4-23-2021

The Effect of Cadmium on Ovarian Adenocarcinoma Cell Lines: An Investigation of the Possible Mechanism of Action

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The Effect of Cadmium on Ovarian Adenocarcinoma Cell Lines: An Investigation of the Possible Mechanism of Action

A Senior Thesis Presented in Partial Fulfillment of the Bellarmine University Honors Program

Submitted by:

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on

April 23, 2021

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ABSTRACT

Cadmium, a heavy metal and carcinogen, is an environmental and workplace contaminate. As a known endocrine disruptor, it can mimic the proliferative effects of estrogen and is classified as a metalloestrogen. While the proliferative effect of estrogen on cancerous cell growth has been well established, the effects of cadmium have not been fully examined. To determine if cadmium stimulates growth in two human ovarian adenocarcinoma cell lines, OVCAR3 and SKOV3, cells were treated for 48 hours with varying concentrations of cadmium, 0.001 μM – 10 μM, and growth was measured using a cell proliferation assay. Both cell lines showed a peak in cellular proliferation at 0.1 μM, and cell death was induced at 10 μM. Further, cadmium was shown to activate phosphorylation of ERK1/2, a key protein involved in estrogen signaling. To determine if cadmium-induced phosphorylation of ERK1/2 uses a similar signaling pathway as estrogen, inhibitors were used to block two key proteins in the estrogen signaling pathway including the estrogen receptor (α and ß) and MEK. Following treatment with each inhibitor, cells were treated with cadmium for five minutes, and immunoblot analysis was used to measure the level of ERK1/2 phosphorylation. Preliminary results suggest the inhibition of MEK decreases ERK1/2 phosphorylation in SKOV3 and OVCAR3 cell lines. However, results demonstrate that inhibiting the estrogen receptors α and ß does not inhibit phosphorylation of ERK1/2, suggesting cadmium induces cellular changes using a different pathway than estrogen.

INTRODUCTION

Ovarian cancer is the most fatal gynecologic cancer and the fifth most fatal cancer in females, causing more death than breast cancer. Each year, over twenty thousand women are newly diagnosed with ovarian cancer, and fourteen thousand women will lose their lives to this silent killer.[1] Unfortunately, due to lack of research and funding, ovarian cancers remain hard to diagnose and many are not found until they've reached stage III or IV, significantly decreasing chance of survival.[1] In fact, the fatality rate of ovarian cancer continues to be 50% due to late-stage diagnosis and commonality of recurrence after treatment.[2, 3] While estrogen has been shown to play a key role in the proliferation of both breast and ovarian cancer, ovarian adenocarcinoma cells have failed to respond to anti-estrogen therapy in the same manner as breast cancer, leading to further questions of the pathway of endogenous and exogenous estrogens in ovarian cells.[3] The purpose of this study is to provide more insight into the response and activation of cellular proliferation in ovarian adenocarcinoma cells due to cadmium, an exogenous contaminate able to mimic estrogenic effects.

Ovarian Cancer

The American Cancer Society estimated 21,750 women in the United States would be diagnosed with ovarian cancer in the year of 2020.[4] Of those women, it was estimated 13,940 would die due to the cancer.[4] Epidemiologically, rates are highest among white, non-Hispanic, post-menopausal women with diagnosis rates peaking between 50-70 years of age.[5] A 46% survival rate continues to be a harsh reality many women diagnosed with ovarian cancer face, and most of these deaths could have been avoided had there been an effective method to screen for early-stage diagnosis.[1, 5] It is the continued goal of researchers to better understand preventative strategies, risks, manifestation, screening, and treatment of ovarian cancer.

The three main types of ovarian cancers are epithelial, germ cell, and sex-cord-stromal with epithelial ovarian cancer (EOC) making up about 95% of all ovarian cancer types.[1] EOC can be further divided into four subtypes, serous, endometrioid, mucinous, and clear cell, with serous EOC being further divided into high-grade serous carcinomas (HGSC) and low-grade serous carcinomas.[1] HGSC's account for 90% of all ovarian cancer tumors, and there is only a 30% survival rate for women with this subtype.[1] These statistics have remained relatively unchanged over the past thirty years, indicating a huge gap in scientific knowledge.[1]

Originally, it was believed all EOC's originated in the outer epithelium layer of the ovaries, but it was later found there are three points of origin.[1] Malignant growths can start in the epithelium of the ovaries, fallopian tubes, or other sites in the pelvis, making it hard to pinpoint the genetic and environmental triggers for EOC.[1] These malignant growths then proceed in a manner like other cancers, staying benign or metastasizing to other areas of the body. It is metastasized tumors which present such a concern since they become widespread and almost untreatable.

Researchers have identified a wide variety of risks leading to EOC. Most likely, it is a combination of several of these risk factors which lead to the manifestation of the disease. Firstly, several genes have been identified to increase the likelihood of ovarian cancer.[1, 5] Most notable is the BRCA mutation often associated with breast cancer. The presence of BRCA 1 is associated with a 40-50% of EOC development by the age of seventy while BRCA 2 accounts for a 20% risk increase.[5] When a woman with a BRCA mutation develops EOC, the tumor is referred to as Type II and is highly associated with fatal outcomes.[1, 5]

When a tumor cannot be linked to a genetic mutation, it is referred to as a Type 1 tumor and is theorized to be caused by increased inflammation due to the repeated breakdown and

repair of the epithelial layer.[5] The age women begin menstruating, when they reach menopause, and the number of pregnancies along with duration of breastfeeding all have been shown to factor into the development of EOC.[1, 5, 6] The American Cancer Society attributes increased number of ovulation cycles to an increase in risk for EOC. Unfortunately, the average age women begin menstruating has decreased from 16 years to 12.5 years meaning their ovulation cycles, on average, are four years longer than they used to be.[5] Anything which halts ovulation, such as oral contraceptives, pregnancy, and breastfeeding, are associated with decreasing the risk of ovarian cancer due to decreased stress on reproductive systems related to menstruation.[5] However, it is important to note these are not preventative strategies for ovarian cancer as the use of oral contraceptives, pregnancy, and breast-feeding are lifestyle burdens not every woman wishes to undergo.

The lack of consistent and accurate screening for ovarian cancer remains a large factor contributing to its mortality rate. Late-stage EOC, which includes both stage III and IV cancer, is difficult to treat and often recurs after initial treatment.[1, 6] Further, the symptoms associated with ovarian cancer are non-specific and occur commonly in women for a wide variety of non-cancer related issues such as abdominal bloating, abdominal pain, nausea, frequent urination, change in bowel movements, back pain, loss of weight, and fatigue.[6] In the past, health care professionals screened for the cancer antigen 125 (CA125) as a tumor marker for ovarian cancer, but it has recently been shown that this marker lacks specificity and sensitivity as it was not elevated in 50% of patients during early stage EOC.[1] Since the CA125 screen has been proven to be unreliable, it is increasingly harder to make an early diagnosis. Currently, health care professionals rely on a combination of knowing a patient's genetic risks, their symptoms, and a transvaginal sonography scanning for pelvic masses to determine if there are any masses which

require biopsy.[1] Once a mass has been identified, only a biopsy can confirm the diagnosis of ovarian cancer. Unfortunately, even this procedure comes with risks as the core-needle biopsy used to collect tissue can result in abdominal wall metastasis.[1] Most biopsies will result in a diagnosis of stage III EOC.

