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ORIGINAL ARTICLE

Arsenite and cadmium promote the development of mammary tumors

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Abstract

Previous studies demonstrate that the heavy metal cadmium and the metalloid arsenite activate estrogen receptor-alpha in breast cancer cells by forming a high-affinity complex with the ligand-binding domain of the receptor and that environmentally relevant doses of cadmium have estrogen-like activity *in vivo*. The present study showed that in estrogen-receptor positive cells, arsenite and cadmium increased the global expression of estrogen-responsive genes and that an environmentally relevant dose of arsenite also had estrogen-like activity *in vivo*. Similar to estrogens, exposure of ovariectomized animals to arsenite induced the expression of the progesterone receptor, GREB1, and c-fos in the mammary gland and the expression of complement C3, c-fos, and cyclin D1 in the uterus and the increase was blocked by the antiestrogen ICI-182,780. When virgin female animals were fed a diet, that mimics exposure to either arsenite or cadmium, and challenged with the chemical carcinogen dimethylbenzanthracene, there was an increase in the incidence of mammary tumors and a decrease in the time to tumor onset, but no difference in the total number of tumors, tumor multiplicity, or total tumor volume. Together with published results, these data showed that environmentally relevant amounts of arsenite and cadmium had estrogen-like activity *in vivo* and promoted mammary tumorigenesis.

Introduction

The global incidence rate of breast cancer has increased continuously over the past several decades (1) with the most rapid increase occurring in economically developing countries (1). In 2012, approximately 1.7 million new cases of the disease were diagnosed making breast cancer the most commonly diagnosed cancer in women in both developed and developing countries (2,3). Although breast cancer is a global health problem, the causes of the disease are still largely unknown. Past and current endocrine status is the most prominent risk factor for developing breast cancer. Early age at menarche (4), late age at menopause (5), later age at first full-term pregnancy (6), and menopausal exposure to exogeneous estrogens and progestin increase the risk of developing the disease (7). Family history (8) and a history of benign breast disease are also risk factors for developing breast cancer. However, taken together, these risk factors account for only about 40% of breast cancer cases (9). Childhood exposure to ionizing radiation (10), adult alcohol consumption (11), and obesity (12) are additional risk factors but are unlikely to account for the remaining cases (13) suggesting that there are unidentified risk factors for developing breast cancer.

The prominence of endocrine status in the etiology of the disease has led to the suggestion that exposure to environmental estrogens may increase the risk of developing breast cancer. Although a number of chemicals in the environment have estrogen-like activity, such as the naturally occurring phytoestrogen genistein and the synthetic xenoestrogen bisphenol

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Abbreviations

BPA	bisphenol A;
DMBA	dimethylbenzanthracene;
ERα	estrogen receptor-alpha;
FBS	fetal bovine serum;
IMEM	improved minimal essential medium

A (BPA), the contribution of phytoestrogens and xenoestrogens to the etiology of the disease is not clear. Less clear is the contribution of metals and metalloids with estrogen-like activity (14–23). Referred to as metalloestrogens, these metals and metalloids fall into two subgroups, bivalent cations that include cadmium, calcium, lead, mercury, cobalt, copper, nickel, chromium, and tin and oxyanions that include arsenite, nitrite, selenite, and vanadate (14–23). Many metalloestrogens are prevalent environmental contaminants. For example, the metalloid arsenic is an environmental contaminant that has no known physiological function but is present in the body as a result of occupational and nonoccupational exposures (24). The major source of occupational exposure to arsenic is through employment in smelters, the production of wine, and the manufacture of insecticides, pesticides, fungicides, and pharmaceuticals, whereas the primary source of nonoccupational exposure is through food and drinking water. The Joint Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) Expert Committee on Food Additives set the health concern level for arsenic as 2.0–7.0 μg/kg bw/day (25), yet some populations may exceed these exposures. The estimated daily intake of arsenic from food ranges from 0.01 to 5.6 μg/kg bw/day and from drinking water ranges from 0.2 to 0.539 μg/kg bw/day but may be as high as 12.5 μg/kg bw/day in some areas (26). In addition to dietary intake, the estimated exposure to inhaled arsenic ranges from 0.02 to 0.2 μg/kg bw/day in populations living in rural areas and from 0.4 to 0.6 μg/kg bw/day in populations living in cities (27) and may be higher in populations living near smelters and power plants that burn coal containing high concentrations of arsenic. Cigarette smoke is also a source of inhaled arsenic and exposures range from 0.7 to 6.0 μg/day (28). Prior to the ban of arsenic in pesticides, smokers in the 1950s inhaled more than 100 μg/day. Arsenic rarely occurs as an element but occurs as an organic compound with carbon and hydrogen or an inorganic compound with oxygen, chlorine, and sulfur. We have previously shown that arsenite, an oxyanionic form of arsenic, activates estrogen receptor-alpha (ERα) in transfected cells; promotes the growth and increases the transcription of estrogen-regulated genes in estrogen-dependent breast cancer cells (18); and mimics the *in utero* effects of estrogen in the mammary gland of female offspring (29). The ability of arsenite to activate ERα is due to a high-affinity interaction with the ligand-binding domain of the receptor (18), suggesting that arsenite is an environmental estrogen that may contribute to the risk of developing breast cancer.

