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Evidences for involvement of estrogen receptor induced ERK1/2 activation in ovarian cancer cell proliferation by Cadmium Chloride

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ABSTRACT

Cadmium (Cd) as a human carcinogen and one of the most toxic industrial and environmental pollutant mimics the estrogenic effects in cell proliferation. So, it might have a role in the incidence and etiology of hormone-related cancers such as ovarian cancer as the most lethal gynecologic malignancy. This study aimed to evaluate the estrogenic effect and underlying mechanism of Cd in ovarian cancer cell line proliferation. OVCAR3 and SKOV3 cell lines were treated with different concentrations of $CdCl_2$ (0- 50 μM). Cell proliferation was analyzed using MTT and BrdU assay. To evaluate the estrogenic effect of Cd, the cells were pre-incubated with estrogen receptor (ER) antagonist ICI 182,780. The expression of ER was determined using western blotting method. Real-time RT-PCR method was used to assess c-fos, c-jun and FOXO3a mRNA level. The results showed that Cd has an estrogenic proliferative effect at nM concentration range and ICI 182,780 significantly reversed the $CdCl_2$-induced cell proliferation. Cd also increased the expression of ERs. Cd exposure induced activation of p-ERK1/2 in these cells. Cd also intensified c-jun, c-fos, and FOXO3a mRNA expression. Taken together, the current work suggests that Cd induces ovarian cancer cell proliferation in an ER-dependent mechanism induced ERK1/2 activation pathway. Understanding of downstream targets by which Cd deregulates cell proliferation can be noteworthy to define its underlying carcinogenesis mechanism.

1. Introduction

Cadmium (Cd), a heavy metal and one of the most toxic transition metals, has been classified as an important human carcinogen by the International Agency for Research on Cancer (IARC) and the United States National Toxicology Program (NTP) (Aquino et al., 2012; Brama et al., 2007; Nagata et al., 2013). Following the expansion of urbanization and industrialization, the amount of environmental Cd has increased (Nampoothiri and Gupta, 2006). Different investigations have shown that many countries and regions in the world are threatened to varying degrees of Cd contamination (Byrne et al., 2013; Chan et al., 2006). One of Cd chemical forms, Cadmium Chloride ($CdCl_2$), is used extensively in chemical engineering, electroplating, and nuclear industries and it can easily release into the environment and contaminate water, air, foods, and plants. The presence of this toxic metal in cigarettes and environmental air pollution is well documented and suggests its role in the increased incidence of several human cancers. It accumulates and persists 15–20 years in the human soft tissues and leads irreversible damage to many organ systems including the female reproductive systems (Satarug et al., 2011). It can promote the proliferation of cancer cells and induce cancer by multiple mechanisms such as oxidative stress, aberrant gene expression, inhibition of DNA repair and apoptosis (Julin et al., 2011; Jin et al., 2003). Moreover, some studies have demonstrated that Cd as a metalloestrogen can bind to estrogen receptor (ER) and mimics the estrogen activity (Aquino et al., 2012; Johnson et al., 2003; Siewit et al., 2010). Exposure to environmental endocrine disruptors and the ability of these pollutants to bind ER were drawn the hypothesis of their role in the carcinogenesis, incidence, and etiology of hormone-related cancers such as breast, uterine, prostate and ovarian cancers (Brama et al., 2007; Byrne et al., 2013). Ovarian cancer as the most lethal gynecologic malignancy causes over 140,000 deaths annually worldwide (Sieh et al., 2013) and the survival rate of this disease is poor with 50% case fatality rate because of the diagnosis in the advanced stages (Jemal et al., 2008; Vargas, 2014). Epidemiological data have demonstrated that endogenous and exogenous estrogens can be effective in ovarian cancer pathogenesis (Lau et al., 1999).

Two isoforms of intracellular ER, ERα, and ERβ, after binding to estrogens and translocate to the nucleus, activate some transcription factors and signaling pathways. The deregulation of estrogenic

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pathways can elevate transcriptional activity contributed to the development of cancer. Expression of ER is associated with a degree of differentiation and cell proliferation speed of tumor (Guo and Sonenshein, 2004; Oh et al., 2001).

Several investigations indicated that Cd as a metaloesterogen can bind and activate ER and induce not only expression of specific ER target genes but also other estrogen-like effects such as increasing the growth of breast cancer cells (Brama et al., 2007), ovarian cancer cells (Nampoothiri and Gupta, 2006) and increased cell proliferation in rat uterine cells (Aquino et al., 2012). Some reports show that Cd stimulates transcription of several estrogen-inducible genes like progesterone receptor, some proto-oncogenes and transcription factors such as c-fos, c-jun, and FOXO3a. Moreover, Cd up-regulates some signaling pathway kinases and increases phosphorylation of extracellular signal-regulated kinases involved in regulation of cell growth/proliferation, metastasis, and apoptosis (Brama et al., 2007). The deregulation of ERK/MAPK pathway plays a critical role in carcinogenesis and provides estrogen-dependent signaling in tumor growth (Filardo et al., 2002). Recent studies have shown that ERK cascade activity increases almost in one-third of all human cancers. Moreover, the inhibition of components of this cascade can be consider as an important anti-tumor strategy (Roskoski, 2012). Although some studies have demonstrated that Cd accumulates and concentrates in ovarian tissue, there are still no studies on the association between Cd exposure and the etiology of ovarian cancer. Furthermore, the mechanism whereby Cd causes the cell proliferation remains poorly understood and no uniform outcome has been pulled out with respect to the cellular signaling pathways suggested in proliferation induced by Cd. So this study aims to evaluate the estrogenic effect of Cd on the cell proliferation of ovarian cancer and determine the underlying mechanism.

2. Material and methods

2.1. Chemicals and antibodies

Culture media and growth supplements were purchased from Gibco, Germany. $CdCl_2$, Estradiol (E2) and PD 98059 were obtained from Sigma–Aldrich Company. BrdU kit, cDNA synthesis kit and RNX-Plus were purchased from Roche (Mannheim, Germany), Takara Bio INC and Sinaclon (RN7713C) respectively. ICI 182,780, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) human antibody (G-9: sc-365,062), goat anti-mouse IgG1-HRP (sc-2005), mouse monoclonal IgG1 ERα (sc-73,479) were purchased from Santa cruse and mouse anti-human monoclonal IgG1 ERβ and phosphorylated-ERK1/2 (p-ERK1/2) mouse monoclonal anti-human from Biorad and Cell signaling respectively.

2.2. Cell cultures

Human ovarian cancer cell lines, OVCAR3 and SKOV3, were obtained from National Cell Bank of Iran (NCBI. Pasture Institute of Iran) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2, 95% air at 37 °C. The experiments were done in RPMI medium supplemented with cFBS (charcoal-treated FBS) 1%. For the preparation of stock solutions, $CdCl_2$ was dissolved in deionized water; E2 and ICI 182,780 were dissolved in ethanol. The stock solutions were sterilized by filtration and diluted by RPMI medium.

2.3. MTT viability assay

The viability of treated cell lines was determined by MTT assay. 5×10^3 cells/well were seeded in 96-well plates in RPMI medium containing 10% FBS and incubated for 24 h to reach 60–80% confluence. To evaluate Cd effect on cell viability, fresh medium containing 1% FBS was added and the cells were treated by $CdCl_2$ (0.00001–50 µM) for 48 h. Afterward, 20 µl MTT tetrazolium salt (2 mg/ml) was added to wells and the cells were incubated for 4 h. MTT tetrazolium salt was converted to color-dense formazan crystals by mitochondrial dehydrogenases of viable cells. After incubation, the medium was removed and DMSO was added to the wells. It dissolved the insoluble formazan crystals and made a violet color. Each point represents four repeats, each triplicate. To determine the percentage of viable cells, absorbance values at 570 nm was measured and the cell viability was calculated by the formula (mean OD of treated cells/mean OD of control cells) × 100.