The most common treatment for ovarian cancer involves a debulking surgery followed by chemotherapy.[1] With debulking surgery, surgeons remove the majority of the tumor to increase the effectiveness of chemotherapy since there will be less malignant cells present. With advanced cell EOC, chemotherapy begins with the administration of carboplatin and paclitaxel over six to eight therapeutic cycles.[1, 6] Despite these treatment steps, remission is not ensured, and patients who do go into remission have up to a 95% rate of recurrence in late-stage ovarian cancers.[1] Understanding how this deadly cancer functions is vital to create better screening and treatment for the women who develop this disease.

Estrogen

Estrogens are hormones vital to the development of sexual and reproductive functions in both males and females. In females, androstenedione is produced from cholesterol in the theca cells adjacent to granulosa cells in the ovaries.[7] This androgen is then transported to granulosa cells with high concentrations of the enzyme aromatase responsible for converting androstenedione to estrogen.[7] From there, estrogen is converted to its most potent biologically active form, estradiol, and can then circulate in the blood stream to induce effects on various tissues.[7] Endogenous estrogen production is highest in pre-menopausal women and has a wide variety of regulatory functions within the body, with the most important being breast, mammary gland, and ovary development and ovulation maintenance.[7-9] However, elevated levels of estrogen in post-menopausal women have been associated with the development of breast and

ovarian cancer.[8] Due to this, it is normal and desirable for estrogen production to decrease in age, especially after a woman reaches menopause.

The interaction of estradiol with estrogen receptors (ERs) is responsible for cellular changes associated with estrogen, including cellular proliferation. Estrogen receptors, such as ER-α and ER-β, are part of a steroid receptor subfamily of transcription factors which play a role in breast, uterine, and ovarian cancer.[10] Previously, the nuclear membrane was the accepted location of ERs. In recent years, the presence of plasma membrane estrogen receptors has opened up a wider avenue for estrogen action.[11]

Each ER is functionally distinct, and recent research has proposed ER-β opposes the action of ER-α.[9, 12] In fact, the ratio of ER-α to ER-β has been suggested as a potential marker for carcinogenesis in several estrogen-sensitive cancers.[3, 13] Within ovarian cancer, 67% percent of patients are ER positive and have a higher ratio of ER-α to ER-β, suggesting increased levels of ER-α are pro-tumorigenic.[3] Furthermore, a decrease or lack of ER-β in estrogen-sensitive cancer has led researchers to investigate the anti-proliferative effects of ER-β, suggesting the binding of estradiol to ER-β can inhibit pathways activated by ER-α.[3, 12] Furthermore, tumors can decrease the expression of ER-β but the mechanism by which they achieve this remains largely unknown.[12] Some have even begun to speculate if ER-β can be used in tandem with estrogen antagonists for the treatment of estrogen-sensitive cancers.[12]

Nuclear estrogen receptors are intracellular receptors with a dimerized structure of cysteine residues regulated by a zinc binding domain.[14, 15] When activated by estrogen or an estrogen-like-chemical, the ER protein binds to DNA at specific nucleotide sequences termed oestrogen response elements.[15] ER-α and ER-β were found to be evolutionarily conserved

structurally with a highly conserved DNA binding domain, COOH-terminal ligand binding domain, and variable NH_2-terminal domain.[9]

With the recent discovery of membrane-bound ER, the process of estrogen signaling in ovarian cells has become more complicated. Membrane-bound ERs function in a different manner and have been shown to interact with a G-protein coupled estrogen receptor 1 (GPER1) to activate a protein kinase cascade eventually leading to DNA regulation (Figure 1).[3, 11] The crosstalk between ERα and GPER1 has become a large point of study in ovarian adenocarcinomas and may provide a possible avenue to explain estrogen resistance in these cancers.

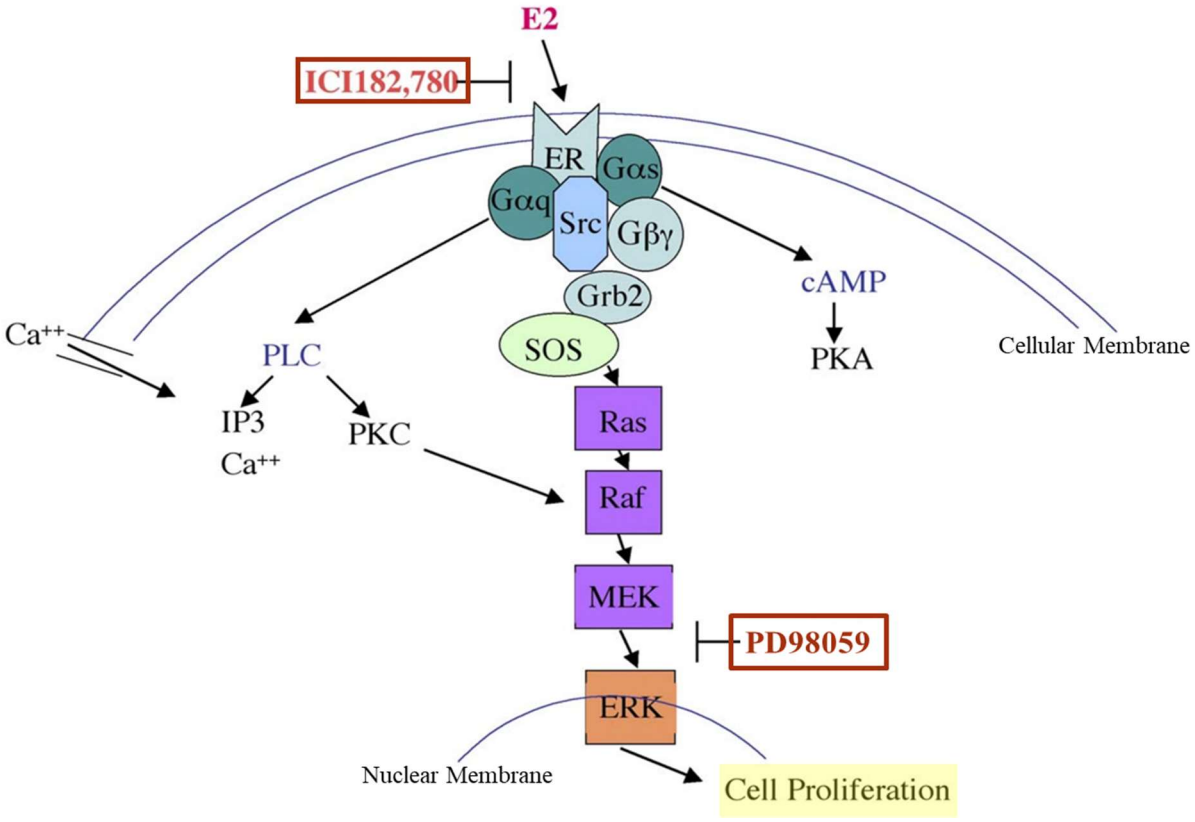

Figure 1: Estrogen signaling pathway kinase cascade through membrane-bound ER activation. ICI 182,780 and PD98059 are shown by the step which they inhibit. Figure adapted from Levin *et al*. (2008).[11]