Cadmium is also an environmental contaminant with estrogen-like activity that has no known physiological function but is present in the body as a result of occupational and nonoccupational exposures. Dust and fumes are the primary sources of occupational exposure to the metal. Cadmium is used in galvanizing and electroplating, in batteries, in electrical conductors, in the manufacture of alloys, pigments, and plastics (e.g. added to stabilize PVC pipes), and in the stabilization of phosphate fertilizers. The main sources of nonoccupational exposure are diet, cigarette smoking, and, to a lesser degree, drinking water (30,31). Dietary exposure to cadmium is estimated to range from 0.12 to 0.49 μg/kg bw/day (30,31). In infants 0–1 year of age, exposure to the metal is estimated to range from 0.37 to 2.2 μg/kg bw/day (32,33) which exceeds the WHO Provisional Tolerable Weekly Intake of 7 μg/kg bw/week. Due to the high efficiency of pulmonary absorption, cigarette smoke is an important source of exposure to cadmium (34,35). The estimated intake from one pack of cigarettes is 2–4 μg/day. The concentration of cadmium in drinking water is generally <1 μg/l of cadmium but may be as high as 10 μg/l as a result of industrial discharge and leaching from metal and plastic (PVC) pipes. There is increasing *in vitro* and *in vivo* evidence that cadmium is a potent metalloestrogen that activates the genomic and nongenomic pathways of ERα (reviewed in [36]). For example, cadmium induces the proliferation of estrogen-dependent breast cancer cells (15,20), increases the transcription and expression of estrogen-regulated genes (15,37), activates ERα in transfection assays (15,16,20), and increases signaling through the ERK1/2 and Akt pathways (37). In immature and ovariectomized rats and mice, environmentally relevant amounts of cadmium also mimic the effects of estrogens in target organs. For example, in ovariectomized rats, cadmium (5 μg/kg bw) increases the expression of estrogen-regulated genes in the uterus and mammary gland and the increase is blocked by an antiestrogen (14).

Arsenic and cadmium are associated with several cancers and are classified by the International Agency for Research on Cancer (IARC) as category I carcinogens (reviewed in [38,39]). Exposure to arsenic is associated with an increased risk of developing skin, bladder, kidney, lung, and liver cancers and possibly prostate cancer and exposure to cadmium is associated with an increased risk of developing lung, prostate, and kidney cancers and possibly liver, pancreatic, and stomach cancers. Arsenic and cadmium are not direct acting mutagens but increase DNA damage through the production of reactive oxygen species and the inhibition of DNA repair. In addition to causing genomic damage, arsenic and cadmium promote the development and growth of tumors through the activation of the Ras-Raf-MEK-ERK, p38MAPK, and JNK pathways. The goals of the present study were to determine whether arsenite and cadmium mimic the effects of estradiol on gene expression in ERα positive cells, whether exposure to an environmentally relevant amount of arsenite also mimics the effects of estrogens in estrogen target organs, and whether dietary exposure to arsenite or cadmium promotes mammary tumorigenesis. The results show that in ERα positive cells, arsenite and cadmium mimic the effects of estradiol on gene expression. Arsenite also increases the expression of estrogen-regulated genes in ovariectomized animals and the increase is blocked by an antiestrogen. In carcinogen challenged animals, dietary exposure to either arsenite or cadmium increases the incidence and decreases the latency of mammary tumors. Together with published data, these results suggest that arsenite and cadmium are environmental estrogens that contribute to the risk of developing breast cancer.