2.4. BrdU cell proliferation assay

Cell proliferation was measured by BrdU kit, a colorimetric immunoassay based on bromodeoxyuridine incorporation. The method was performed according to the manufacturer's protocol. 5 × 103 cells/well was seeded in 96-well plates. After 24 h, the cells were treated by $CdCl_2$ (0.001, 0.01, 0.1 µM) and E2 (1 µM) in the presence and absence of ICI 182,780 (10 µM) for 48 h. Then 10 µl of BrdU labeling solution was added to each well and the cells were incubated again for 4 h. During the labeling period, BrdU as a pyrimidine analogue of thymidine is incorporated into DNA during S-phase of the cell cycle. After the removal of BrdU labeling solution, cells were fixed and denatured by the kit's FixDenta solution for 30 min at room temperature. FixDenta solution denaturized DNA. To the incorporated, BrdU was accessible for detection by the peroxidase-conjugated anti-BrdU antibody. So cells were incubated for 90 min by peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD). Anti-BrdU-POD bound to BrdU incorporated into newly synthesized cellular DNA. After washing the unbound anti-BrdU-POD, the color reaction was developed for 3–5 min with the substrate solution, and stopped by adding 25 µl of sulfuric acid (1 M), and optical density of the samples were determined using a spectrophotometric micro plate reader at 370 nm. Each point represents 3 repeats, each triplicate.

2.5. Real time RT-PCR

mRNA was isolated from the cells using Trizol reagent (Invitrogen, USA) and reversely transcribed using the first-strand cDNA synthesis kit according to the manufacturer's protocol. Quantitative real-time PCR assay of cDNA (1 µl) was carried out using the SYBR Green kit (Qiagen) in an ABI step one plus system. 40 cycles of PCR were performed under the condition of denaturation at 95 °C for 15 s, annealing at 60 °C for 25 s, and elongation at 72 °C for 20 s. Amplification specificity was confirmed by a melting point curve generated at the end of each PCR reaction. The relative expression level of mRNA genes was normalized by the endogenous housekeeping gene GAPDH and determined using the $2^{-\Delta\Delta Ct}$ analysis method. Each point represents 2 repeats, each duplicate. The used primers for Real-time PCR are shown in Table 1.

2.6. Western blot analysis

ERα, ERβ, and p-ERK1/2 protein contents were detected by western

Table 1
Sequences of the primer pairs genes used for Real-time-PCR.

Oligo Name	Oligo Sequence 5'— > 3'
GAPDH forward	CTCCCGCTTCGCTCTCTG
GAPDH reverse	TCCGTTGACTCCGACCTTC
FOXO3a forward	CCC AGC CTA ACC AGG GAA GT
FOXO3a reverse	AGC GCC CTG GGT TTG G
c-fos forward	GGATAGCCTCTCTTACTACCAC
c-fos reverse	TCCTGTCATGGTCTTCACAACG
c-jun forward	AAGGAAGCTGGAGAGAATCG
c-jun reverse	CTGTTTAAGCTGTGCCACCT

blotting method. The cells were incubated with various concentrations of $CdCl_2$ and E2 for 24 h. The cells were suspended in an ice-cold RIPA lysis buffer (20 mM Tris–HCl pH 7.5, 0.5% Nonidet P-40, 0.5 mM PMSF, 100 mM b-glycerol 3-phosphate and 0.5% protease inhibitor cocktail) and were vortexed every 15 min for 2 h. The extracts were centrifuged (14,000 rpm, 10 min, 4 °C). The protein concentration of each lysate was determined by Bradford Protein Assay (Bio-Rad Laboratories, Inc., USA). An Equal amount of protein in each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech.). Membranes were incubated with blocking buffer containing 5% non-fat dry milk in PBST (PBS + 0.1% Tween 20) for 2 h at room temperature. Membranes were incubated overnight with mouse monoclonal antibody as a primary antibody against ERα, ERβ, p-ERK1/2 at 4 °C and washed three times with PBST. Then Membranes were incubated with goat anti-mouse IgG1-HRP (sc-2005) as a secondary antibody for 2 h at room temperature. After washing with PBST, the proteins were detected by ECL detection reagent from Bio-Rad. The expression of GAPDH was used as an internal reference. Quantitative analysis of relative level of ERs expression was performed by image j software.

2.7. Statistical analysis

Mean of replicate wells and then, mean of independent repeats from measurements was calculated. Data were presented as means ± standard deviation (S.D.). The statistical analysis was performed using SPSS18.0 for a nonparametric test of variance between groups (ANOVA) followed by Dunnett's test and independent sample *t*-test. The significant difference was statistically considered as $p < .05$.

3. Results

3.1. Cadmium had a biphasic effect on cell proliferation of ovarian cancer cell lines

Some studies have shown that Cd can stimulate cell proliferation (Byrne et al., 2013; Nagata et al., 2013), so this study evaluates the stimulation of ovarian cancer cell lines proliferation by $CdCl_2$. OVCAR3 and SKOV3 cell lines were seeded in RPMI medium containing 1% FBS and 5% charcoal and exposed to $CdCl_2$ (0.01 nM- 50 μM) for 48 h. Cell growth was analyzed using MTT assay. As shown in Fig. 1A and B. nM concentrations of $CdCl_2$ significantly enhanced the cell growth compared to control (Cd 0 μM). The maximum viability was observed at 1 nM (0.001 μM) of $CdCl_2$ (133 ± 9 in OVCAR3; $p < .05$ and 132 ± 3.4 in SKOV3; $p < .05$). However, higher doses of $CdCl_2$ (μM range) revealed the cytotoxic effect on OVCAR3 and SKOV3 cell lines and displayed a significant reduction in cell growth compared to control (72 ± 7.7–28% in OVCAR3 and 79 ± 8.2–21% in SKOV3 cells at 50 μM $CdCl_2$; $p < .05$). A significant difference was observed between the proliferation of cells at highest (50 μM) and lowest (0.001 μM) $CdCl_2$ concentrations ($p < .05$). Therefore Cd via biphasic dose-response phenomenon induced cell proliferation at nM concentrations, but considerably inhibited cell growth at higher concentrations.

3.2. Cadmium increased proliferation in ovarian cancer cell lines through ERs

Since Cd significantly increased ovarian cancer cell proliferation at nM concentrations, this range was selected for the subsequent experiments. BrdU incorporation assay also determined cell proliferation. The results showed (Fig. 2A and B) that $CdCl_2$ significantly stimulated cell proliferation at lower concentrations (0.001–0.1 μM) in comparison with control in OVCAR3 (143 ± 15 for 0.1 μM, 154 ± 8.9 for 0.01 μM, 166 ± 9.3 for 0.001 μM and 177.6 ± 6.6 for E2) and SKOV3 (105 ± 2.8 for 0.1 μM, 112.7 ± 4.8 for 0.01 μM, 132 ± 11.7 for 0.001 μM and 117 ± 6.6 for E2). The maximum proliferative response was observed at 0.001 μM $CdCl_2$. We also used E2 as a natural agonist of ER to compare with proliferative effects of $CdCl_2$ in ovarian cancer cell lines.