Under normal conditions, estradiol binds to membrane-bound ER-α located in lipid domain rafts within the cellular membrane and initiates a cascade of events that results in increased cellular proliferation.[16] Upon estrogen binding, ER-α interacts with transmembrane growth factors, including the epidermal growth factor receptor (EGFR). EGFR, not shown in Figure 1, then activates Src kinase which in turn activates MEK. MEK activation induces the phosphorylation of ERK1/2 which then phosphorylates a number of signaling proteins that induce cellular proliferation.[16, 17] It is thought EGFR, Src and MEK also play important pro-tumorigenic roles in cancerous cell growths, making them possible key players in estrogen and estrogen-like interaction and activation of cellular proliferation in ovarian cancer.[17-19]

One common method used to treat estrogen-sensitive cancers is the use of anti-estrogens to block the proliferative effect of ER-α. In breast cancer, the anti-estrogen tamoxifen is the first line of treatment for hormone dependent, ER^+ breast cancer.[8, 9] Due to its success, it was proposed as a therapy in ER^+ ovarian cancers. However, EOC has shown a large resistance to anti-estrogen treatment due to estrogen resistance in these cell lines.[3, 12] One emerging theory suggests that because tamoxifen blocks both ER-α and ER-β, ER-β is unable to halt cellular proliferation.[12] Furthermore, estrogen signaling pathways are able to interact with several oncogenic pathways, promoting cellular proliferation even when the effects of estrogen are blocked by the ER-antagonist tamoxifen.[3] Crosstalk between estrogen signaling pathways most likely results in the lack of success seen in anti-estrogen treatment for EOC. The speculation behind anti-estrogen treatment resistance in EOC demonstrates the complexity of hormone signaling and possible role in ovarian cancers.

Endocrine Disruptors and Metalloestrogens

Along with endogenous hormones produced by the body, there are a wide variety of natural and synthetic compounds that can interact with hormonal function and are referred to as endocrine disruptors (EDs).[20] These endocrine disruptors can interfere or react with receptor binding and the metabolism of hormones to increase or decrease the effects of hormones within the body.[20, 21] Many are familiar with the endocrine disruptor DDT which was once used as a common pesticide before it was determined to have hazardous effects on the environment and humans. It was classified as an ED due to its ability to interfere with several hormonal functions, including estrogen and androgen action.[20] Since then, significantly more EDs have been discovered and classified.

The issue with endocrine disruptors is their ability to interfere with the natural homeostatic processes within the body that rely on hormonal signaling.[20] When exposure occurs early in life, EDs can significantly impact development and fertility of many organisms, including humans.[20, 21] Due to this, it is important to identify EDs and limit their release into the environment to decrease exposure to developing organisms. While DDT has been banned due to its adverse effects as an ED, there remain many common pollutants in this category.[20]

Many are familiar with bis-phenol-a (BPA) as a stabilizing agent commonly used in plastics. Over recent years, concern was raised over the estrogenic effects of BPA on the body, especially since exposure rates from plastic bottles was so high.[22] Levy *et al.* (2004) discovered BPA can interact with ER as an exogenous estrogen and induce feminizing effects on tadpoles, a key study in raising public awareness to the dangers of EDs and BPA.[22] In this study, it was shown that under normal conditions, the ratio of male to female tadpoles is 50:50.[22] When exposed to the environmental contaminant of BPA, the percentage of females rose as high as

80%, demonstrating the estrogenic effects of BPA could determine the sex of tadpoles at the larval stage.[22] This has serious implications in ruining the balance of natural environments by altering the natural ratio of male to female frogs. Furthermore, it is possible BPA also has adverse effects on fertility which were not examined in this study.

While it is hard to determine exposure events due to the wide pollution of EDs in the environment, Rattan *et al.* (2017) studied how exposure to EDs could affect female fertility in adults. It is known that reproductive functions in females are highly dependent on a balance of hormones, mainly estrogen. Any increase in exogenous hormone exposure is likely to cause adverse effects, including increased risk for ovarian cancer.[21, 23] Identifying endocrine disruptors and limiting exposure is highly important for the health of females.

Recent studies have demonstrated heavy metals can have estrogenic effects on the body and that this action may occur through a nonspecific interaction with the estrogen receptor. Since ERs have a large ligand binding cavity, many EDs are able to interact and increase negative hyper-estrogenic effects in individuals exposed to contaminants.[9] This recently defined group of heavy metal EDs, termed metalloestrogens, are naturally occurring chemicals which mimic or interfere with the actions of estrogen within the body.[15] It is also known heavy metals are common carcinogens. These carcinogens with estrogen-like activity may increase carcinoma growth by stimulating estrogen receptor pathways responsible for increasing cell proliferation.[15] How and which pathways metalloestrogens interact with are largely still under investigation in several types of adenocarcinomas, including EOC.

Over the years, heavy metal environmental contamination has increased due to urbanization and industrial processes.[24] Cadmium (Cd) remains an environmental contaminant of concern due to the effect it has on the body; causing oxidative stress, DNA damage, and

inhibition of DNA repair mechanisms.[18] Combined, these effects can increase the risk of cancer in individuals exposed to Cd. This exposure can occur through environmental contamination of water, air, food, and plants, occupational exposure, or through an individual's use of cigarettes.[2] Cd persists in soft tissues for 15-20 years and has even been linked to increased proliferation in lung cancers.[2, 18] The mechanism of cadmium action that results in cellular proliferation has earned it a spot in the emerging class of metalloestrogens.

Originally, studies focused on the cytotoxic effects of cadmium and other heavy metals demonstrated exposure increased the susceptibility of cancer manifestation due to the inhibition of several key cellular functions.[25] However, a person is more likely to be exposed to chronic low levels of Cd than singularly being exposed to high concentrations. In 2006, Brama *et al*. showed that exposure of breast cancer cells to 10 μM $CdCl_2$ induced cellular proliferation through the interaction of Cd with ER-α.[26] This research provided evidence that metalloestrogens could mimic the effects of estradiol in low concentrations and with an estrogen dependent cancer, such as breast cancer, cellular proliferation of cancerous cells could be increased in women chronically exposed to Cd.

Zang *et al.* (2009) provided further evidence for the interaction of Cd with ER and the activation of ERK1/2.[27] After ten minutes of 1 μM Cd exposure, breast cancer cells showed increased phosphorylation of ERK1/2.[27] Through treatment with the ER inhibitor ICI 182,780, cadmium-induced phosphorylation of ERK1/2 was inhibited, suggesting an ER dependent pathway of activation.[27] Other studies, discussed below, have also shown estrogen dependent cancers increase in proliferation upon Cd exposure and support an ER dependent mechanism.