Materials and methods

Cell culture and RNA preparation

The MCF-7 breast cancer cell line was maintained in improved minimal essential medium (IMEM) containing 2 mM glutamine and 5% fetal bovine serum (FBS). For estrogen free experiments, the cells were cultured in phenol red-free IMEM containing 5% charcoal stripped calf serum (CCS) for 48 h. The cells were treated with estradiol (1 nM), arsenite (1 μM), or cadmium (1 μM) in the absence or presence of the antiestrogen ICI-182,780 (500 nM) for 24 h. Total RNA was extracted and purified using miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and the quality of the RNA was analyzed using

Agilent Bioanalyzer (Agilent). MCF-7 cells were obtained from the Tissue Culture and Biobanking Shared Resource at Georgetown and authenticated using PowerPlex 16. Arsenite ($NaAsO_2$) and cadmium ($CdCl_2$) were purchased from Sigma.

Microarray analysis

One hundred nanograms of total RNA were converted to cDNA and then cRNA using Agilent LowInput QuickAmp Labeling Kit (Agilent). The resulting cRNA was labeled with Cy3 and purified. The concentration and incorporation of dye was measured using NanoDrop ND-100 spectrophotometer. The labeled cRNA was hybridized to Agilent SurePrint G3 human whole genome 8x60K microarray (Agilent) following the manufacturer's protocol. Microarrays were scanned with an Agilent DNA microarray scanner. Feature Extraction (11.5.1.1; Agilent) was used to filter, normalize, and calculate the signal intensity and ratios. Raw data were imported, quantile normalized and analyzed in GeneSpring software (12.6.1; Agilent), and visualized using Genesis (genome.tugraz.at). Identification of differentially expressed genes was performed by filtering the dataset using $P < 0.05$ and treatment-to-control ratio ≥2 and by one-way ANOVA statistical analysis followed by Benjamin–Hochberg multiple testing. Microarray analysis was performed on at least three replicates of each treatment. The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database at https://www.ncbi.nlm.nih.gov/geo (accession no. GSE136595).

Animals

Female Sprague-Dawley rats were obtained from Harlan Laboratories, Indianapolis, IN and housed under a 12-h light–dark cycle. All animal studies were conducted in accordance with the Georgetown University Animal Care and Use Committee.

To study the estrogen-like effects of arsenite, virgin female rats were ovariectomized at 28 days of age; maintained on a purified phytoestrogen-free diet that contained no detectable arsenic or cadmium and was not supplemented with copper, chromium, and selenium (Tekland Lab Animal Diets TD02343). The animals recovered for 2 to 3 weeks prior to treatment for 4 days with arsenite (5 μg/kg bw/day by intraperitoneal injection; Sigma, St. Louis, MO), ethinyl estradiol (50 μg/kg bw/day by oral gavage; Sigma), and/or ICI-182,780 (500 μg/kg bw/day by subcutaneous injection; Tocris, Baldwin, MO) (n = 13–15 animals/group). The animals were then euthanized and the uterus and mammary gland were removed and frozen.

To study the effects of arsenite and cadmium on mammary tumorigenesis, virgin female rats at 25 days of age were placed on the control diet (TD02343; n = 20) or the control diet containing 0.2 mg/kg diet of arsenite (TD02345; n = 20) or cadmium (TD02344; n = 20) to mimic dietary exposure to the metalloid and metal. Based on typical food intake, the estimated intake of arsenite or cadmium was approximately 10 μg/kg bw/day. At 49 days of age, the animals were challenged with dimethylbenzanthracene (DMBA, 50 mg/kg bw by oral gavage; Sigma). To determine whether arsenite or cadmium promote the development of mammary tumors, the dose of DMBA was chosen to yield an approximately 50% incidence of tumors in the control animals. As an additional control, animals were also fed the control diet (n = 10) or the control diet containing either arsenite (n = 10) or cadmium (n = 10) but not treated with the carcinogen. Animals remained on the respective diets throughout the study. Tumor development was monitored weekly by palpation beginning 11 weeks after treatment with DMBA. The number, position, and size of the tumors were recorded. Tumor volume was calculated as volume = largest diameter × (smallest diameter)^2 × 0.4 (40). Animals were euthanized when the tumors were 10% of total body weight.

