Cadmium Promotes Breast Cancer Cell Proliferation by Potentiating the Interaction between ER{alpha} and c-Jun

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Cadmium is an environmental contaminant that enters the body through diet or cigarette smoke. It affects multiple cellular processes, including cell proliferation, differentiation, and apoptosis. Recently, cadmium has been shown to function as an endocrine disruptor, to stimulate estrogen receptor α (ERα) activity and promote uterine and mammary gland growth in mice. Although cadmium exposure has been associated with the development of breast cancer, the mechanism of action of cadmium remains unclear. To address this deficit, we examined the effects of cadmium treatment on breast cancer cells. We found that ERα is required for both cadmium-induced cell growth and modulation of gene expression. We also determined that ERα translocates to the nucleus in response to cadmium exposure. Additionally, we provide evidence that cadmium potentiates the interaction between ERα and c-Jun and enhances recruitment of this transcription factor complex to the proximal promoters of cyclin D1 and c-myc, thus increasing their expression. This study provides a mechanistic link between cadmium exposure and ERα and demonstrates that cadmium plays an important role in the promotion of breast cancer. (Molecular Endocrinology 24: 981–992, 2010)

Breast cancer is one of the most common malignancies that occur in women in the United States, accounting for one in four female cancers. Breast cancer develops as a result of abnormal proliferation of cells in the mammary gland. Normal growth of mammary epithelial cells is modulated by circulating levels of estrogen, a female hormone produced by the ovaries. The activity of estrogen is mediated by the estrogen receptors (ERs), ERα and ERβ. These two isoforms of ER display considerable homology in their DNA binding domains and COOH-terminal domains but are highly variable in their NH2-terminal trans-activation function-1 domains (3, 4). Both ERα and ERβ bind to the active form of estrogen, 17β-estradiol (E2), with similar affinities. They function as ligand-gated transcription factors that control the expression of genes necessary for growth, development, and homeostasis. However, the two forms of ER play very different roles in breast cancer. ERα has long been known as a crucial regulator of growth and development of the mammary gland and also as a key prognostic marker for breast cancer development. Although ERβ has been reported to be present in breast cancer cells, less is known about its role in breast cancer development.

Breast cancer can be classified as either ER positive or ER negative, depending on the presence or absence of ERα. Development of ER-positive breast cancer is controlled by the activity of the ERs and the circulating levels of 17β-E2. The role of ERα in hormone-refractory breast tumorigenesis and the underlying mechanism of this role are unclear. Endocrine disruptors, including heavy metals such as cadmium (Cd), may be involved in ERα-mediated modulation of hormone-refractory breast tumorigenesis (1, 2, 5–7). Although Cd exposure has been associated

Abbreviations: AP, Activating protein; Cd, cadmium; CdCl2, Cd chloride; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CTD, cathepsin D; CycD1, cyclin D1; CycE, cyclin E1; E2, estradiol; ER, estrogen receptor; FBS, fetal bovine serum; ICI, ICI 182,780; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; siRNA, small interfering RNA; PPT, propyl pyrazole triol; Ri, SiRNAi.
with the development of breast cancer, the nature of this relationship remains unclear (1, 2, 5–7).

Cd is derived from various industrial sources, including present and former mining activities and the production of alloys, batteries, fertilizers, pigments, and combustion byproducts. Human exposure results from consumption of contaminated water and food or inhalation of cigarette smoke and metal smoldering fumes. Cd is part of the family of heavy metals that exists as a natural component of the Earth’s crust. Thus, it is present in varying concentrations in all ecosystems. In general, heavy metals cannot be degraded and often accumulate in biota, water, and soil. Because heavy metals form complexes with organic compounds, high levels of heavy metals have been shown to be dangerous to all living organisms. Heavy metals often alter the activity of biological molecules and hinder their ability to function properly by binding to nitrogen-, oxygen-, or sulfur-containing groups, ultimately interfering with cellular processes. Common toxicities affecting higher eukaryotic cells include impaired mitochondrial function, enhanced levels of free radicals, and DNA damage, all of which can result in death or malignant transformation. Exposure to elevated levels of Cd and other heavy metals has been associated with an increased incidence of breast cancer development (6, 8). In particular, Cd, nickel, mercury, and selenium have been shown to have estrogenic effects and are collectively referred to as metalloestrogens.

