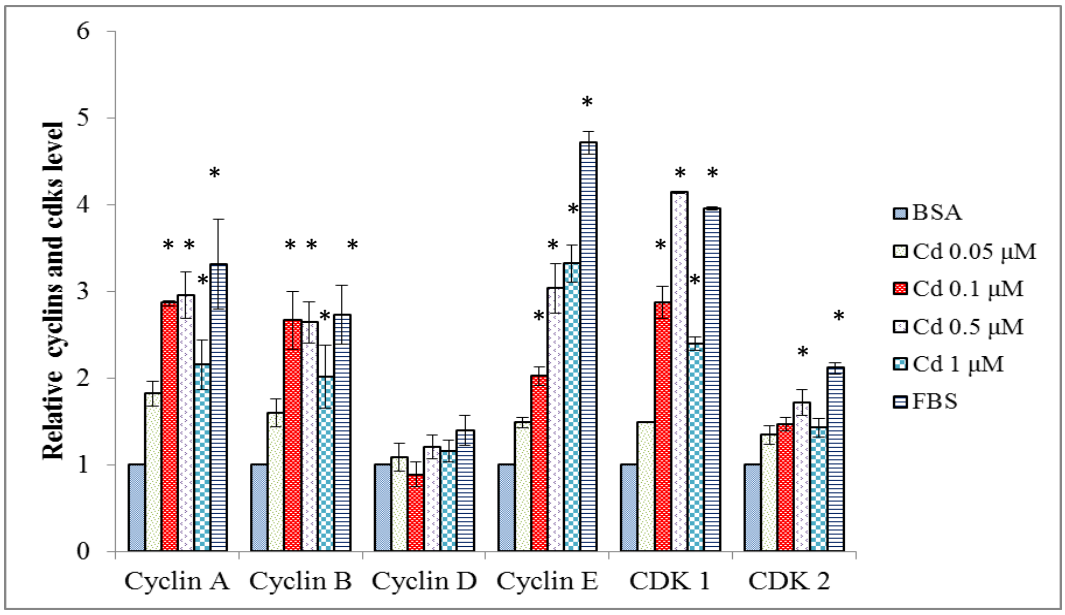
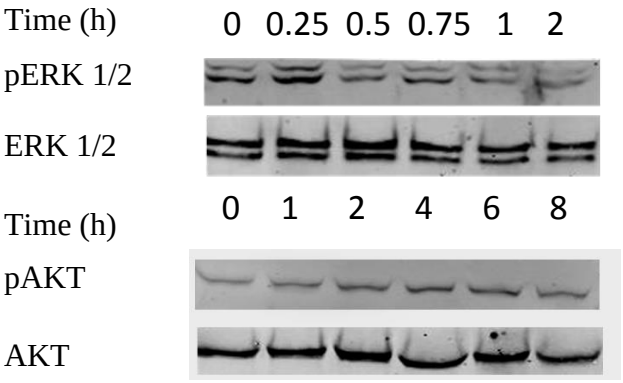


Fig. 4. Cyclin and CDK expression in response to Cd treatment. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.05, 0.1, 0.5 or 1 μM $CdCl_2$ for 48 h. Cell lysates were analyzed for cyclins A, B, D,and E, and CDK 1 and 2. Band intensity relative to the BSA control was plotted as bar graph showing mean ± SE. *Significantly different from the BSA control group ($p<0.05$, n=3).

A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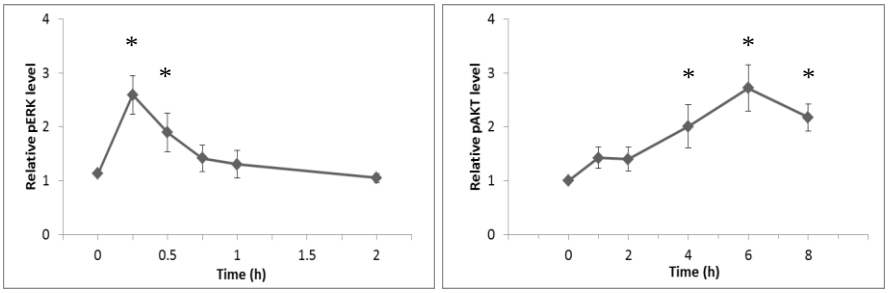
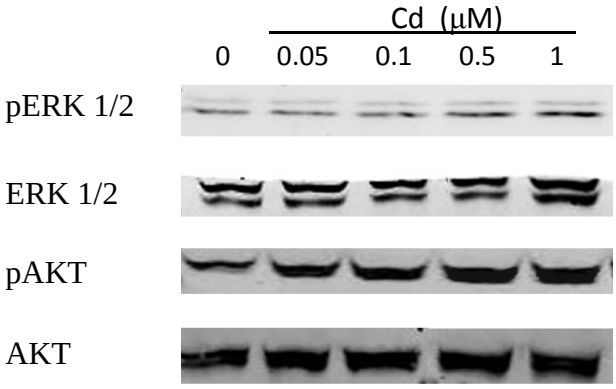
pERK 1/2
ERK 1/2
Time (h)
0 1 2 4 6 8
pAKT
AKT