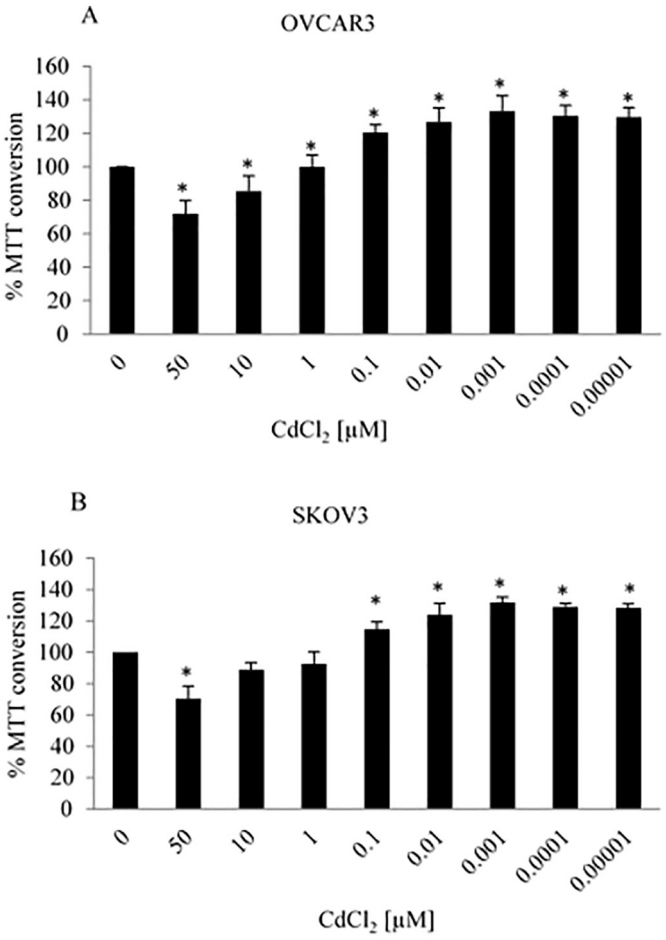

Fig. 1. $CdCl_2$ showed a biphasic effect on cell viability of ovarian cancer cell lines. OVCAR3 (A) and SKOV3 (B) cells were treated with $CdCl_2$ (0.00001 - 50 μM) and cell viability was analyzed by MTT assay. Results are represented as mean ± S.D. * shows significant difference vs. Cd [0] ($p < .05$).

Moreover, it has been reported that Cd could mimic some functions of estrogen via ER in the hormone-dependent cancers (Geffroy et al., 2005), so we also assessed the estrogenic activity of Cd in ovarian cancer cell lines. To evaluate an ER-dependent mechanism in the Cd-induced ovarian cell proliferation, the cells were pre-incubated with ICI 182,780 (10 μM) as an ER antagonist for 1 h. ICI 182,780 significantly inhibited $CdCl_2$-induced proliferation when compared to $CdCl_2$-treated cells without ICI 182,780. As illustrated in Fig. 2A following exposure to ICI 182,780, the growth effects of $CdCl_2$ on OVCAR3 cell lines significantly reduced (% 68 for 0.1 μM, %65 for 0.01 μM, %88 for 0.001 μM and %100 for E2). ICI 182,780 also inhibited cell proliferation in SKOV3 cell (%30 for 0.001 μM, %14 for 0.01 μM, %6.5 for 0.1 μM and %18.5 for E2; Fig. 2B). The inhibitory effect of ICI 182,780 on $CdCl_2$-induced proliferation as well as E2 suggests that $CdCl_2$ increases cell proliferation by an ER-dependent mechanism.

3.3. Cadmium induced ERα and ERβ protein expression in ovarian cancer cell lines

Since our data showed that Cd mimicked E2 action in OVCAR3 and SKOV3 cells and ICI182, 780 blocked the Cd-stimulated effects; we determined whether this metal can modulate ERs expression. With this aim, cell lines were incubated for 24 h with different concentrations of

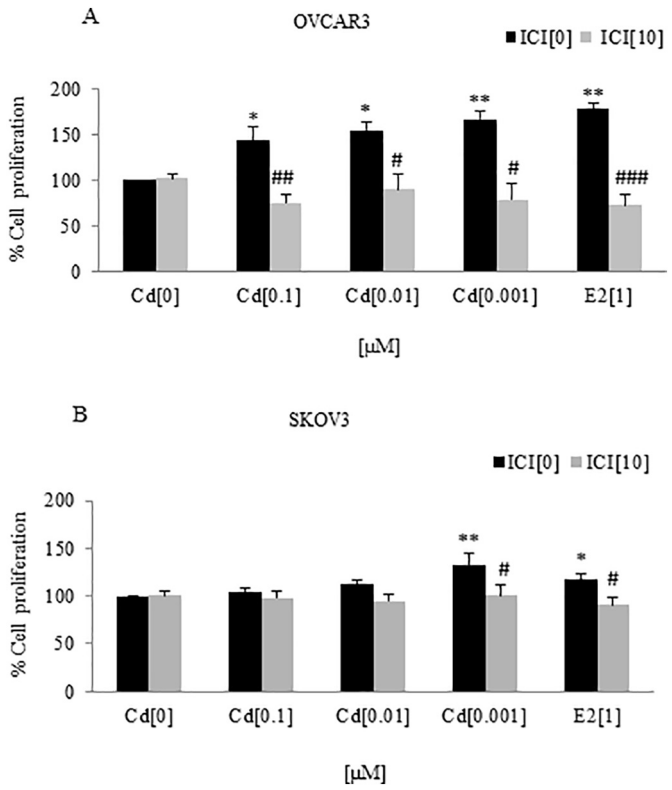

Fig. 2. $CdCl_2$ enhanced the cell proliferation of ovarian cancer cell lines in an ER-dependent manner. The cells were treated with $CdCl_2$ (0.1, 0.01, 0.001 μM) and Estradiol (1 μM) alone or in the presence of ICI (10 μM). Cell proliferation was evaluated by BrdU assay in OVCAR3 (A) and SKOV3 (B) cell lines. Data are presented as mean ± SD. * and ** show significant difference vs. Cd [0] ($p < .05$ and $p < .01$, respectively). #, ## and ### show significant difference between the cells treated with ICI and corresponding cells without ICI ($p < .05$, $p < .01$ and $p < .001$; respectively). Cd=$CdCl_2$, E2 = Estradiol, ICI=ICI 182,780.

$CdCl_2$ and the expression of ERα (Fig. 3A and C) and ERβ (Fig. 3B and D) were measured by western blot analysis. Quantitative analysis of the relative level of ERα expression by "image j" software demonstrated that Cd significantly increased ERα expression in both OVCAR3 and SKOV3 cell lines compared to control.

The simulative effects of $CdCl_2$ on the expression of ERα increased in a dose-dependent manner (4 folds at 0.1 μM, 7 folds at 0.01 μM, 6 folds at 0.001 μM in OVCAR3 and 2 folds at 0.1 μM, 3.7 folds at 0.01 μM, and 5.3 folds at 0.001 μM in SKOV3 vs. control). The expression of ERβ was also increased (1.98 folds at 0.1 μM, 3.47 folds at 0.01 μM, 3.54 folds at 0.001 μM in OVCAR3 and 0.9 folds at 0.1 μM, 2 folds at 0.01 μM, and 3 folds at 0.001 μM in SKOV3 vs. control).

Modulation of ERs expression by estrogen-like effects of Cd was confirmed in the presence and absence of ICI182,780. The results demonstrated that ICI182,780 significantly reduced the expression of ERα in OVCAR3 cells (%75 for 0.1 μM, %69 for 0.01 μM, %33 for 0.001 μM and %63.3 for E2) and in SKOV3 cells (%33 for 0.1 μM, %59 for 0.01 μM, %66 for 0.001 μM and %66.6 for E2). ICI182,780 also significantly decreased ERβ expression in both OVCAR3 (%59.5 for 0.1 μM, %57 for 0.01 μM, %46 for 0.001 μM and %50 for E2) and SKOV3 cells (%33.7 for 0.1 μM, %51 for 0.01 μM, %66.6 for 0.001 μM and %68 for E2).