Recent studies have suggested that metalloestrogens may function as endocrine disruptors, perturbing the normal hormonal cycle and altering the development of the mammary gland, resulting in neoplastic growth (1, 2). Cd is the best characterized of the metalloestrogens. Specifically, animal studies have shown that Cd exposure can increase uterine weight and density of the mammary gland, as well as induce changes in the lining of the uterus, which are all early signs of tumorigenesis (2). Cd can mimic the action of estrogen in breast cancer cells by binding to nitrogen-, oxygen-, or sulfur-containing groups, ultimately interfering with cellular processes. Common toxicities affecting higher eukaryotic cells include impaired mitochondrial function, enhanced levels of free radicals, and DNA damage, all of which can result in death or malignant transformation. Exposure to elevated levels of Cd and other heavy metals has been associated with an increased incidence of breast cancer development (6, 8). In particular, Cd, nickel, mercury, and selenium have been shown to have estrogenic effects and are collectively referred to as metalloestrogens.

This study aims to further investigate the mechanism of Cd-induced breast cancer cell proliferation and gene expression and to determine the role of ERα in this process. We provide several lines of evidence that suggest that the effects of Cd on breast cancer cell growth and gene expression are dependent on ERα. Our results show that Cd promotes the nuclear localization of ERα and enhances the binding of ERα to the promoters of cyclin D1 (CycD1) and c-myc. We also demonstrate that Cd potentiates the interaction between ERα and c-Jun, a member of the activating protein (AP)-1 family of transcription factors. Taken together, our results suggest that Cd promotes the ERα/c-Jun interaction and increases recruitment of the ERα/c-Jun complex to promoter regions, thereby directly regulating the expression of Cd-inducible genes.

Results

To determine whether Cd has an effect on ER-positive breast cancer cell growth, three ER-positive breast cancer cell lines, MCF-7, T-47D, and ZR-75-1, were plated in six-well plates in hormone-deprived media and treated with $10^{-6}$ M cadmium chloride (CdCl$_2$), $10^{-8}$ M 17β-E$_2$, or were mock treated with PBS (Fig. 1A). For each cell type, the optimal Cd concentration was determined using a titration assay in which cells were plated in a 96-well plate and treated with varying concentrations of CdCl$_2$, ranging from $10^{-12}$ M to $10^{-2}$ M (data not shown). The results shown in Fig. 1A indicate that Cd enhanced the growth rate of all three ER-positive breast cancer cell lines (MCF-7, T-47D, and ZR-75-1), relative to the mock control. As expected, cells treated with E$_2$ displayed a significant increase in cell growth, compared with mock-treated cells. These results were further confirmed by indirect cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (data not shown). Although Cd affects cell proliferation to a lesser degree than E$_2$, our results do indicate that Cd stimulates ER-positive breast cancer cell growth.

To dissect the molecular mechanism of Cd’s action on breast cancer cell growth, we examined whether Cd was able to induce ER target gene expression. MCF-7 cells were hormone deprived and treated with $10^{-6}$ M CdCl$_2$. Total RNA was isolated, and the expression levels of several ER target genes were analyzed by semiquantitative RT-PCR (Fig. 1B). Cd-treated MCF-7 cells expressed higher levels of CycD1 and c-myc as early as 24 h after exposure, whereas cathepsin D (CTD) displayed a more delayed response (48 h). These three genes have been shown to be important in the development and progression of breast cancer (11–17). Cd exposure had no detectable effect on the expression level of pS2, an estrogen-responsive gene that is commonly used as a prognostic marker of breast cancer (18, 19). We also examined the expression of p21, a cell cycle inhibitor that has been shown to be regulated by ERα (12, 20) and found that expression of p21 was decreased by Cd exposure. Consistent with its inhibitory role in the cell cycle, reduction of p21 levels may serve as an alternative mechanism by which Cd promotes cell proliferation. Alternatively, re-
duced p21 levels may work in conjunction with elevated levels of CycD1 and c-myc to promote cell growth.

The observation that Cd stimulated breast cancer cell proliferation led us to expand our analysis to other genes directly involved in cell cycle regulation. To this end, we included cyclins and cyclin-dependent kinases (CDKs) in our analysis. Our results show that expression of cyclin E1 (CycE), cdk2, and cdk4 increased after Cd treatment, whereas the other cyclins and CDKs showed no significant changes in their expression levels after treatment (Fig. 1B).