In 2016, Huff *et al.* reported their findings on the effect of Cd on several lung adenocarcinoma cell lines. In these studies, it was found that in cell lines derived from female

patients, Cd induced cellular proliferation and caused increased levels of phosphorylated ERK1/2, both of which were inhibited when the estrogen receptors were blocked by the addition of an estrogen receptor antagonist, ICI 182,780.[18] Through inhibitor studies, it was found that Src, epidermal growth factor (EGFR) and GPER were necessary for cadmium-induced ERK1/2 phosphorylation.[18] This study suggests Cd interacts with G-protein coupled ERs in female lung adenocarcinomas to stimulate cellular proliferation through the activation of the ERK1/2 pathway.

Purpose of Study

Based on previous studies, cadmium has demonstrated estrogen-like activity in a number of different tissues, inducing cellular proliferation in an ER-dependent manner.[15] It seems likely other estrogen-sensitive tissues, including ovarian tissue, might also respond to Cd in a similar way. Using two different ovarian cell lines, OVCAR3 and SKOV3, preliminary results in the Huff lab suggested Cd may slightly increase cellular proliferation in OVCAR3 while the SKOV3 cell line does not. Studies conducted by Pujol e*t al*. (1998) have shown that SKOV3 cells have a deleterious mutation in ER-α, truncating the resulting protein and rendering it unable to bind estrogen.[13] This difference in a functional ER-α might explain the differential response to Cd that has been observed.

Furthermore, Biochemistry and Molecular Biology student Kira Steinke showed that 0.1 μM of Cd activated ERK1/2 in both OVCAR3 and SKOV3 cell lines within 10 minutes of treatment. These results show there is ERK1/2 phosphorylation occurring in OVCAR3 and SKOV3 upon treatment with Cd. However, it has not been confirmed this phosphorylation induced by Cd works in an ER dependent manner. It could be possible the phosphorylation in OVCAR3 occurs through ERs while another pathway is responsible for the phosphorylation in

SKOV3 due to the mutation in ER-α. Pathways of Cd interaction in ovarian cancer have yet to be identified.

The purpose of this study is to first extend the proliferation studies to determine if these two cell lines demonstrate differential responses to Cd and then study ERK1/2 phosphorylation after inhibition of ERs and MEK. The research should provide insight into the signaling pathways that are activated by Cd to cause a cellular cascade in OVCAR3 and SKOV3 ovarian adenocarcinoma cell lines.

METHODS

Cellular Proliferation Assays

Two human ovarian adenocarcinoma cell lines, OVCAR3 and SKOV3, were obtained from ATCC (Manassas, VA) and grown following manufacturer's directions. To conduct treatments with cadmium and estradiol, cells were plated at 5,000 cells per well in a 96-well plate. For SKOV3, cells were plated in 200 µL of McCoy's 5a containing 10%(v/v) FBS (ATCC) and 1% penicillin/streptomycin (Thermo-Fisher, Waltham, MA) growth media. For OVCAR3, cells were plated in 200 µL of RPMI 1640 containing 10%(v/v) FBS, 0.01 mg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (ThermoFisher). Cells were allowed to grow for 24 hours before the growth media was changed to the appropriate experimental media lacking hormones and supplemented with 10% charcoal-stripped fetal bovine serum. After another 24 hours, cells were treated in quadruplicate with varying cadmium ($CdCl_2$) concentrations ranging from 0.001 µM to 10 µM or estradiol concentrations ranging from 0.001 µM to 0.1 µM. No treatment was used as the control for the cadmium studies while 1% ethanol was used as the control for the estradiol studies. Cells were treated for 48 hours, and cellular

proliferation was measured using the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Madison, Wisconsin) following manufacturer's instructions. A BioRad 680 plate reader (Hercules, CA) was used to determine the absorbance readings at 490 nm. These experiments were repeated 4-5 times for each cell line.

Inhibitor Studies

OVCAR3 and SKOV3 cell lines were grown at 400,000 cells in 3 mL of respective growth media on a 60 cm^3 petri dish for 48 hours. The media was then replaced with 3 mL of media lacking hormones and containing charcoal-stripped fetal bovine serum for 48 hours. To inhibit the estrogen receptors alpha and beta, cells were pretreated with 10 μM ICI 182,780 dissolved in DMSO from Tocris Bioscience (Minneapolis, MN) for one hour. For the inhibition of MEK, cells were pretreated with 50 μM PD98059 (Cell Signaling Technology, Danvers, MA) dissolved in DMSO for one hour. Cells were then treated with 0.1 μM $CdCl_2$ for 10 minutes, and cell lysates were prepared. To prepare whole cell lysates, media was removed, and cells were washed with ice-cold phosphate buffered saline two times. Cells were then treated with 60 μL of ice-cold cell lysate buffer prepared with 1 mL RIPA buffer (Sigma), 1 μL Phosphatase Inhibitor Cocktail 2 (Sigma), 1 μL of Phosphatase Inhibitor Cocktail 3 (Sigma), 1 μL Protease Inhibitor Cocktail (Sigma), and 10 μL of 100 mM PMSF. Cells were scraped from the petri dish on ice and collected in microcentrifuge tubes. Each lysate was sonicated for 1 second two times using a Microson™ ultrasonic cell disruptor (Barcelona, Spain) before being centrifuged for 10 minutes at 4°C. The supernatant was transferred to clean microcentrifuge tubes and stored at -80°C.

SDS Gel Electrophoresis and Immunoblot Analysis

Proteins (25 μg) were separated on a 4-20% polyacrylamide SDS gel at 300 V for 15 minutes and transferred to a nitrocellulose membrane for immunoblot analysis. The blot was blocked in 5% milk in TTBS (20 mM Tris pH 7.4, 140 mM NaCl, 0.1% Tween-20) for one hour at room temperature. The blot was washed with TTBS 1x15 minutes and 3x5 minutes and then incubated with a primary polyclonal antibody at a 1/1000 dilution raised to phospho-ERK1/2 from Cell Signaling Technology overnight with shaking at 4°C. Following washes as described above, the blot was treated with a 1/1000 dilution of goat anti-rabbit conjugated with horseradish peroxidase polyclonal antibody from ThermoFisher. After washing, the blot was exposed to a 1:1 dilution of the ECL reagents from BioRad for five minutes before being imaged on a BioRad Chemidoc system. The membrane was then stripped for ten minutes using Stripping Buffer from Thermo Scientific and re-probed with a 1/1000 dilution of antibody raised to total ERK1/2 from Cell Signaling Technology. The proteins were exposed to ECL reagents as previously described and imaged once more. The images were analyzed by densitometry using Unscan-it software (Silk, Orem, UT). The images were reported as relative amounts of P-ERK1/2 to total ERK1/2.