3.4. Cadmium stimulated ERK1/2/MAPK activation in ER-dependent manner

To characterize the mechanism by which Cd-induced the proliferation of ovarian cancer cell line, we evaluated the ER-dependent activation of ERK1/2 as a key player in cell signaling proliferation via ERs (Geffroy et al., 2005). OVCAR3 and SKOV3 cells were treated with various concentrations of $CdCl_2$ (0.001–0.1 μM) and after 10 min, the level of p-ERK1/2 was determined by western blot analysis using p-ERK1/2 monoclonal antibody. "Image j" software quantitatively analyzed the relative level of p-ERK1/2. As depicted in Fig. 4A and 4B, 0.001 μM of Cd significantly induced activation of p-ERK1/2 in OVCAR3 cells (2.64 fold, $p < .01$) and in SKOV3 cells (2.2 fold, $p < .001$). Furthermore, ICI 182,780 as ER antagonist partially blocked the Cd-induced activation of p-ERK1/2 (%54.5 in OVCAR3 and %61.3 in SKOV3) suggesting that an ER-dependent mechanism is involved in this signaling.

3.5. ERK1/2 inhibitor (PD 98059) inhibited Cd-induced ovarian cancer cell proliferation

To more assessment whether the increased level of p-ERK1/2 could involve in Cd-induced cell proliferation, OVCAR3 and SKOV3 cells were incubated with $CdCl_2$ (0.001–0.1 μM) alone or in the presence of ERK1/2 inhibitors PD 98059 (0.5 μM) for 48 h. As shown in Fig. 5A and Fig. 5B, BrdU assay showed that PD 98059 significantly decreased the $CdCl_2$-induced cell proliferation in OVCAR3 (%18 for 0.1 μM, %22.7 for 0.01 μM, %25 for 0.001 μM and %26 for E2) and in SKOV3 cells (%28 for 0.1 μM, %26.6 for 0.01 μM, %33 for 0.001 μM and %63.3 for E2). Also, the level of p-ERK1/2 was evaluated by western blot in Cd-treated cells in the presence and absence of PD 98059 (Fig. 6A and B). The results demonstrated that PD 98059 (in Cd-treated cells in the presence of PD 98059 relative to Cd-treated cells without PD 98059 incubation) significantly decreased the level of p-ERK1/2 in OVCAR3 (%5 for 0.1 μM, %51.6 for 0.01 μM, %39 for 0.001 μM and %37 for E2) and SKOV3 (%52 for 0.1 μM, %55.5 for 0.01 μM, %42.5 for 0.001 μM and %40.5 for E2). Therefore, since PD 98059 reduced both increased level of p-ERK1/2 and subsequently, it inhibited cell proliferation in Cd-treated cells, it can be suggested that ERK1/2 could involve in Cd-triggered proliferation.

3.6. Cadmium induced the transcription of c-jun, c-fos and FOXO3a by ER

Recent studies have suggested that Cd increases proliferation of some cancer cells by stimulation of c-fos and c-jun as proto-oncogene (Aquino et al., 2012; Roskoski, 2012). Moreover, FOXO3a was known as a transcription factor that regulates ER and cellular proliferation. It has been identified as a key regulator of ER and E2- induced cell growth. The deregulation of FOXO3a activity is often seen in cancers and is associated with MAPK/ERK pathway (Johnson et al., 2003; Spillman et al., 2010). To evaluate whether $CdCl_2$ can stimulate cell proliferation by induction of c-fos, c-jun and FOXO3a expression, OVCAR3 and SKOV3 cells were treated with $CdCl_2$ (0.001–0.1 μM) for 1, 4, 6, 8, 10, 12 and 24 h and modulation of c-fos, c-jun and FOXO3a mRNA was determined by Real-time PCR. The results demonstrated that $CdCl_2$ significantly intensified the expression of these genes in comparison with control. The highest expression of c-jun was observed after 4 h, c-fos after 8 h and FOXO3a after 6 h treatment (data not shown). As shown in Fig. 7A, C and E, maximum response was also seen at 0.001 μM $CdCl_2$ in OVCAR3 cells 3.6 folds for c-jun, 4.5 folds for c-fos and 2.85 folds for FOXO3a and in SKOV3 cells 4.5 folds for c-jun, 8 folds for c-fos and 4.54 folds for FOXO3a (Fig. 7B,D and F, respectively). E2 increased c-fos and c-jun and FOXO3a transcription similarly to $CdCl_2$. The involvement of ERs in the induction of these genes was confirmed using ICI 182,780. The cells were exposed to 0.001–0.1 μM of $CdCl_2$ and E2 (1 μM) alone or in the presence of ICI 182,780, and mRNA expression was determined. As shown in Fig. 7, the antagonist significantly inhibited mRNA level of c-jun, c-fos and FOXO3a. The mRNA expression decrease in ICI 182,780 treated cells was calculated in OVCAR3 (%30 for 0.1 μM, %83 for 0.01 μM, %44 for 0.001 μM and