Western blot analysis was used to determine whether the protein levels of these genes were affected by Cd treatment (Fig. 1C). Cells treated with $10^{-6}$ M CdCl$_2$ also displayed higher CycD1 and c-myc protein levels at 24 h after treatment. However, no significant change in the ERα protein level was observed. Cd also induced the expression of CycE and cdk2 proteins. These results establish that Cd promotes MCF-7 cell proliferation through induction of ER target genes (i.e. CycD1 and c-myc) and non-ER target genes (i.e. CycE and cdk2), and that these increases are not dependent on elevated levels of ERα (Fig. 1C).

These results led us to question whether the Cd-induced effect requires ERα. To establish a link between Cd-induced cell proliferation and ERα, we treated MCF-7 cells with ICI 182,780 (ICI) to decrease endogenous levels of ERα. ICI is a pure antiestrogen that binds to the ER and promotes receptor degradation. MCF-7 cells were treated with either $10^{-6}$ M CdCl$_2$ or $10^{-6}$ M CdCl$_2$ in the presence of $10^{-8}$ M ICI, or were mock treated with PBS. Cells were harvested and submitted to direct cell counting and Western blot analysis to monitor cell proliferation and ERα levels, respectively (Fig. 2, A and B).

Cells treated with ICI (I or Cd+I) expressed significantly lower levels of ERα after 24 h of treatment (Fig. 2B). Reduced levels of ERα corresponded with decreased Cd-induced cell proliferation, suggesting that the effect of Cd treatment is, in part, dependent on the presence of ERα (Fig. 2, A and B). Consistent with the growth results, ICI diminished the effect of Cd on CycD1 and c-myc expression at both the protein and
mRNA levels (Fig. 2B and Supplemental Fig. 1A published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).

To determine whether Cd-induced breast cancer cell proliferation is dependent on ERα, we silenced ERα expression using siRNA, and cell growth was analyzed 2, 3, and 4 d after treatment (Fig. 2C and data not shown). Cells transfected with siRNA directed against ERα (Ri-ERα) did not grow in response to the Cd or estrogen (E2), whereas cells transfected with a scrambled siRNA control (Ri-control) continued to be stimulated by Cd and estrogen treatment (Fig. 2C). Consistent with the growth results, Ri-ERα also diminished the effects of Cd-induced CycD1 and c-myc expression at the mRNA level (Fig. 2D).

Next, we examined the role of ERβ in Cd-induced cell growth using small interfering RNA (siRNA) to deplete endogenous ERβ expression. Cell growth was monitored 2, 3, and 4 d after treatment (Fig. 2E and data not shown). Interestingly, cells transfected with Ri-ERβ displayed an increase in cell proliferation under all three conditions (mock, Cd, and estrogen treated). Furthermore, our results also suggest that ERβ depletion has no effect on Cd or estrogen response (Fig. 2E). Western blot analysis was used to monitor the ERβ expression. Cells transfected with Ri-ERβ expressed significantly lower levels of ERβ 48 h after treatment; however, depletion of ERβ did not have any significant effects on the expression of CycD1 and c-myc at the protein level (Fig. 2F).

To further assess the role of ERα in Cd-induced cell proliferation, we analyzed the effects of Cd on the growth of MDA-MB-453 breast cancer cells. MDA-MB-453 cells are ERα negative but ERβ positive. The cells were treated with varying concentrations of CdCl₂ ranging from 10⁻¹² M to 10⁻⁷ M, or were mock treated with PBS (Supplemental Fig. 1B). Cell proliferation was measured with the MTT assay 4 and 6 d after treatment. As expected, Cd exposure did not promote growth of MDA-MB-453 cells. Taken together, our results demonstrate that Cd-
induced breast cancer cell growth is dependent on ERα, and not ERβ.