RESULTS

Cellular Proliferation

The ovarian adenocarcinoma cell lines OVCAR3 and SKOV3 were used to determine the proliferative effect of Cd and estradiol on EOC. Both cell lines are epithelial in origin and were cultured from post-menopausal females, making them an ideal representative for ovarian cancer response in these studies. As previously stated, there is a difference in ER-α expression between the two cells lines, with SKOV3 containing a 32 bp mutation hypothesized to be responsible for

estrogen-insensitivity in this cell line. Cells were treated for 48 hours with Cd concentrations ranging from 0.001 µM to 10 µM, and cellular proliferation was determined using Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Experiments were repeated in quadruplicate and averaged for six trials. As shown in Figure 2A, OVCAR3 demonstrated slight, but insignificant, cellular proliferation when treated with nanomolar concentrations of Cd when compared to the control. This proliferation peaked with 0.1 µM Cd treatment at 106% ± 4% S.E.M of the control. When treated with 10 µM Cd, OVCAR3 cells experienced significant cell death with only 61% ± 8.8% S.E.M proliferation compared to the control. This suggests there is a fairly significant line between chronic low doses of Cd which may induce proliferation and doses which induce death.

SKOV3 also demonstrated slight but insignificant cellular proliferation when treated with low concentrations of Cd, as demonstrated in Figure 2B. Cellular proliferation peaked with a treatment of 0.01 µM Cd at 105% ± 2.9% S.E.M of the control. As with OVCAR3, treatment of SKOV3 with 10 µM Cd induced cell death with proliferation only 87% ± 4.5% S.E.M. of the

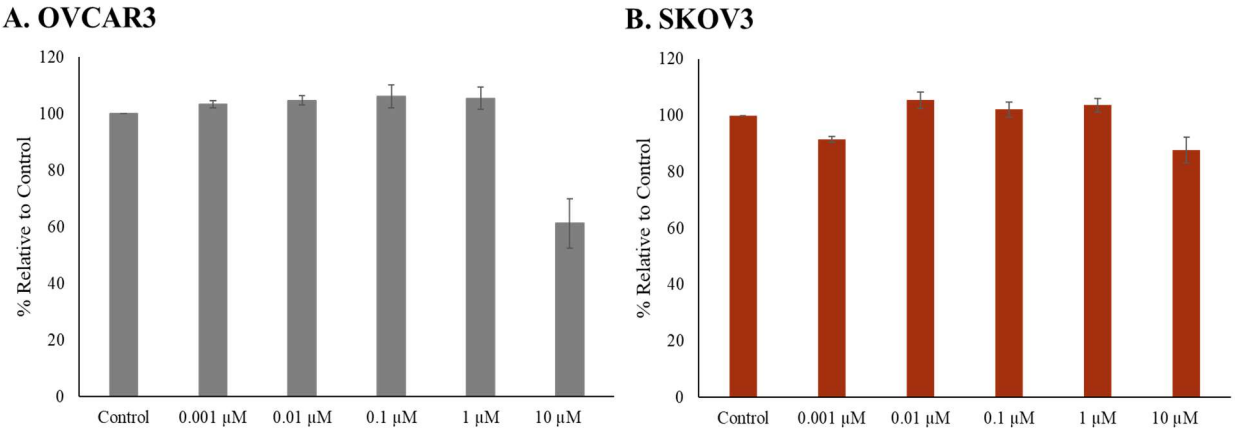

Figure 2: Treatment with Cd does not significantly affect cellular proliferation in (A) OVCAR3 and (B) SKOV3 cell lines. Cells were treated with 0.001 µM Cd to 10 µM Cd. Results are reported as relative to untreated control.

control. What was unexpected was treatment with 0.001 µM Cd consistently showed a decrease in cellular growth as compared to the control (91% ± 1% S.E.M proliferation).

Cells were treated for 48 hours with estradiol (E2) concentrations ranging from 0.001 µM to 0.1 µM, and cellular proliferation was determined using Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Experiments were repeated in quadruplicate and averaged for six trials. As shown in Figure 3A, OVCAR3 showed no significant increase in proliferation when compared with the control, even though these cells are derived from estrogen-responsive tissues. This data suggests estrogen does not have a proliferative role in OVCAR3 cells.

More interestingly, SKOV3 responded differently to estradiol treatment as shown in Figure 3B. Treatment with 0.001 µM and 0.01 µM estradiol was comparable to the control of ETOH, while treatment with 0.1 µM showed decreased cellular proliferation of 88% ± 5% S.E.M of the control. Perhaps, because SKOV3 does not contain a functional ER-α, estradiol is activating ER-β and causing some, but not significant, arrest in cellular proliferation.

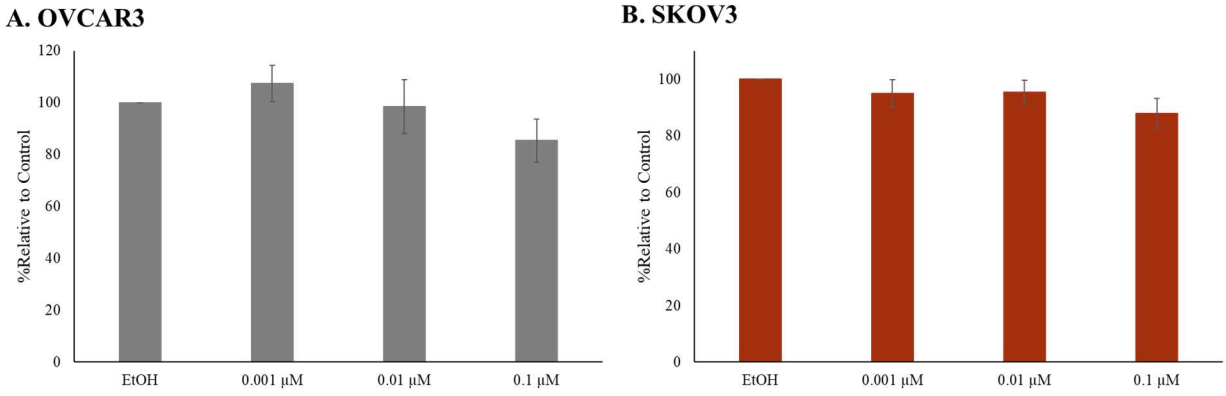

Figure 3: Treatment with Estradiol (E2) does not significantly affect cellular proliferation in (A) OVCAR3 and (B) SKOV3 cell lines. Cells were treated with 0.001 µM E2 to 0.1 µM E2. Results are reported as relative to untreated control.