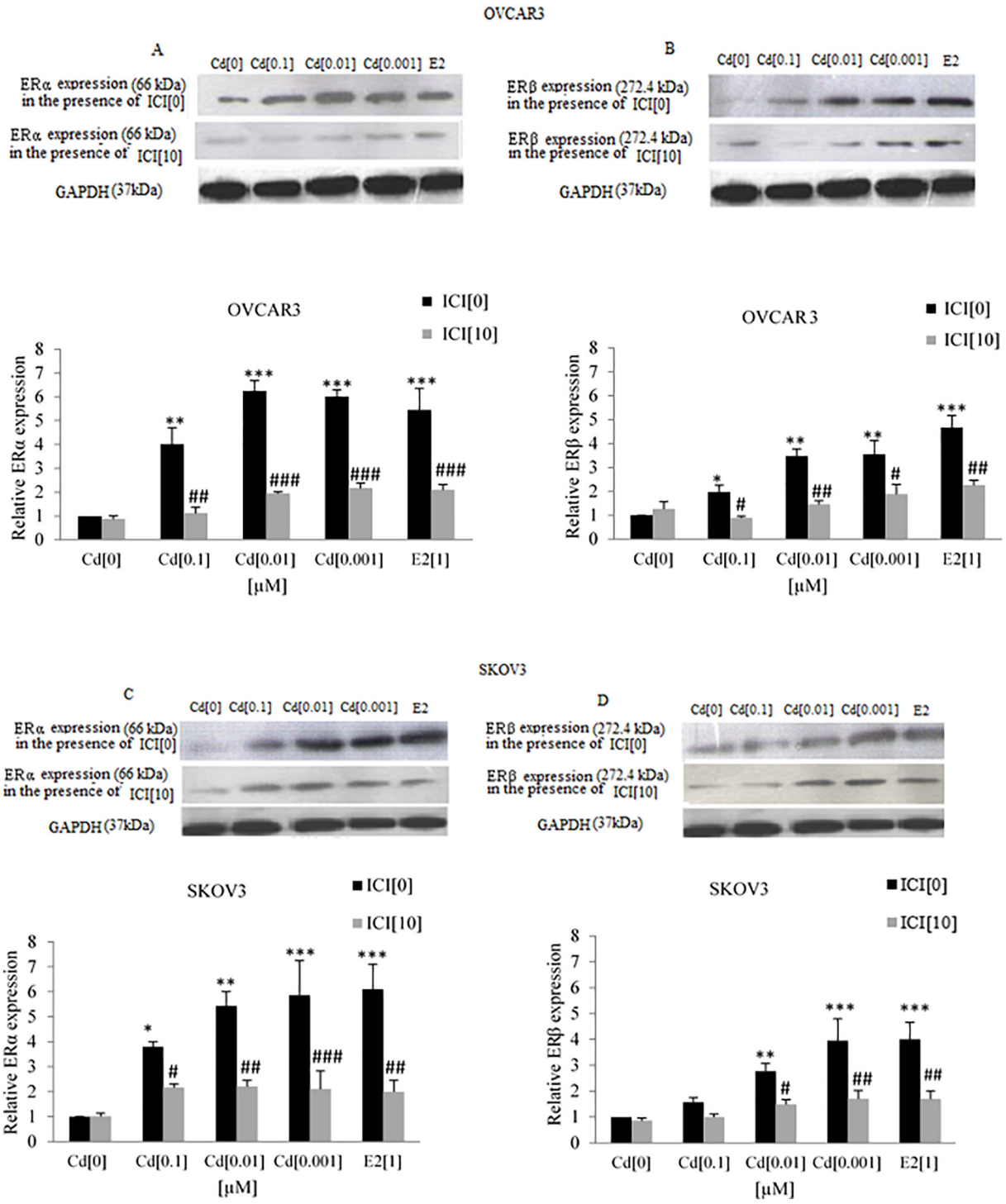

Fig. 3. $CdCl_2$ induced ERα and ERβ protein expression in ovarian cancer cell lines. The cells were treated with $CdCl_2$ (0.1, 0.01, 0.001 μM) and E2 (1 μM) in the presence of ICI (10 μM) and without ICI. The expression of ERα and ERβ in OVCAR3 (A, B) and SKO3 (C, D) were evaluated using western blotting method. Quantitative analysis of relative level of ERα and ERβ were performed by image j software.*, ** and *** show significant differences vs. Cd [0] without ICI ($p < .05$, $p < .01$ and $p < .001$, respectively). #, ## and ### show significant differences between the cells treated with and corresponding cells without ICI ($p < .05$, $p < .01$ and $p < .001$, respectively). Cd=$CdCl_2$, E2 = Estradiol, ICI=ICI 182,780.

%47.6 for E2) and in SKOV3 (%22.6 for 0.1 μM, %62.5 for 0.01 μM, %64.5 for 0.001 μM and %67.8 for E2) for c-jun. ICI 182,780 decreased c-fos mRNA in OVCAR3 (%60 for 0.1 μM, %60 for 0.01 μM, %48.8 for 0.001 μM and %57 for E2) and in SKOV3 (%52.6 for 0.1 μM, %69.5 for 0.01 μM, %56 for 0.001 μM and %54 for E2). Additionally, mRNA expression of FOXO3a was reduced in OVCAR3 (%23.5 for 0.1 μM, %25 for 0.01 μM, %33 for 0.001 μM and %34.4 for E2) and in SKOV3 (%39 for 0.1 μM, %36 for 0.01 μM, %46.7 for 0.001 μM and %38 for E2).

4. Discussion

Cd was introduced as a xenoestrogen which mimics estrogen effects, disrupts the endocrine system (Joseph, 2009), activates the ER, alters the expression of various estrogen target genes (Johnson et al., 2003;

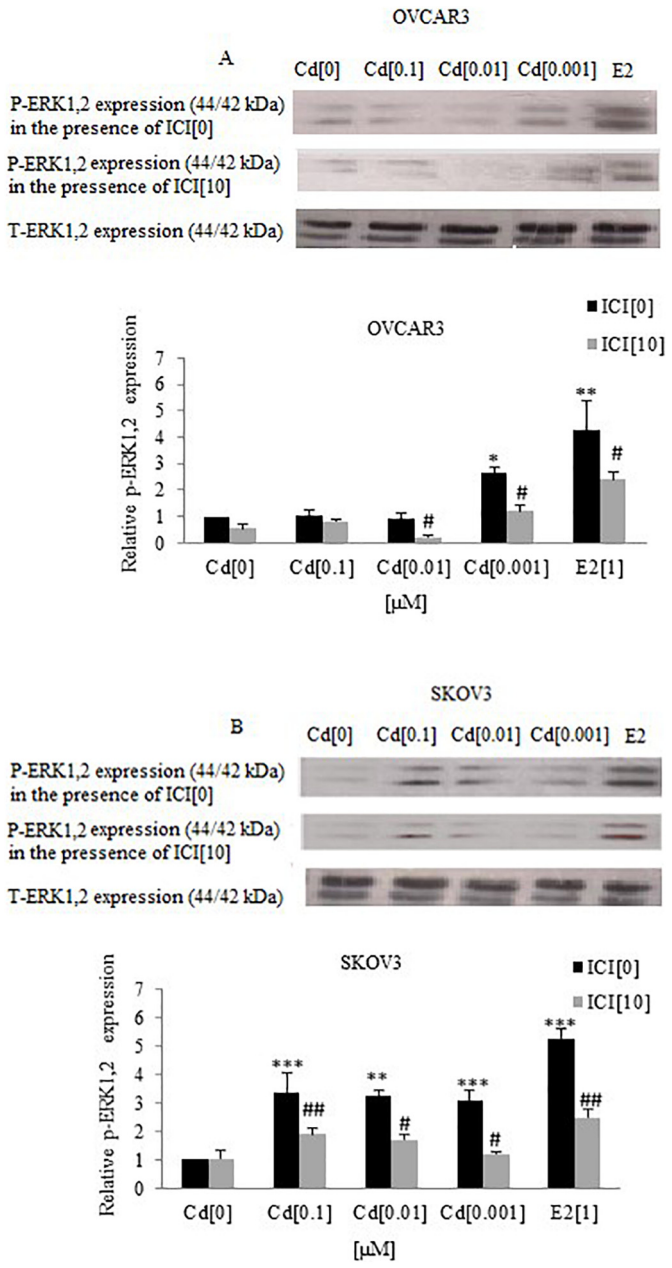

Fig. 4. $CdCl_2$ increased p-ERK1/2 in ER-dependent manner. The expression of p-ERK1/2 was evaluated by western blot analysis after exposure of OVCAR3 (A) and SKOV3 (B) cells with $CdCl_2$ (0.1, 0.01, 0.001 μM) and E2 (1 μM) in the presence or absence of ICI (10 μM). Quantitative analysis of relative level of p-ERK1/2 was performed by image j software. ** and *** show significant differences vs. Cd [0] without ICI ($p < .01$ and $p < .001$, respectively). # and ## show significant differences between the cells treated with ICI and corresponding cells without ICI ($p < .05$ and $p < .01$, respectively). Cd=$CdCl_2$, E2 = Estradiol, ICI=ICI 182,780.