Real-time reverse transcriptase-polymerase chain reaction

For RNA extraction, frozen tissue was pulverized in liquid nitrogen using Spectrum Bessman Tissue Pulverizers. One milliliter of Trizol reagent (Invitrogen) was added and the tissue was then homogenized using Omni Tip Soft Tissue Homogenizer. The homogenate was centrifuged for 10 min at 12,000 rpm at 4°C, the lipid layer removed, and the supernatant collected. After a 5 min incubation at room temperature, chloroform (0.2 ml) was added for each 1 ml of sample. The sample was vortexed and incubated at room temperature for 3 min. The sample was centrifuged for 15 min at 12,000 rpm at 4°C. The upper phase was collected and an equal volume of isopropanol was added. The sample was incubated at –20°C for 2 h to overnight and centrifuged for 15 min at 12,000 rpm at 4°C. Following centrifugation, the pellet was washed with 1 ml of 75% ethanol, centrifuged for 5 min at 7,000 rpm at 4°C, dried, and resuspended in 50 μl of DEPC treated water. The 260:280 ratio and concentration were determined. Samples were aliquoted and stored at –80°C.

For the reverse transcriptase reaction, deoxyribonuclease I (2 μl, Invitrogen) and 2.5 μl of 10× buffer (200 mM Tris–HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl; Invitrogen) was added to 2 μg of RNA and incubated at room temperature for 15 min. Then 2 μl of EDTA (25 mM; Invitrogen) was added and the sample incubated for 15 min at 65°C. Each 70 μl RT reaction contained 7 μl of ×10 Taqman RT Buffer (500 mM KCl, 100 mM Tris–HCl pH 8.3), 15.4 μl of 25 mM $MgCl_2$, 14 μl of dNTPs, 3.5 μl of random hexamers, 1.4 μl of RNase inhibitor, 1.75 μl of MultiScribe reverse transcriptase (Applied Biosystems), 2 μg of RNA, and DEPC treated water to 70 μl. The mixture was incubated in the thermal cycler for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C.

For the real-time polymerase chain reaction, each 10 μl reaction contained 5 μl of Sensimix II Probe Mastermix (Bioline), 0.5 μl of ×20 Assay on Demand (Applied Biosystems) and 4.5 μl of cDNA; or 5 μl of Sensimix SYBR (Bioline), 0.25 μl of 20 μM forward or reverse primer, and 4.5 μl of cDNA. Samples were then run on the 7900HT (Applied Biosystems) and the data analyzed by the $2^{-\Delta\Delta Ct}$ method using the SDS 2.1 software (Applied Biosystems).

Statistical analysis

The unpaired, two-sided Student's t-test determined the statistical significance of differences in means of the gene expression data. For each gene, separate comparisons and t-tests were conducted for ethinyl estradiol (EE) to control (C), arsenite (As) to C, and arsenite plus the antiestrogen ICI-182,780 (As+ICI) to As. Kaplan–Meier analyses compared time to tumor onset with differences in survival curves evaluated by the log-rank test with two-sided P values. Kruskal–Wallis and ANOVA analyses evaluated the differences in tumor incidence, number, and multiplicity.