Relative pERK level
Time (h)
Relative pAKT level
Time (h)

B


Cd (μM)
0 0.05 0.1 0.5 1
pERK 1/2
ERK 1/2
pAKT
AKT

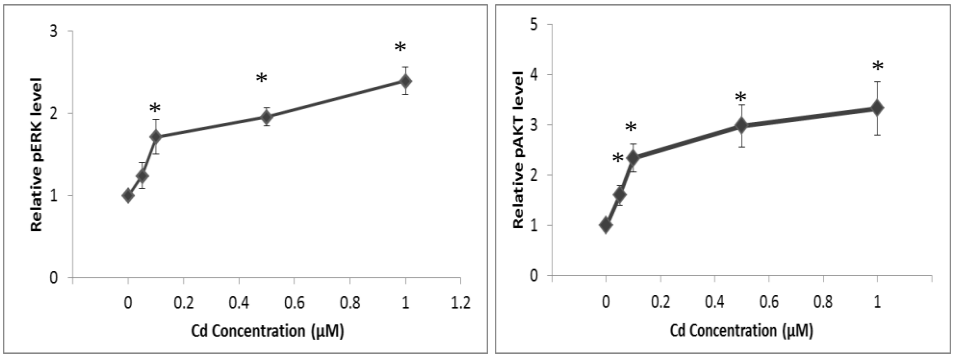

Relative pERK level
Cd Concentration (μM)
Relative pAKT level
Cd Concentration (μM)

Fig. 5. Time-course and concentration-response patterns of Cd-induced ERK and AKT phosphorylation. (A) Time-course. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.5 μM $CdCl_2$ for 0.25, 0.5, 0.75, 1 or 2 h. Cell lysates were analyzed for pERK 1/2. Cell lysates for cells treated with 0.5 μM $CdCl_2$ for 1, 2, 4, 6 or 8 h were analyzed for pAKT. (B) Concentration-response. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.05, 0.1, 0.5 or 1 μM $CdCl_2$. Cells were harvested after 15 min to analyze pERK 1/2 and at 6 h to analyze pAKT. Band intensity relative to the control was plotted as mean ± SE. *Significantly different from the control group ($p<0.05$, $n=3$).