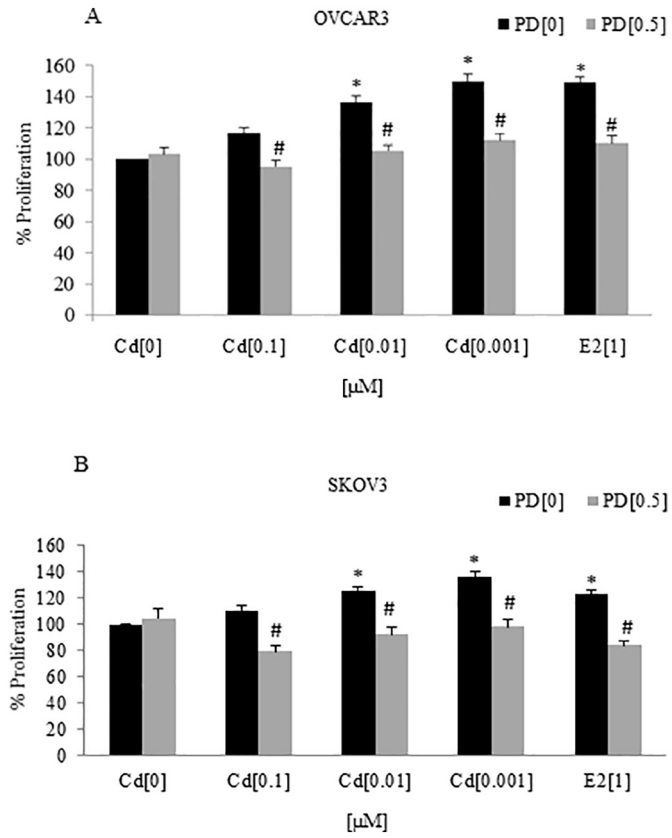

Fig. 5. ERK1/2 inhibitor (PD 98059) decreased Cd-induced ovarian cancer cell proliferation. OVCAR3 (A) and SKOV3 (B) cells were treated with $CdCl_2$ (0.1, 0.01, 0.001 μM) and E2 (1 μM) alone or in the presence of PD (0.5 μM). Cell proliferation was measured by BrdU assay. * shows significant difference vs. Cd [0] ($p < .05$). # shows significant difference between the cells treated with PD and corresponding cells without PD ($p < .05$). Cd=$CdCl_2$, E2 = Estradiol, PD = PD 98059.

Siewit et al., 2010) and also associates with the occurrence and development of typical estrogen-related cancers such as breast, endometrial and ovarian cancers (Adams et al., 2014; Hartwig, 2013). Cd is known as a reproductive toxicant which accumulates in ovary and uterus and causes some toxicity effects (Nampoothiri and Gupta, 2006). The function of estrogens in the etiology of ovarian cancer and estrogen-mimicking properties of Cd suggest a role of this metal in ovarian malignancy (Spillman et al., 2010; Syed et al., 2001). In the present study, we investigated the effect of various concentrations of Cd on the proliferation of ovarian cancer cell lines. Our results show that Cd significantly increased cell proliferation at nM concentration but markedly inhibited cell growth at μM concentration in a biphasic dose-response manner. Previous studies have shown a biphasic effect of Cd on cell proliferation in human embryo lung fibroblast cells and human embryonic kidney cells (Jiang et al., 2008, 2009). The results of Hao et al. demonstrated that Cd and Hg stimulated cell proliferation at 0.05 and 0.5 μM but inhibited it at 50 and 500 μM in breast cancer cell lines (Hao et al., 2009). Gao et al. explored that $CdCl_2$ stimulated the proliferation of uterine cancer cell line at lower concentrations (0.1 μM and 10 μM) and it inhibited proliferation at concentrations ≥50 μM (Gao et al., 2015). Khojastehfar et al. concluded that nM concentration of Cd enhanced MCF7 cell proliferation but μM concentration of this heavy metal-induced cell apoptosis by elevation of ROS level in a dose-dependent manner (Khojastehfar et al., 2015). More recent studies in breast, prostate, mesangial and lung epithelial cell lines indicated that low concentrations of Cd promoted cell growth (Bakshi et al., 2008; Kundu et al., 2009; Wei et al., 2015). The results of Huff et al. showed that both $CdCl_2$ and NaAsO2 at nM concentrations, environmentally relevant concentrations, have proliferative effect in some types of lung cancer cell lines (Huff et al., 2016).

Here, we examined whether Intracellular ER may mediate Cd-induced ovarian cancer proliferation. To determine this relationship, we used ICI 182,780 as a general inhibitor of ERα and ERβ. The results suggest a metalloestrogenic effect of Cd in ovarian cancer cell lines. Inconsistency with our results, Stoica et al. and Byrne et al. observed that Cd functionally acted like E2 in breast cancer cells as a result of its ability to bind to the ligand-binding domain of ERα with high affinity (Byrne et al., 2009; Stoica et al., 2000). Brama et al. claimed that CdCl2

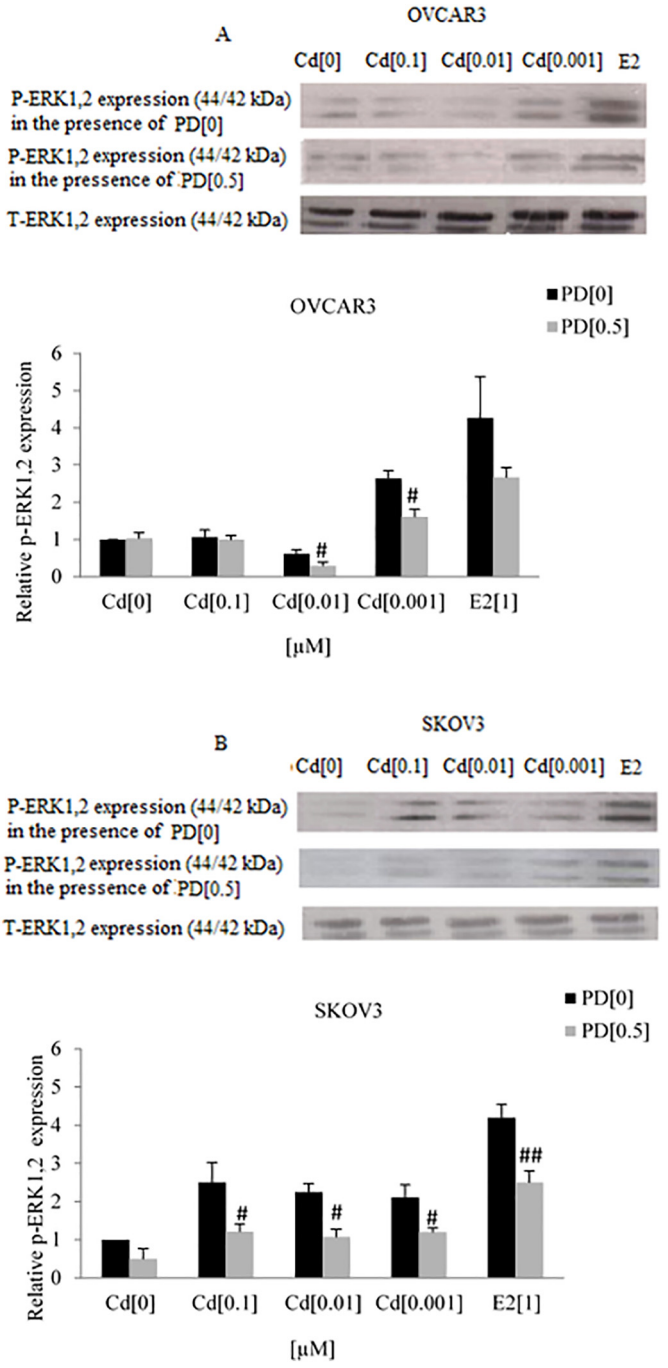

Fig. 6. PD 98059 inhibited Cd-induced phosphorylation of ERK1/2 in ovarian cancer cell lines. The expression of p-ERK1/2 was evaluated by western blot analysis after exposure of OVCAR3 (A) and SKOV3 (B) cells with $CdCl_2$ (0.1, 0.01, 0.001 μM) and E2 (1 μM) in the presence or absence of PD (0.5 μM). Quantitative analysis of relative level of p-ERK1/2 was performed by image j software. #, ## show significant differences between the cells treated with PD and corresponding cells without PD ($p < .05$ and $p < .01$, respectively). Cd=$CdCl_2$, E2 = Estradiol, PD = PD 98059.