Inhibitor Studies

Previous studies performed by Kira Steinke in the Huff lab showed that ERK1/2 was activated by 0.1 µM Cd within 10 minutes of treatment (unpublished data). To determine if Cd-induced activation of ERK1/2 involves membrane-bound ERs, SKOV3 and OVCAR3 cells were treated with the ER antagonist, ICI 182,780, one hour before treating the cells with Cd for ten minutes. As shown in Figure 4, Cd increased ERK1/2 phosphorylation alone in both SKOV3 and OVCAR3 cells. When the ER antagonist was added, there was a 50% increase in activation in OVCAR3 cells. In contrast, SKOV3 cells showed a 23% decrease in ERK1/2 phosphorylation

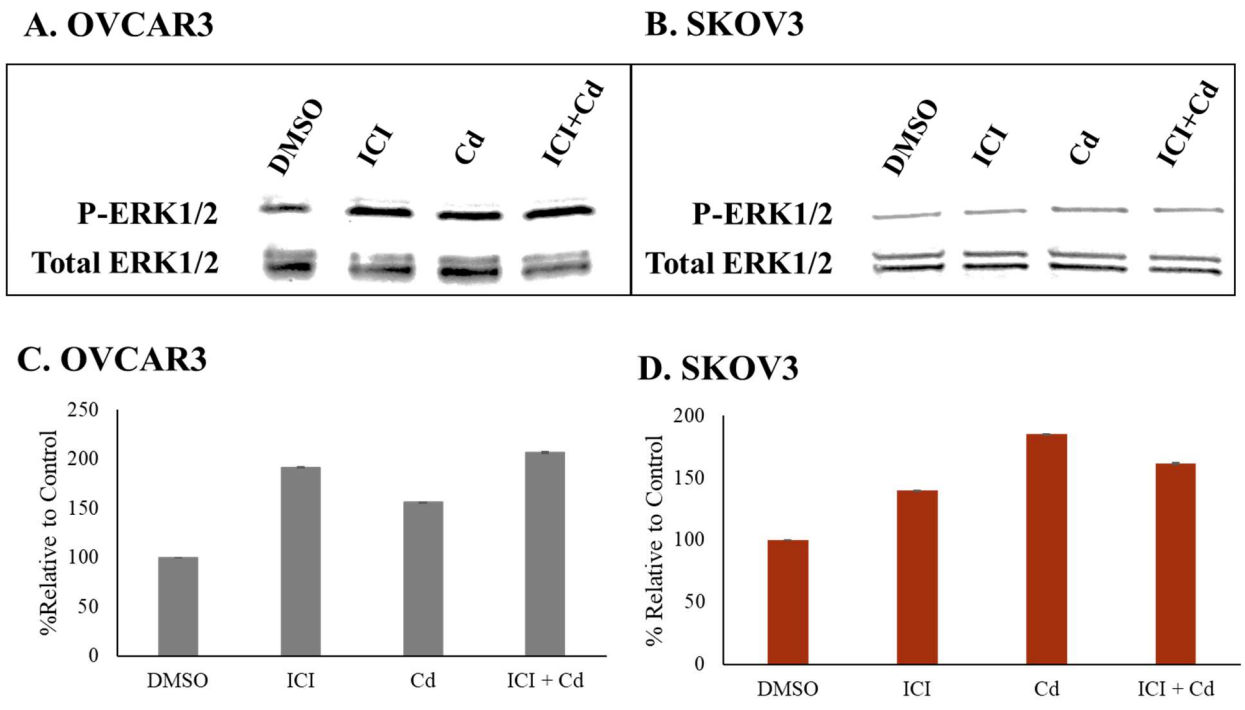

Figure 4: Inhibition of ER with ICI 182,780 does not inhibit Cd-induced ERK1/2 phosphorylation in OVCAR3 but has some affect in SKOV3. (A&B) Results of immunoblots for OVCAR3 and SKOV3, respectively. (C&D) Quantitative analysis of relative P-ERK1/2 performed using Unscan-it software. Cells were treated with 10 µM ICI 182,780 (ICI) for one hour before a ten-minute treatment with 0.1 µM Cd. Results are reported as % P-ERK/Total ERK relative to the untreated control and averaged for three trials.

when first treated with the ER antagonist. These results suggest ICI inhibits some of the Cd-induced ERK1/2 phosphorylation in SKOV3 cells but not in OVCAR3 cells.

Next, mitogen-activated protein kinase (MEK) was inhibited using PD98059 to confirm the activation of ERK1/2 upon Cd treatment. While it is likely that the ERs are not involved in the activation, inhibiting the signaling protein before ERK1/2 phosphorylation will begin to confirm the steps leading to phosphorylation. The results are shown in Figure 5. Once again, ERK1/2 phosphorylation was increased by Cd alone in both SKOV3 and OVCAR3 cells. When the MEK antagonist was added, there was a 131% decrease in activation in OVCAR3 cells and a 238% decrease in activation in SKOV3 cells. These results suggest MEK plays a key role in Cd-induced ERK1/2 phosphorylation.

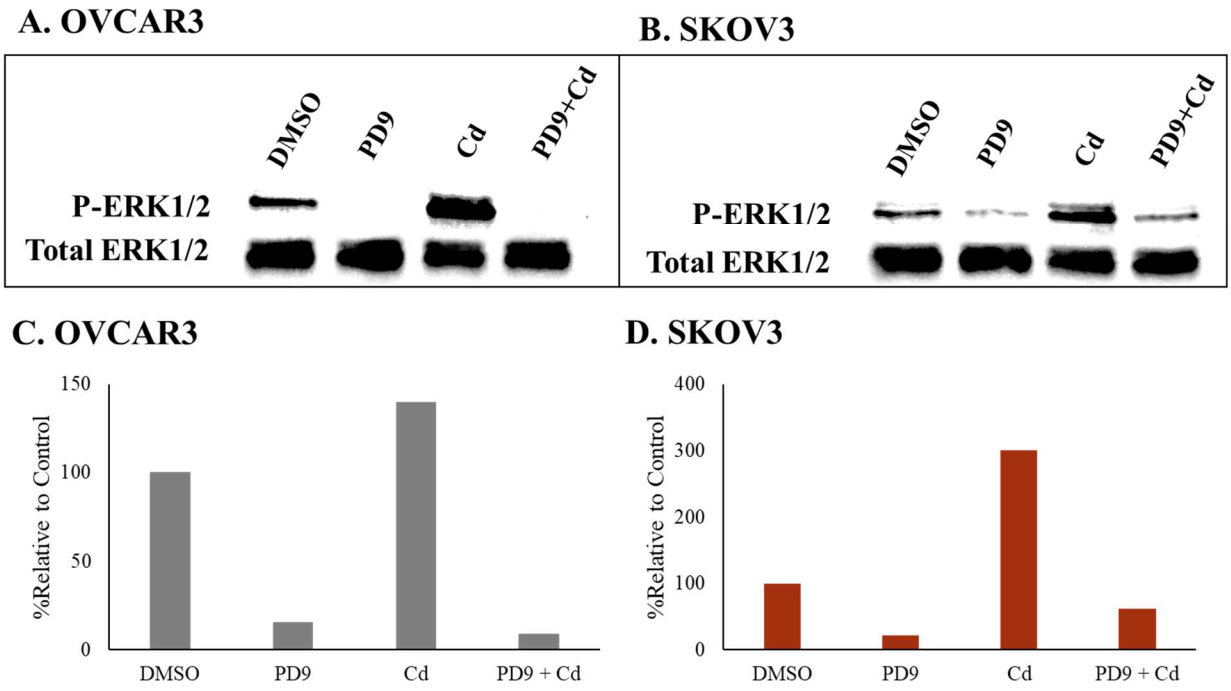

Figure 5: Inhibition of MEK with PD98059 inhibits Cd-induced ERK1/2 phosphorylation in OVCAR3 and SKOV3 cell lines. (A&B) Results of immunoblots for OVCAR3 and SKOV3, respectively. (C&D) Quantitative analysis of relative P-ERK1/2 performed using Unscan-it software. Cells were treated with 50 µM PD98059 (PD9) for one hour before a ten-minute treatment with 0.1 µM Cd. Results were reported as % P-ERK/Total ERK relative to untreated control for one trial.