Given that Cd-induced cell proliferation and gene expression may be dependent on ERα, we postulated that Cd may stimulate ERα nuclear localization. To determine whether Cd promotes the nuclear translocation of ERα, MCF-7 cells were hormone deprived for 2 d and treated with 10^{-6} M CdCl_2 (Cd) or 10^{-8} M E_2, or were mock treated for 0.5, 2, and 4 h (Fig. 3). Cells were harvested by fixation in formaldehyde, and ERα was examined by immunofluorescence and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei. ERα proteins are normally localized in both the cytoplasm and nucleus; however, when cells are hormone deprived, a majority of the ERα proteins are found in the cytoplasm (Fig. 3). As shown in Fig. 3, Cd promotes the nuclear translocation of ERα, starting at 1 h and peaking at 4 h after treatment (Fig. 3). As expected, E_2 treatment also stimulates the translocation of ERα to the nucleus. However, the translocation effect was much quicker under estrogen condition, with maximum translocation observed after 30 min of treatment (Fig. 3). These data suggest that Cd acts similarly to estrogen in promoting ERα nuclear localization. Once inside the nucleus, the Cd-ERα complex may regulate the expression of Cd-induced genes (i.e. CycD1 and c-myc).

Because Cd promotes translocation of ERα to the nucleus, we questioned whether ERα is required for the expression of Cd-induced genes. To address this issue, we analyzed the trans-activation of CycD1 and c-myc promoters in the presence and absence of ERα. We performed a reporter gene assay by transfecting MCF-7 cells with a plasmid containing a luciferase gene directly linked to either the CycD1 (1.7 kb) or the c-myc (2.1 kb) promoter sequences along with siRNA directed against ERα or a scramble siRNA control. Cells transfected with an empty vector served as a negative control. After transfection, cells were treated with either Cd, propyl pyrazole triol (PPT), a highly selective ERα agonist, or Cd in the presence of PPT. Cells mock treated with PBS served as the negative control. Results in Fig. 4, A and B, demonstrate that Cd treatment modestly increases the transcriptional activity at both the CycD1 and c-myc promoters by 3- and 4-fold, respectively, in cells transfected with the Ri-Control. As expected, PPT also trans-activated the two ERα-regulated promoters (4.5-fold for CycD1 and 5.3-fold for c-myc). Interestingly, the combination of Cd and PPT only resulted in a slightly higher trans-activation, suggesting the absence of synergistic effects between the two agonists (Fig. 4, A and B). Similarly, growth and gene expression analyses of cells treated with both Cd and PPT also indicate these ago-

**FIG. 3.** Cd promotes translocation of ERα to the nucleus. MCF-7 cells were plated in six-well plates containing untreated glass cover-slips under hormone-deprived conditions. Twenty hours later, cells were treated with 10^{-6} M CdCl_2 or 10^{-8} M 17β-E_2. Cells were harvested by fixation in 10% formalin 0, 0.5, 2.0, and 4.0 h after treatment. ERα (FITC) and nuclear staining (DAPI) were analyzed by immunofluorescence.

**FIG. 4.** Cd activates target gene expression through ERα. MCF-7 cells were transfected with either Ri-ERα (black) or Ri-Control (white) along with (panel A) CycD1-luc or (panel B) c-myc-luc. Renilla luciferase construct was cotransfected as an internal control. After transfection, cells were treated with 10^{-6} M CdCl_2, PPT, 10^{-6} M plus PPT, or mock treated [control (C)]. Luciferase activity was measured using a dual luciferase assay. Statistics represent triplicate samples. Panel C, MCF-7 cells were hormone deprived and treated with 10^{-6} M CdCl_2 (Cd) or mock treated with PBS (C), followed by fixation in formaldehyde. ChIP analysis was performed with α-ERα antibodies, and ERα recruitment to target gene promoters was determined by promoter-specific primers and semiquantitative RT-PCR. Primer positions are depicted on the promoter diagram for each gene. Input represents total chromatin before immunoprecipitation. Panel D, Band intensities of PCR product were quantitated using Quantity One software (Bio-Rad) and statistics represent fold changes of three independent experiments.
AP-1 interaction in ERα-positive breast cancer cells by coimmunoprecipitation. MCF-7 cells were treated with 10^{-6} M CdCl_{2}, cell lysates were incubated with anti-ERα antibodies, and the presence of AP-1 proteins was analyzed by Western blot analysis (Fig. 5, A and B). The results in Fig. 5, A and B, show that, although c-Fos is found in complex with ERα, no significant difference was observed between Cd-treated and mock-treated cells. Strikingly, a greater fraction of c-Jun coprecipitated with ERα in the Cd-treated cells. This ERα/c-Jun interaction was confirmed by reverse coimmunoprecipitation with c-Jun. In this case, the amount of ERα found in complex with c-Jun was greater in Cd-treated cells than in the control cells (Fig. 5, C and D). Taken together, these results suggest that Cd promotes ERα/c-Jun complex formation.