stimulated an ER-dependent mitogenic signaling in breast cancer cell line and the ER-antagonist ICI 182,780 blunted it (Brama et al., 2007). Ronchetti et al. reported that nM concentrations of Cd exerted a potential estrogenic effect on cell proliferation of normal and tumor lactotrophs and its effects were blocked by ICI 182,780 (Ronchetti et al., 2013). In contrast to our study, Ali et al. reported that ICI 182,780 could not inhibit the CdCl2-induced proliferation in HepG2, MCF-7and ECC-1 cell lines. They suggested that instead of ER, a membrane G protein-coupled receptor of estrogen, GPR30, mediated these Cd effects (Ali et al., 2015). Moreover, Huff et al. observed that both ER antagonist ICI 182,780 and GPER antagonist G-15 attenuated CdCl2 and E2 stimulated proliferation in lung cancer cell lines (Huff et al., 2016). Although recent studies have suggested that GPR30 possibly has a role in proliferative effects of Cd as a xenoestrogen (Gao et al., 2015; Huff et al., 2016), the activation of ERs was considered as a critical step in metal-induced carcinogenesis (Aquino et al., 2012).

More attention has been given to the roles of ER in etiology, incidence, and pathology and survival rate of ovarian tumors as a prominent hormone-related cancer. (Järvinen et al., 2000). So we also evaluated the effect of various concentrations of $CdCl_2$ on the expression of ERα and ERβ. We found that Cd similar to E2 significantly increased the expression of ERs (α and β), but the expression of ERα was higher than ERβ. These results suggested that both subtypes of ER have possible roles in the Cd-induced proliferation of ovarian cancer cells. Ronchetti et al. claimed that Cd up-regulated mRNA expression of ERα in anterior pituitary cells at nM concentration (Ronchetti et al., 2013). Huff et al. reported that 100 nM $CdCl_2$ and E2 increased both ERα and ERβ expression in lung cancer cell lines (Huff et al., 2016). ERα and ERβ can dimerize and associate with DNA and some transcription factors (Leclercq, 2002). The findings of Dougherty et al. demonstrated that ERβ protein was expressed higher than ERα in lung tumors and cell lines (Dougherty et al., 2006). Lindberg et al. have been suggested that ERα and ERβ were co-expressed. ERα not only was responsible to inducing cell proliferation but also it could up-regulate ERβ (Lindberg et al., 2003).

The high level of ERβ expression in lung tumors and ERα expression in breast tumors can provide a good rationale that ERα and ERβ may play a critical role in cellular proliferation depending on tissue type (Brandenberger et al., 1997). ERK/MAPK, one of the most important intracellular protein kinases play a critical role in cell proliferation by phosphorylation of transcription factors and stimulation of intracellular networks of signaling cascades (Waisberg et al., 2003; Geffroy et al., 2005; Roskoski, 2012). We observed that $CdCl_2$ activated ERK1/2 in OVCAR3 and SKOV3 cells similar to estradiol effects and ICI 182,780 pretreatment reduced ERK1/2 phosphorylation. It suggests that ERK/MAPK pathway might be involved in Cd-induced proliferation and ER is required for ERK1/2 activation. In supporting our findings, Huang et al. reported that 1 μM Cd-induced a sustained activation of ERK1/2 in mouse epidermal cells (Huang et al., 2001). Jiang et al. showed that low concentrations of Cd promoted cell proliferation by increasing in p-ERK1/2 activity whereas, at high Cd concentrations, phospho-p38 activity markedly increased and induced apoptosis in human embryo lung fibroblast cells (Jiang et al., 2009). The results of Hao et al. claimed that Cd in a biphasic effect, at higher concentrations (50 and 500 μM) induces apoptosis by increasing of JNK and P38 phosphorylation but at lower concentrations (50 and 500 nM), activated ERK/MAPK pathway lead to proliferation of HEK293 cell line (Hao et al., 2009). Gao et al. also reported that ERK1/2 /MAPK pathway is essential for Cd-induced cell proliferation in uterine cell lines. They explored that $CdCl_2$ stimulated the growth of uterine cancer cell lines at lower concentrations (0.1 μM and 10 μM) using dose-dependent p-ERK1/2 activation and inhibited the cell proliferation at concentrations ≥5 μM by p-ERK1/2 diminution (Gao et al., 2015). Brama et al. showed that ICI 182,780 in the MCF-7 cells blocked ERK1/2 activation (Brama et al., 2007). Liu et al. described that treatment of MCF-7 cells with 0.5–10 μM $CdCl_2$ caused rapid activation of ERK1/2 similar to E2 and partially blocked by specific siRNA of ERα (Liu et al., 2008). In accordance with previous results, our study demonstrated that low Cd concentrations activated ERK1/2 and subsequent proliferation in ovarian cancer cell lines. So ERK1/2 signal pathway might take part in hormesis phenomenon by phosphorylation of ERK1/2 and activation of dependent genes. Stebbing explained that in the lower dose of metals, cells showed an overcompensation response to protect and adapt against the damage of metals but higher doses caused severe toxic effects to induce cell death