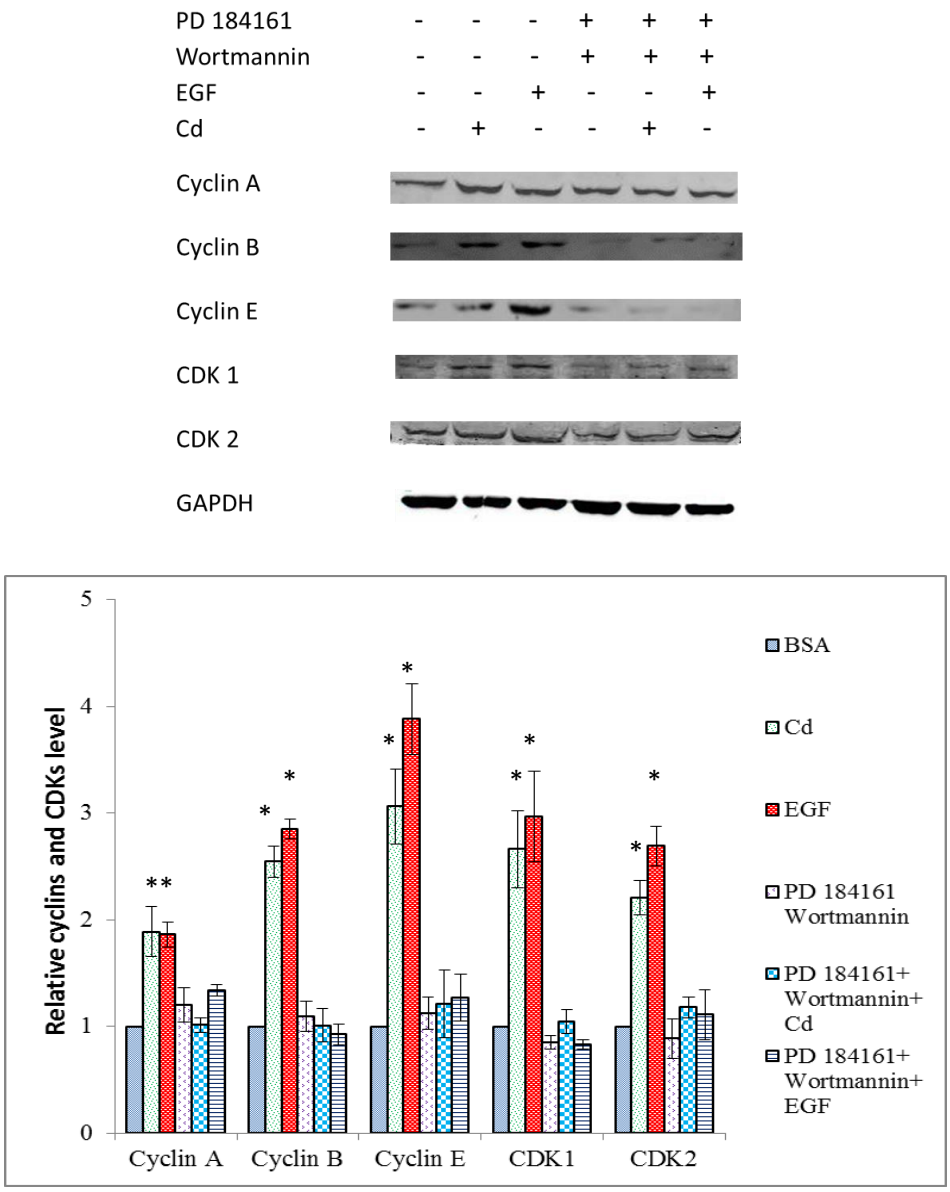


Fig. 6. Effect of ERK and AKT inhibitors on expression of cyclin and CDK. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.5 μM $CdCl_2$ in the presence or absence of 1μM PD 184161 and 1μM Wortmannin for 48 h. Cell lysates were analyzed for cyclins A, B , E and CDK 1, 2. Band intensity relative to the BSA control was plotted as bar graph showing mean ± SE. *Significantly different from the BSA control group ($p<0.05$, n=3).