Having demonstrated that Cd can promote the ERα/c-Jun interaction and that Cd promotes ERα recruitment to the promoters of target genes, we evaluated whether c-Jun is directly involved in ERα-mediated transcription after Cd treatment. To address this question, we examined the recruitment of c-Jun to the promoter regions of CycD1 and c-myc using a second ChIP. The occupancy of c-Jun on the promoter regions of both c-myc and CycD1 is expected because both regions analyzed contain an AP-1 response element; however, the results presented in Fig. 6, A and B, show that higher levels of c-Jun were recruited to the CycD1 and c-myc promoters in Cd-treated cells in comparison with untreated cells. In contrast, c-Jun occupancy was not observed at the U6 RNA promoter. To determine whether c-Jun and ERα are part of the same transcription complex, we performed a ChIP re-ChIP assay. MCF-7 cells were treated with 10^{-6} M Cd, and cells were harvested for ChIP analysis with the first antibody (either ERα or c-Jun). After the first ChIP assay, the DNA-protein complexes were extracted and re-ChIPed with the second antibody (either ERα or c-Jun). Our results confirm that Cd promotes the recruitment of both ERα and c-Jun to the same promoter region, suggesting that they are likely part of the same transcription complex (Fig. 6, C and D).

To understand the role of c-Jun in Cd-induced gene expression, we analyzed the expressions of CycD1 and c-myc after siRNA-mediated silencing of c-Jun (Fig. 7, A–C). Expression analysis at the protein and mRNA lev-
els confirmed that the expressions of CycD1 and c-myc are decreased in cells transfected with Ri-c-Jun. To further discern the role of c-Jun in Cd-induced cell growth, we transfected MCF-7 cells with either Ri-c-Jun or a control siRNA. Cells were treated with either Cd, E2, or mock treated with PBS (C), and growth was monitored 3 d after treatment (Fig. 7D). Although the depletion of c-Jun diminishes the levels of agonist-induced cell growth and gene expression, cells continue to display some level of Cd and estrogen responsiveness. This may be attributed to the incomplete silencing of c-Jun by the siRNA. Taken together, results from this study strongly suggest Cd promotes the binding of ERα to c-Jun to regulate the expression of Cd-induced gene and promote breast cancer cell growth.

Discussion

Cd is an environmental contaminant that is often referred to as an endocrine disruptor due to its ability to mimic the ER and alter the expression of various estrogen target genes. Previous studies have suggested that exposure to Cd can affect mammary gland development in ovariectomized rats (2). Although there is no direct evidence that Cd can promote breast cancer in humans, it has been implicated as a cancer causing agent and has been classified by the International Agency for Research on Cancer as a potential human carcinogen. Studies have demonstrated that Cd can promote growth of MCF-7 breast cancer cells and can activate the ER in vitro (1, 6–8, 21, 22). However, the mechanism of action of Cd is poorly understood. Results from this study confirm that Cd can promote cell proliferation in three human ERα-positive breast cancer cell lines, MCF-7, T-47D, and ZR-75-1 (Fig. 1A). Furthermore, our results demonstrate that Cd does not promote proliferation in ERα-negative breast cancer cells (Supplemental Fig. 1).

To fully establish a link between Cd-induced breast cancer cell growth and the ER, we used ICI and Ri-ERα to reduce the intracellular levels of ERα. We found that both ICI and Ri-ERα were able to block Cd’s action, suggesting that Cd-induced cell growth and gene expression are dependent on the ER (Fig. 2 and Supplemental Fig. 1). Consistent with previous reports, we found that multiple ER target genes (CycD1, c-myc, and CTD) are up-regulated by Cd exposure (Fig. 1, B and C) (1, 6, 7). Additionally, our analysis demonstrates that cells exposed to Cd also expressed higher levels of CycE, cdk2, and cdk4 and decreased levels of p21. All of these genes function as cell cycle regulators that modulate progression of cells through the G1/S transition (Fig. 1, B and C). Overexpression of any one of these cell cycle regulators