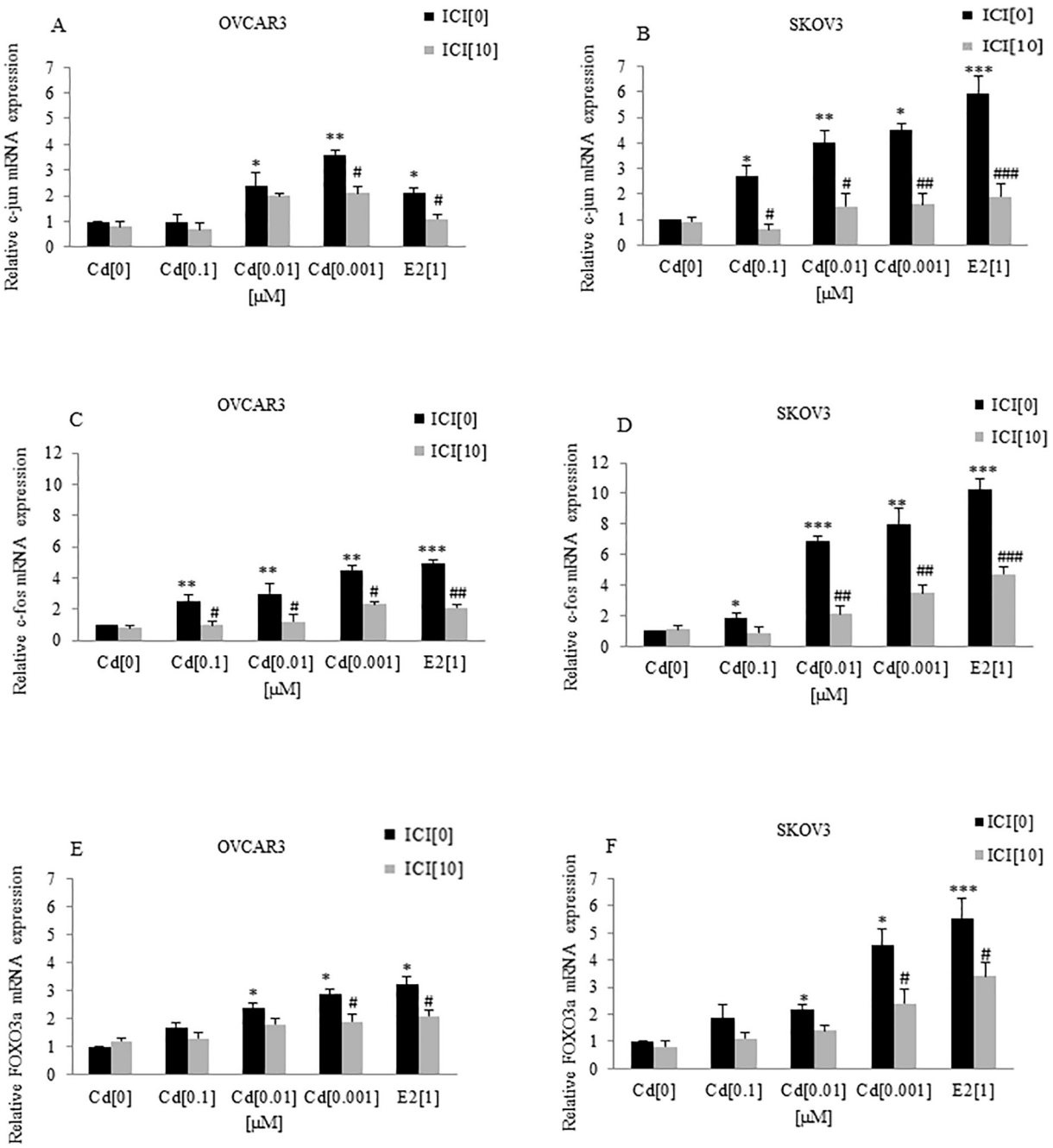

Fig. 7. $CdCl_2$ intensified the transcription of c-jun, c-fos and FOXO3a genes through ER. The cells were treated with $CdCl_2$ (0.1, 0.01, 0.001 μM) and and E2 (1 μM) in the presence or absent of ICI (10 μM). mRNA transcription of c-jun (A, B), c-fos (C, D) and FOXO3a (E, F) was determined by Real time PCR. Results were represented as mean ± S.D. *, ** and *** show significant differences vs. Cd [0]. #, ## and ### show significant differences between the cells treated with ICI and corresponding cells without ICI ($p < .05$, $p < .01$ and $p < .001$, respectively). E2 = Estradiol, Cd=$CdCl_2$, ICI=ICI 182,780.

(Stebbing, 2002).

Furthermore, the determination of downstream target genes induced by $CdCl_2$ is noteworthy and can help to define its underlying carcinogenesis mechanism. Therefore, we evaluated c-fos and c-jun expression which are stimulated by estrogen through ERK/MAPK pathway. These are not only proto-oncogenes which in combination together act as the transcription factor and regulate cell proliferation but also are key components which are activated by mitogenic stimuli and play an important role in many cellular functions to convert extracellular signals to change of gene expression (Adams et al., 2014). Estrogen can stimulate c-fos and c-jun through ERK pathway. We demonstrated that c-fos and c-jun mRNA expression were up-regulated by $CdCl_2$ similar to E2. Cd effects on c-fos and c-jun expression can be mediated by ER involvement. Ronchetti showed that 10 nM Cd enhanced c-fos mRNA expression in an ERα – dependent manner in anterior pituitary cells (Ronchetti et al., 2013). Some data indicated that Cd increased cell proliferation by stimulation of ERK1/2 activity probably through activation of c-fos, c-jun and by an ERα-dependent mechanism (Brama et al., 2007; Roskoski, 2012). Additionally, our analysis demonstrated that cells exposed to Cd also expressed higher levels of FOXO3a compared to control. This gene is a member of FOXO proteins which regulates cell cycle and modulates progression of cells. Some studies reported that FOXO3a expression correlated with some clinical responses of breast cancer such as ER positivity, axillary lymph node negativity and histologic grade (Esteva et al., 2002; Jiang et al., 2013). Recent studies suggested a clear link between the expression of

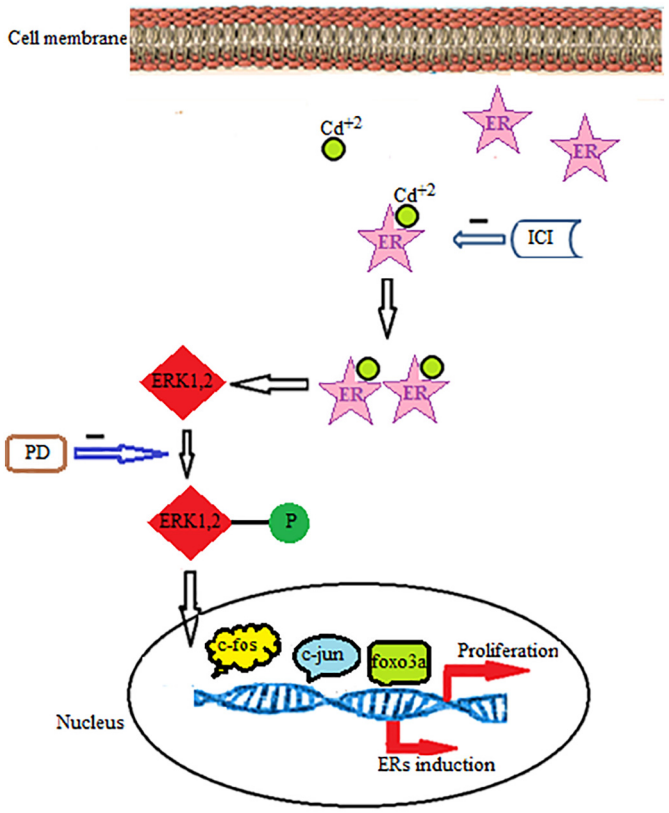

Fig. 8. Schematic representation of $CdCl_2$ mechanism involved in ovarian cancer cell proliferation. After binding to ER, Cd increased the expression of ERs, phosphorylation of ERK1/2 and activation of c-fos, c-jun and FOXO3a transcription factors to induce proliferation of ovarian cancer cells. Cd=$CdCl_2$, PD = PD 98059, ER = Estrogen Receptor, ICI=ICI 182,780.

FOXO3a and ER+ in breast cancer (Guo and Sonenshein, 2004; Jiang et al., 2013). Guo and Sonenshein postulated that overexpression of FOXO3a increased expression of ERα in ERα + NF639 and T47D cells. They identified two strongly binding forkhead sites in ERα promoter and introduced FOXO3a as a key mediator of expression of ERα in breast cancer (Guo and Sonenshein, 2004; Madureira et al., 2006). Furthermore, it is also regulated by the ERK/MAPK pathway (Myatt and Lam, 2007). These results showed that Cd exposure induced the expression of FOXO3a through ERK1/2 signaling pathway and suggested that FOXO3a might regulate cell proliferation in Cd-exposed ovarian cells. Finally, it would be intriguing to design a model of the possible mechanism by which $CdCl_2$ activated intracellular signaling cascade in ovarian cancer cell proliferation in Fig. 8. $CdCl_2$ induced the proliferation of the ovarian cancer cells and expression of ER in an ER-dependent manner by increasing p-ERK1/2 and following activation of c-fos, c-jun, and FOXO3a transcription factors.

5. Conclusion

Our findings suggest that biphasic response of Cadmium at low nM concentrations promotes an estrogenic type of proliferation in ovarian cancer cell lines. Also activation of signals like ERK1/2 and some transcription factors such as c-fos, c-jun and FOXO3a might be involved in Cd-induced proliferation. Perception of downstream targets by which Cd deregulates cell proliferation can be worthwhile and helpful to define its underlying carcinogenesis mechanism.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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