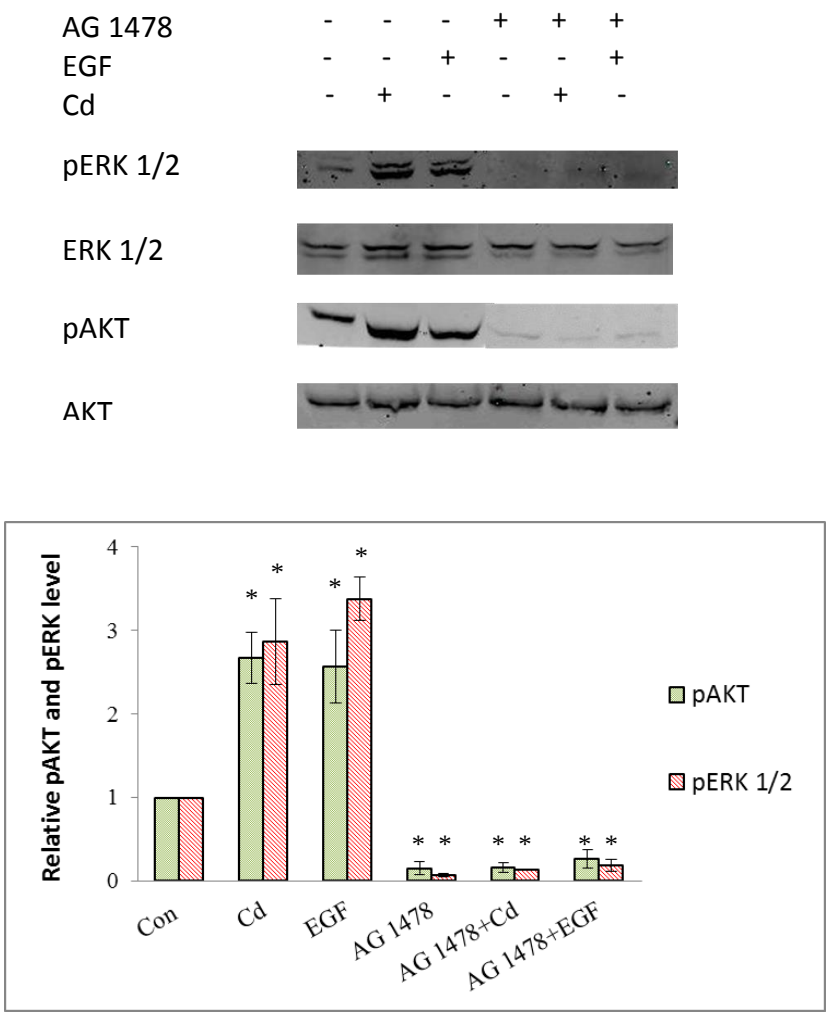


Fig. 7. Effect of EGFR inhibitor on ERK and AKT phosphorylation. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.5 μM $CdCl_2$ in the presence or absence of 2 μM AG 1478 for 15 min for analyzing pERK 1/2 and for 6 h for analyzing pAKT. Band intensity relative to the control was plotted as bar graph with mean ± SE.*Significantly different from the control group ($p<0.05$, n=3).

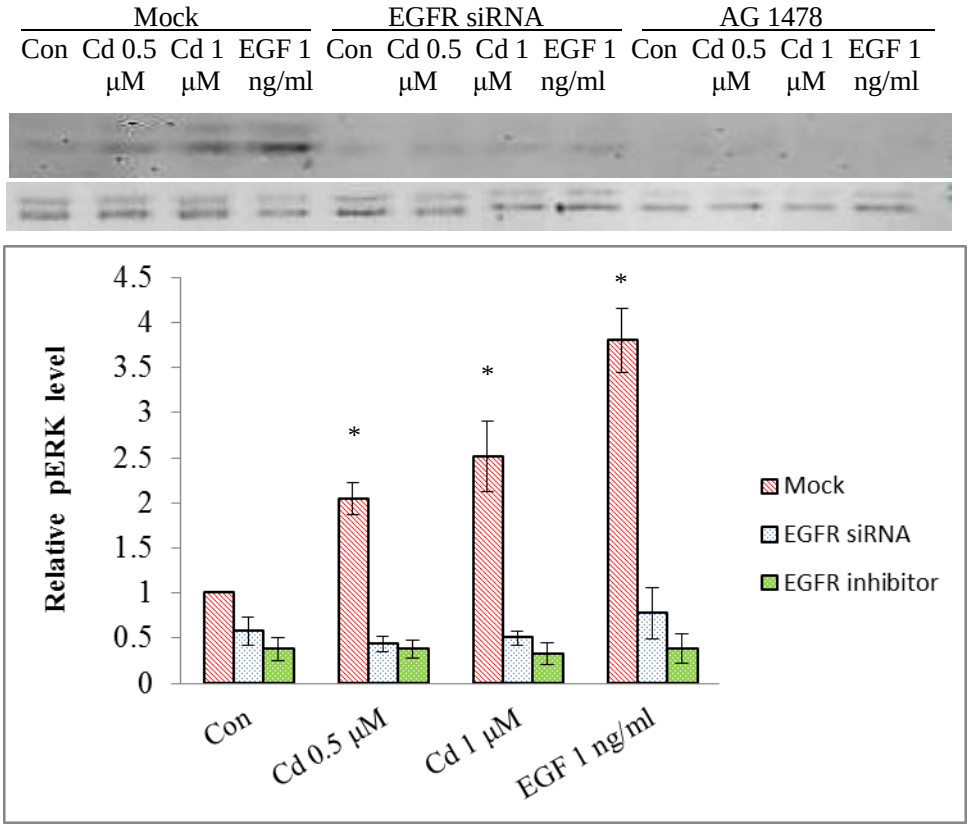
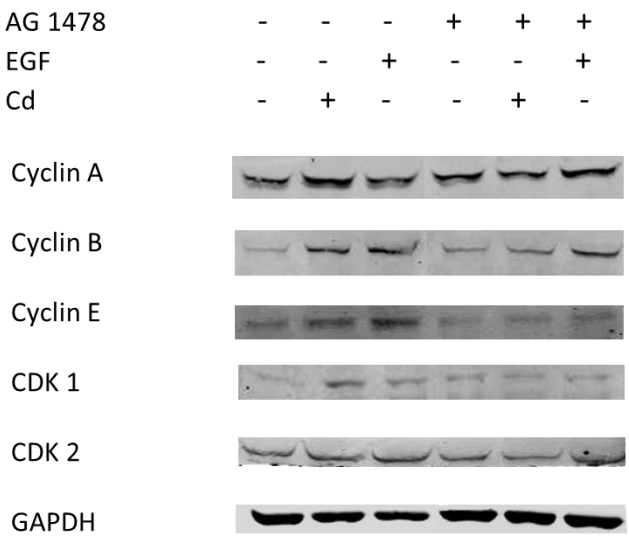