FIG. 6. c-Jun is recruited to target gene promoters. Panel A, MCF-7 cells were hormone deprived and treated with 10⁻⁶ M CdCl₂ (Cd) or mock treated [control (C)]. ChIP analysis was performed with α-c-Jun antibodies, and c-Jun recruitment to target gene promoters was determined by promoter-specific primers using semiquantitative RT-PCR. Panel B, Band intensities of PCR product were quantitated using Quantity One (Bio-Rad). Panels C and D, ChIP re-ChIP was used to determine whether ERα and c-Jun are part of the same promoter complex. ChIP assay was performed as in A with antibodies against ERα or c-Jun. Protein-DNA complexes of the first ChIP were extracted and re-ChIPed with the second antibody. Normal rabbit serum was used as a negative control. The occupancy of ERα/c-Jun complexes on target promoters was analyzed using promoter-specific primers and semiquantitative RT-PCR. Band intensities of PCR products were quantitated using Quantity One (Bio-Rad).
results in the deregulation of growth, often leading to cancer (23–26).

Results from our reporter gene assay further support the argument that Cd stimulates the expression of CycD1 and c-myc by increasing the transcriptional activity of these target promoters. Because CycD1 and c-myc are both ER target genes, we fully expected to observe ERα occupancy at the CycD1 and c-myc promoters. Before this study, it was unclear whether Cd was responsible for stimulating the binding of ERα to these promoters or whether Cd induced nongenomic effects, leading to elevated levels of CycD1 and c-myc. Using ChIP analysis, we demonstrated that Cd enhances the recruitment of ERα to the proximal promoters of CycD1 and c-myc (Fig. 4). This finding is further supported by the observation that Cd promotes the translocation of ERα into the nucleus, consistent with ERα’s role as a transcriptional regulator (Fig. 3).

Both CycD1 and c-myc are nonclassical ER target genes, and their expression, although dependent on ERα, does not require direct binding of ERα to the DNA. Instead, ERα is recruited to their promoters via AP-1 (27, 28). AP-1 is a transcription factor complex that contains proteins encoded by the proto-oncogenes c-Jun, c-Fos, and other AP-1 family members. This complex interacts with AP-1 binding sites in promoters to activate transcription of genes involved in cell growth, differentiation, and development. Previous studies have shown that c-Fos and c-Jun suppress ERα-dependent trans-activation of estrogen response element-containing promoters (29–31) by recruiting ERα to AP-1 promoters. ERα has been shown to cross talk with AP-1 and coregulate genes like CycD1
and c-myc (28, 32). Here, we have demonstrated for the first time that Cd exposure increases the interaction between ERα and c-Jun, but not between ERα and c-Fos (Fig. 5). These data are in accordance with the recent finding that stress induced by arsenic exposure increases ERα-bound c-Jun (33). Like Cd, arsenic is a heavy metal and the ERα/c-Jun interaction has been shown to neutralize heavy metal stress to avoid apoptosis (33). In the case of Cd, this interaction stimulates the expression of genes to mediate breast cancer cell growth.

Our findings demonstrate that Cd enhances the recruitment of c-Jun to the CycD1 and c-myc promoters (Fig. 6, A and B) and that ERα is likely recruited to these promoters via its interaction with c-Jun (Fig. 6, C and D). We extended our analysis using siRNA to deplete the endogenous levels of c-Jun and further demonstrated that c-Jun is necessary for the expression of Cd-induced CycD1 and c-myc expression. This is consistent with previous studies that have reported that Cd exposure increases the binding of c-Jun to the AP-1 site, and subsequently increases the trans-activation of AP-1 promoters (34–36). Together with this report, the data from our study suggest that Cd is involved in facilitating the cross talk between ERα and other transcription factors, such as c-Jun, to regulate expression of Cd-induced genes. In support of our findings, a recent study has also revealed that Cd activates the Erk1/2 and Akt kinases, resulting in the phosphorylation of ERα, which has been shown to promote the ERα/c-Jun interaction (22, 37–39). We anticipate that Cd may function to mediate protein-protein interactions between ERα and other transcription factors, providing an alternative mechanism for activating Cd-induced gene expression. This possible mechanism would also explain the failure of recent studies to demonstrate Cd’s estrogenic activity using a pure estrogen response element in a yeast estrogen screen (40, 41).