DISCUSSION

Chronic exposure to low doses of the environmental contaminant cadmium (Cd) has been linked to cellular proliferation in breast, lung and ovarian cancer.[2, 18, 26] The pathway of Cd action has been well defined in breast and lung cancer and has been shown to interact with ERs leading to phosphorylation of ERK1/2, DNA modification, and cellular proliferation. However, it remains unclear if this same pathway is activated in the estrogen-sensitive ovarian cancer. These results demonstrate that phosphorylation of ERK1/2 occurs through a MEK dependent pathway upon low doses of Cd exposure, but they do not confirm an ER dependent mechanism of action. These results also discovered low doses of Cd exposure did not significantly increase cellular proliferation, demonstrating ovarian adenocarcinomas behave differently from breast and lung adenocarcinomas.

The working hypothesis of this study proposed that Cd would induce proliferation in ovarian cells and that this growth would occur through an ER-dependent mechanism. The functionality of ER-α mRNA transcripts in OVCAR3 was confirmed by Lau *et al*. (1999), and other research shows increased ratios of ER-α to ER-β in OVCAR3 cells compared to human epithelial surface epithelium cells (HOSE), which are non-cancerous ovarian cells.[10, 13, 28] Yet in these studies, OVCAR3 cells showed only slight increase in proliferation compared to the control when treated with 0.1 μM Cd for 48 hours, but this difference was not significant. Since OVCAR3 cells also did not respond to estradiol treatments in this study, it is likely that estrogen signaling does not play a significant role in the proliferation of OVCAR3.

Steinke's data (unpublished) does show increased levels of relative P-ERK/Total ERK in OVCAR3 cells treated with 0.1 μM Cd which persist from 5 to 15-minutes and peaks at the 10-minute mark. This confirms low doses of Cd are activating phosphorylation of ERK1/2, but it

remains unclear what role this is playing on a cellular level since there is no significant increase in cellular proliferation. In this study, inhibition of MEK significantly decreased Cd-induced ERK1/2 phosphorylation in OVCAR3 cells, showing it is a necessary signaling protein involved in the pathway. Further testing focused on the inhibition of suspected key players in the phosphorylation of ERK1/2 by Cd is needed to determine the pathway of its action in OVCAR3 cells.

The results of this study demonstrated that inhibition of ERs using the ER antagonist, ICI 182,780 (ICI), did not affect the activation of P-ERK1/2 in OVCAR3 cells. These results further support that Cd does not act in an ER-dependent manner in OVCAR3 cells to induce phosphorylation of ERK1/2. These results are consistent with previously published data that demonstrated ovarian cells do not respond to anti-estrogen treatments and provides further support that Cd does not act in an ER-dependent manner in this cell line.[3] Since ERs are not playing a significant role in the phosphorylation of ERK1/2 by Cd, this means there are other proteins playing a role in MEK activation in the OVCAR3 cell line.

When cells were treated with the ER antagonist alone, there was an increase in relative P-ERK/Total ERK compared to the control in both cell lines. Since ICI is non-selective and will block both ER-α and ER-β, this may account for the increase that was observed in both cell lines. Lazennec (2005) has proposed ER-β could function as a tumor suppressor protein since its levels are downregulated in ovarian carcinogenesis and ER-α is upregulated.[12] Furthermore, it was shown that the restoration of ER-β decreased cellular proliferation and increased adenocarcinoma apoptosis.[12] Since ER-β was inhibited along with ER-α upon ICI treatment, it is possible this removed the inhibitory action of ER-β and allowed increased signaling cascades from other pathways to be activated leading to phosphorylation of ERK1/2. More research needs

to be conducted to determine the effect of ICI treatment alone on the cellular proliferation of OVCAR3 cells to determine if blocking ER activity may activate, rather than inhibit cell growth.

SKOV3 cells responded in a different manner than OVCAR3, showing a slight increase when treated with 0.01 μM Cd for 48 hours compared to the control, but it was also not significant. Lau *et al*. (1999) discovered a 32 base-pair deletion in SKOV3 ER-α transcripts, suggesting that this resulted in loss of ER-α function rendering these cells estrogen-insensitive.[28] This could account for the lack of response seen in SKOV3 cells when treated with the metalloestrogen Cd. It also accounts for the lack of growth seen in SKOV3 cells treated with estradiol. If Cd is affecting SKOV3 cells, it is not causing an increase in cellular growth within the two days of treatment performed in this study.

Even though Cd does not appear to induce proliferation in SKOV3 cells, low concentrations of Cd significantly increase phosphorylation in ERK1/2 within minutes of treatment. Steinke (unpublished results) demonstrated there was a significant increase in relative P-ERK/Total ERK after 5 minutes of 0.1 μM Cd treatment in SKOV3 cells and this increase persisted until the 10-minute mark. Interestingly, at the 15-minute mark Steinke's data showed a decrease in relative P-ERK/Total ERK compared to the control. Perhaps there are pathways at work within SKOV3 cells to counteract the ERK1/2 activation induced by Cd. This could also account for the lack of results seen in cellular proliferation studies.

Treatment of SKOV3 cells with ICI did decrease ERK1/2 phosphorylation caused by Cd. It is interesting this cell line responded differently to ICI inhibition of ERs than OVCAR3 considering the SKOV3 cell line does not have a functional ER-α. Once again, there was an increase in ERK1/2 phosphorylation when SKOV3 cells were treated with the estrogen receptor inhibitor alone, and this elevated level was also maintained in the cadmium and ICI treatment

group. Even though treatment with an estrogen receptor inhibitor reduced the level of Cd-induced phosphorylation of ERK1/2, it is likely estrogen receptors do not play a significant role in SKOV3 cells treated with cadmium because levels were not returned to the control. However, just like OVCAR3 cells, inhibition of MEK in SKOV3 cells treated with Cd resulted in a significant decrease in phosphorylated ERK1/2. This demonstrates, once again, Cd is inducing the activation of MEK and this activation is necessary for ERK1/2 phosphorylation.

In 2019, Ataei *et al.* published their work on the role of cadmium in ovarian adenocarcinoma. However, the work published in this paper did not align with the preliminary work conducted by the Huff lab. Ataei *et al*. (2019) found low concentrations of Cd, 0.1 μM or less, resulted in significant increases in cellular proliferation.[2] Even when replicating cellular proliferation methods of Ataei *et al*. (2019), the results of this research never confirmed low doses of Cd could induce significant cellular proliferation. Furthermore, Ataei *et al*. (2019) showed treatment with ICI was able to inhibit the cellular proliferation induced by low concentrations of Cd, 0.001 μM to 0.1 μM.[2] This data lead to the hypothesis ICI would inhibit the phosphorylation of ERK1/2 in SKOV3 and OVCAR3 cell lines, but again this was not confirmed in this study. Even though the same cell lines are being utilized, it is possible the discrepancy in results could be due to different lab environments. Overall, these differences show how difficult it is to understand ovarian adenocarcinomas and why they remain such a troubling disease.