Results

Estrogen-like effects of arsenite and cadmium on whole genome gene expression

We previously showed that treatment of MCF-7 cells with arsenite or cadmium for 24 h increases the expression of progesterone receptor (PgR) and pS2 (TFF1) which is blocked by the antiestrogen ICI-182,780 (15,18) suggesting that arsenite and cadmium activate the classical ERα genomic pathway. In addition to the genomic pathway, estradiol activates nongenomic pathways through ERα and G-protein estrogen receptor (GPER) (reviewed in [41,42]). In contrast to ERα which is activated by estradiol and inhibited by ICI-182,790, GPER is activated by estradiol but not inhibited by the antiestrogen. To address whether the metalloid and metal mimic the global effects of estradiol on gene expression, MCF-7 cells were treated for 24 h with estradiol (1 nM), arsenite (1 μM), or cadmium (1 μM) in the absence or presence of ICI-182,780 (500 nM) and mRNA expression profiles were determined. To assess the effects of estradiol, arsenite, and cadmium on global gene expression profiles, a microarray analysis was conducted. To highlight common transcriptomic changes, genes were selected for two-dimensional clustering that showed significant differences (i.e. more than 2-fold change with $P < 0.05$) in three or more treatments. As shown in Figure 1A, the response profiles of cells treated with arsenite or cadmium were similar to the response profiles of cells treated with estradiol whether in the absence or presence of ICI-182,790. In the absence of the antiestrogen, 1103 genes were identified that were significantly regulated by estradiol and arsenite; 1429 genes were identified