In summary, the results from this study demonstrate for the first time that Cd promotes breast cancer cell growth and gene expression by potentiating the interaction between ERα and c-Jun and enhancing the binding of these transcription factors to target gene promoters. We speculate that Cd mediates ERα cross talk with other transcription factors, including Sp1, E2F, and NFκB family members, to regulate additional Cd-induced genes. It would be very interesting to investigate a possible transcription factor complex or complexes involved in regulating the expression of CycE, cdk2, and other non-ER target genes that are up-regulated in response to Cd treatment (Fig. 1, B and C). The role of ERβ in Cd-mediated growth and gene expression appears to be minimal because siRNA-mediated depletion of ERβ did not alter the Cd responsiveness of the cells (Fig. 2, E and F). The results presented in this study have established a direct link between Cd-induced ERα-regulated gene expression and breast cancer, and underscore the importance of Cd exposure in breast carcinogenesis.

Materials and Methods

Cell culture

Human MCF-7, T-47D, ZR-75-1, and MDA-MB-453 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). All cells, except T-47D, were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics. T-47D cells were maintained in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, and antibiotics. All cell lines were subcultured every 3–4 d.

Cell proliferation analysis

Cells were plated at a density of 2 × 10^5 per well in a six-well plate and hormone deprived for 2 d. Hormone deprivation is performed by maintaining cells in media containing 10% charcoal-dextran stripped FBS (CDS). Cells were treated with 10^{-6} M CdCl₂ (Sigma, St. Louis, MO) or 10^{-8} M 17β-E₂ (Sigma), or were mock treated with 1X PBS. Cell number was determined every 2 d by cell counting in triplicate using a hemacytometer. Presented data are representative of several experiments. In the ER degradation experiments, cells were treated with 10^{-6} M CdCl₂ alone or 10^{-6} M CdCl₂ and 10^{-8} M ICI (Sigma), or were mock treated. For the RNAi silencing, MCF-7 cells were plated in 24-well plates and transfected with siRNA directed against ERα or c-Jun (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using siRNA transfection reagent (Santa Cruz Biotechnology, Inc.). A scrambled siRNA sequence was used as a negative control. Twenty-four hours after transfection, cells were treated with 10^{-6} M CdCl₂, 10^{-7} M 17β-E₂ or mock treated with PBS. Cells were counted in triplicates and harvested for protein and gene expression analysis 2, 3, and 4 d after treatment.

For the indirect cell proliferation assay, 2000 cells were plated per well in a 96-well plate in hormone-deprived media. Twenty-four hours later, cells were treated with 10^{-6} M CdCl₂ or 10^{-8} M 17β-E₂, or were mock treated. Proliferation was monitored every 2 d by incubating cells with 0.5 mg/ml MTT (Sigma) for 1.5 h. MTT crystals were dissolved in dimethylsulfoxide (Sigma) and read at 595 nm in a plate reader (Bio-Rad, Hercules, CA).

Western blot analysis

MCF-7 cells were plated and treated as described above and harvested 24 and 48 h after treatment. Cells were lysed in 1% sodium dodecyl sulfate-HEPES buffer, and the total protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad). Total protein was separated via SDS-PAGE and transferred to a poly(vinylidene difluoride) membrane. Protein expression was monitored using protein-specific antibodies: α-ER (BD, Franklin Lakes, NJ), α-ERβ, α-CycE, α-cdk2, α-p21, α-E2F1, α-c-Jun (Santa Cruz Biotechnology, Inc.), α-CycD1 (Transduction Laboratories, Lexington, KY), and α-actin (Sigma).
Commmunoprecipitation

MCF-7 cells were plated and treated as previously described. They were harvested 48 h after Cd treatment. Cells were lysed in a 0.5% Igepal solution buffer and sonicated for 30 sec. Cell lysates were immunoprecipitated with α-ERα (1:1 ratio of ERα antibodies: HC-20 and MC-20; Santa Cruz Biotechnology, Inc.), α-c-Jun (Santa Cruz Biotechnology, Inc.), or normal rabbit IgG for 1.5 h and then incubated with protein A for 1 h. Protein complexes were washed three times with PBS and then separated by SDS-PAGE followed by Western blot analysis with α-ERα (Neomarkers, Fremont, CA), α-c-Jun (Santa Cruz Biotechnology, Inc.), α-c-Fos (Santa Cruz Biotechnology, Inc.), and α-rabbit IgG (Bio-Rad) antibodies.