To further understand how Cd activates ERK1/2 in these cell lines, future studies will be designed to examine the inhibition of other key proteins suspected to be involved in Cd-induced signaling. The non-receptor protein-tyrosine kinase, Src, has been studied for years due to its upregulation in a variety of human cancers and its role as a protooncogene.[17] Src plays a key role

in cellular proliferation and its increased expression in breast cancer has been linked to another key protein, epidermal growth factor receptor (EGFR).[17] While it is known to be overexpressed in ovarian cancers, it remains unclear its role in the production of ovarian neoplasms.[17] By inhibiting Src in SKOV3 and OVCAR3 cells with PP2, it can be determined if it plays a role in the phosphorylation of ERK1/2 by Cd.

Another key protein to study as its possible involvement in Cd-induced phosphorylation is EGFR. EGFR is part of a tyrosine kinase receptor family, plays a role in cellular proliferation and is often seen to be overexpressed in human cancers.[19] If Cd is activating MEK through the

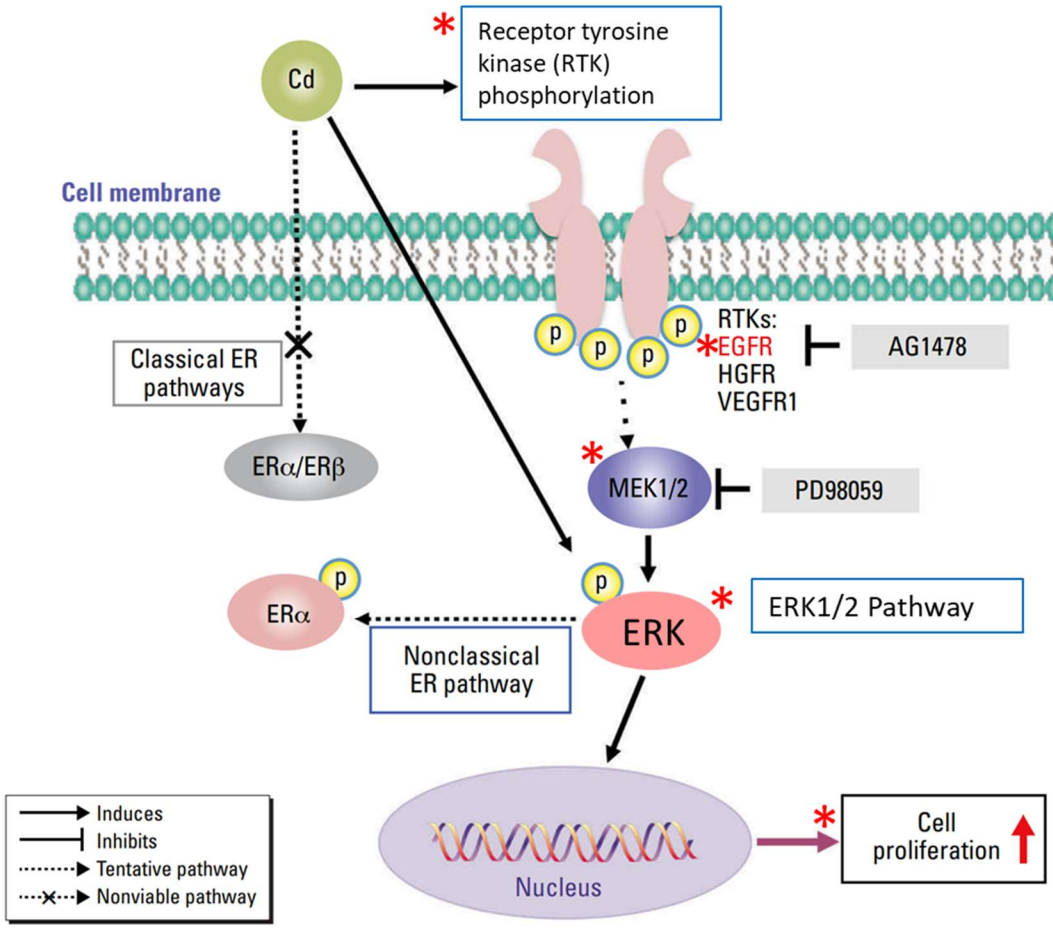

Figure 6: Representation of ERK1/2 phosphorylation due to binding of Cd to receptor tyrosine kinases in uterine cells. * marks the possible path of Cd action in ovarian cancer cells by binding to EGFR, activating MEK, and phosphorylating ERK1/2. Adapted from Gao *et al*. (2015).[29]

involvement of Src, it is most likely doing so through EGFR. To determine the role of this protein in the pathway of Cd-induced ERK1/2 phosphorylation, SKOV3 and OVCAR3 cells could be pretreated with the EGFR inhibitor AG1478. Identifying how these cellular signaling proteins work together with Cd to induce phosphorylation of ERK1/2 would aid in the understanding of Cd effects on ovarian adenocarcinomas.

In 2015, Gao *et al*. published their results on the effect of Cd on uterine cells, another estrogen-sensitive tissue. Cd was shown to induce significant proliferation in uterine cells, and it was found classical ER pathways did not induce ERK1/2 phosphorylation.[29] Instead, their results showed inhibition of EGFR and MEK significantly reduced ERK1/2 phosphorylation induced by Cd.[29] Considering the similar location of uterine and ovarian cells, it is possible ovarian cells follow similar pathways of ERK1/2 phosphorylation. Figure 6, adapted from Gao *et al*. (2015), demonstrates the alternate pathway of Cd action in uterine cells, which could be present in ovarian cells. Further research would help determine the action of receptor tyrosine kinases in Cd-induced ERK1/2 phosphorylation in ovarian cancer cells.

The results of this research contribute to the ever-growing knowledge of Cd and ovarian cancer. Due to its carcinogenic effects and ability to persist in soft tissues for decades, Cd will remain an environmental contaminant of concern. Even if it is not acting as a metalloestrogen in ovarian adenocarcinoma cell lines, it is still important to determine its mechanism of action to understand the full effect of low dose Cd exposure. Furthermore, increased understanding of cellular pathways within ovarian cancer can help guide research regarding early-stage diagnosis and treatment options. Until then, this disease will unfortunately remain a “silent killer.”

ACKNOWLEDGEMENTS

I would like to thank Dr. Mary Huff for being a great advisor and offering never-ending support during my time working towards a thesis in her lab. I would also like to thank my readers, Dr. Amanda Krzysiak and Dr. Steven Wilt for providing insightful feedback and guidance throughout this process. I would like to thank Dr. Jon Blandford as well as the Bellarmine University Honors Program for their support in completing the Honors Senior Thesis requirement. I would like to thank the Bellarmine Biochemistry and Molecular Biology Program and the Biology Department for providing additional space, resources, and lab equipment. I would like to thank the Student Government Association for awarding me an Undergraduate Research Grant for funding needed to complete this research. I would also like to thank Joe and Angela Schmidt for providing funding for the Southern Regional Honors Conference through the Honors Research Award. Finally, I would like to thank my family, peers, and other faculty mentors for providing unyielding support through this process during a global pandemic.

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