Fig. 8. Effect of EGFR inhibitor and siRNA on ERK phosphorylation. HCC 1937 cells were seeded in RPMI 1640 containing 10% FBS for 24 h and transfected with EGFR siRNA for 48 h. Cells were serum starved in 0.2% BSA for 12 h before treating with 0.5, 1μM $CdCl_2$ or 1 ng/ml EGF in the presence or absence of 2 μM AG 1478. Cells were harvested after 15 min treatment for analyzing pERK 1/2. Band intensity relative to the control was plotted as bar graph with mean ± SE.*Significantly different from the control group ($p<0.05$, n=3).



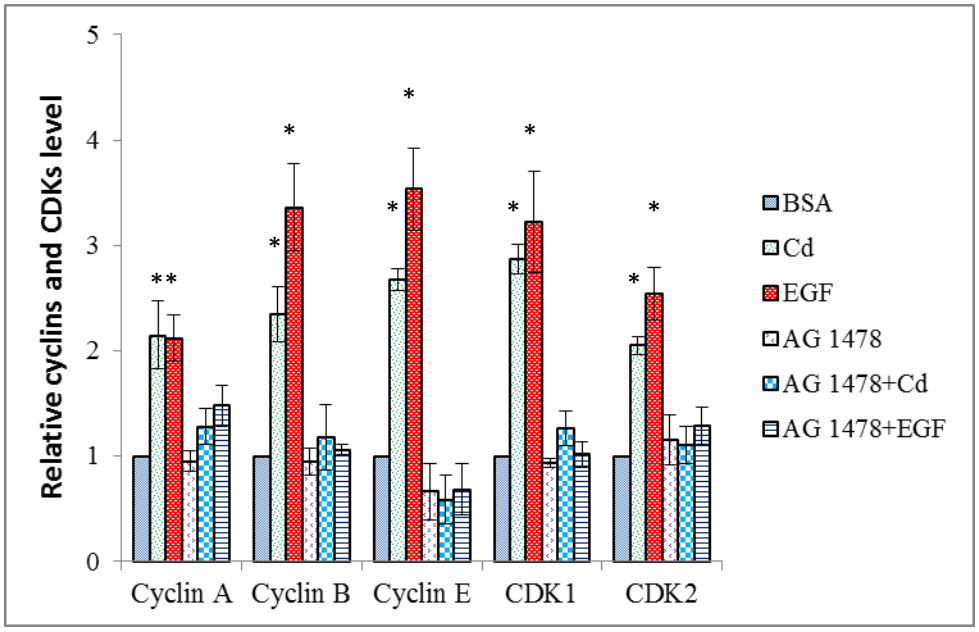


Fig. 9. Effect EGFR inhibitor on the expression of cyclins and CDKs. HCC 1937 cells were seeded in 10% FBS for 24 h and serum-starved for 48 h. Cells were treated with 0.5 μM $CdCl_2$ with or without 2 μM AG 1478 for 48 h and analyzed for cyclins A, B, and E, and CDK 1 and 2,. Band intensity relative to the control was plotted as bar graph with mean ± SE.*Significantly different from the control group ($p<0.05$, n=3).

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