RT-PCR analysis

MCF-7 cells were plated and treated as previously described. Cells were harvested for mRNA analysis 24 and 48 h after treatment. Total RNA was isolated using the Trizol reagent according to the manufacturer’s protocol. Three micrograms of total RNA, Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA), and oligo-dT18 primers were used in the RT reaction. Gene expression was monitored using 3 μl of cDNA and gene-specific primers by 28 cycles of semiquantitative PCR reaction. Gene expression was monitored using 3

Reporter gene assay

The reporter gene assay with promoter-luc was performed by transfecting MCF-7 cells with the firefly luciferase reporter plasmid, pBV-myc promoter (2.1 kb) or pA3-CycD1 promoter (1.7 kb), and with the pRL-SV40 Renilla luciferase plasmid (Promega) using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Five hours after transfection, the medium was changed to hormone-deprived media containing 10−6 M CdCl2, 10−7 M β-E2, or 1× PBS. Cells were harvested at 48 h after transfection and analyzed using a dual luciferase assay kit (Promega). All reporter gene assays were performed in triplicate, with the entire experiment repeated at least three times. pBV-myc (2.1 kb) was obtained from Addgene and the pA3-CycD1 promoter (1.7 kb) was a kind gift of Dr. Richard Pestell (9).

For the RNAi silencing experiments, MCF-7 cells were seeded in 24-well plates and transfected firefly luciferase reporter plasmid, pBV-myc promoter (2.1 kb) or pA3-CycD1 promoter (1.7 kb), and with the pRL-SV40 Renilla luciferase plasmid (Promega, Madison, WI) in the presence of either ERα, c-Jun, ERβ, or control siRNA using siRNA transfection reagent (Santa Cruz Biotechnology, Inc.). Five hours after transfection, the medium was changed to hormone deprived, and 24 h later, cells were treated with 10−6 M CdCl2, 10−7 M 17β-E2, or mock treated with PBS. Cells were harvested at 48 h after treatment and analyzed using a dual luciferase assay kit (Promega).

ChIP

MCF-7 cells were plated in 10-cm plates and hormone deprived for 1.5 d. Cells were treated with 10−6 M CdCl2 for 30 h. After treatment, the cells were fixed with formaldehyde, scraped, and collected by centrifugation. ChIP was performed as previously described (10). Briefly, equal numbers of cells were lysed and sonicated in lysis buffer to break the chromatin down to 1- to 2-kb fragments. The chromatin mixture was immunoprecipitated with antibodies specific for the proteins of interest [1:1 mixture of α-ER antibodies: HC-20 and MC-20 or c-Jun (Santa Cruz Biotechnology, Inc.).] Antibody complexes were purified using either protein A or G beads. The complexes were reverse-cross-linked to release the chromatin fragments, and the DNA was purified using QiaQuick columns (QIAGEN, Valencia, CA). Occupancy at a specific promoter was detected by PCR using the promoter-specific primers listed below. PCR products ranging 300–500 bp were separated by agarose gel electrophoresis and visualized using the ChemiDoc system (Bio-Rad). The sequences of the ChIP primers are listed below:

For quantitative RT-PCR analysis of gene expression, gene sequences were amplified in the presence of SYBR Green fluorescent (Superarray, Frederick, MD) and detected using the iCycler real-time PCR equipment (Bio-Rad). Fluorescent values after each elongation step were collected along with a melting curve analysis at the end of the PCR. Fold difference (N) was calculated using the equation: n = E(CT-treated − CT control) between treated cells and mock-treated (control) cells. E is defined as the PCR efficiency of the specific primer pair used and was determined by the standard curve [E = 10(1/slope of standard curve)]. CT is the threshold cycle, which is defined as the minimum cycle number at which the fluorescence of the PCR product is first detected.

ChIP re-ChIP

For the re-ChIP assay, protein A-antibody complexes resulting from the first ChIP were extracted twice by incubation with 25 μl of 10 mM dithiothreitol for 20 min each at 37 C. The supernatants were pooled and diluted 10 times with re-ChIP buffer (20 mM Tris-HCl, pH 8.1; 2 mM EDTA; 150 mM NaCl; and 0.1% Triton X-100). The chromatin mixture was re-ChIPed with the second antibody: α-ER (1:1 mixture of α-ER antibodies: HC-20:MC-20), α-c-Jun, or normal rabbit IgG,
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