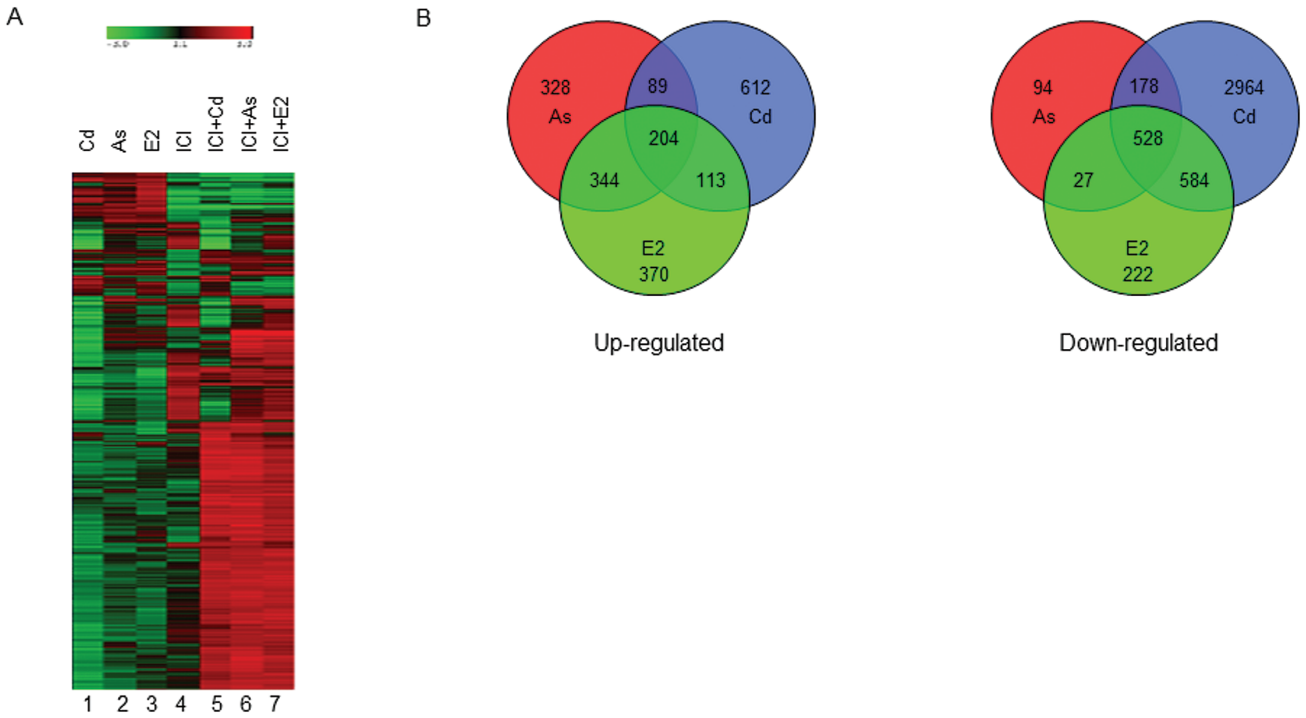

Figure 1. Gene expression profile in MCF-7 cells treated with estradiol, arsenite, or cadmium. MCF-7 cells were treated for 24 h with estradiol (E2; 1 nM), arsenite (As; 1 μM), or cadmium (Cd; 1 μM) in the absence or presence of the ERα antagonist ICI-182,780 (ICI; 500 nM). (**A**) Two-dimensional clustering of genes responsive to treatment with estradiol, arsenite, or cadmium in absence and presence of ICI-182,780. 2,167 genes were selected that showed significant difference, i.e. more than 2-fold with ($P < 0.05$), in three or more treatments. (**B**) Venn diagram comparing the number of significantly up-regulated and down-regulated genes in cells treated with estradiol, arsenite, or cadmium. ($n = 4$ for treatments in the absence of ICI-182,780; $n = 3$ for treatments in the presence of ICI-182,780).

that were regulated by both estradiol and cadmium; and 732 genes that were regulated by estradiol, arsenite, and cadmium. The 732 genes that were common to estradiol, arsenite, and cadmium included 204 genes that were up-regulated and 528 genes that were down-regulated (Figure 1B). The response profile of cells treated with the antiestrogen was the inverse of the response profile of cells treated with estradiol. To further define whether arsenite and cadmium mimic estradiol, the expression of genes that are known to be regulated by estradiol was examined (Figure 2). For this analysis, 108 estrogen-regulated genes were selected from the Qiagen SuperArray gene list and the Ingenuity Pathways Knowledge Base. The response profile of the 108 genes was similar in cells treated with estradiol, arsenite, or cadmium (Figure 2A). A subset of 17 genes, with the greatest fold change in expression following treatment with estradiol such as PgR, GREB1, and c-fos, is shown in Figure 2B. The response profile of the 17 genes was similar in cells treated with estradiol, arsenite, or cadmium and reversed by the antiestrogen suggesting that arsenite and cadmium activate ERα. In addition to genes regulated by estradiol and reversed by ICI-182,780, 3812 genes were identified that were significantly regulated by estradiol and arsenite and 2546 genes were identified that were regulated by estradiol and cadmium but not inhibited by the antiestrogen (Figure 1A and Supplementary Figure S1, available at *Carcinogenesis* Online) suggesting that similar to estradiol, arsenite and cadmium also activate pathways that are independent of ERα including the GPER pathway.

Estrogen-like effects of arsenite in ovariectomized animals

To determine whether *in vivo* exposure to a low, environmentally relevant dose of arsenite also demonstrates estrogen-like activity in target organs, female Sprague–Dawely rats were ovariectomized on postnatal day 28 and allowed to rest for 2 to 3 weeks prior to treatment. The animals were then treated for 4 days with arsenite (5 μg/kg bw/day; approximately 38.5 nmol/kg bw/day) in the presence or absence of the antiestrogen ICI-182,780 (500 μg/kg bw/day). As a positive control, animals were treated with ethinyl estradiol (50 μg/kg bw/day). To ask whether arsenite mimics the effects of estrogens on the expression of genes controlled through the genomic pathway, the ability of the metalloid to induce PgR and Greb1 was measured in the mammary gland (Figure 3A and B). As expected, in animals treated with ethinyl estradiol, there was an approximately 3.5- and 3.2-fold increase in PgR and Greb1 mRNA, respectively. In animals treated with arsenite, there was a 2.6- and 2.7-fold increase in PgR and Greb1 mRNA, respectively, that was blocked by the antiestrogen suggesting that the effects of arsenite on gene expression in the mammary gland are mediated through the ERα genomic pathway. To determine whether arsenite also mimics the effects of estrogens on the expression of genes controlled through the nongenomic pathway, the ability of the metalloid to induce c-fos was measured (Figure 3C). There was an approximately 1.5-fold increase in c-fos mRNA in response to treatment with ethinyl estradiol and an approximately 1.9-fold increase in c-fos mRNA in response to arsenite that was blocked by the antiestrogen suggesting that the effects of arsenite in the mammary gland are also mediated by the ERα nongenomic pathway. To ask whether arsenite also mimics the effects of estrogens on the expression of genes in the uterus, the ability of the metalloid to induce complement C3, c-fos, and cyclin D1 was measured (Figure 4A–C). There was an approximately 17.5- and 2.3-fold increase in C3 and c-fos mRNA, respectively, in the ethinyl estradiol treated animals and an approximately 2.4-,

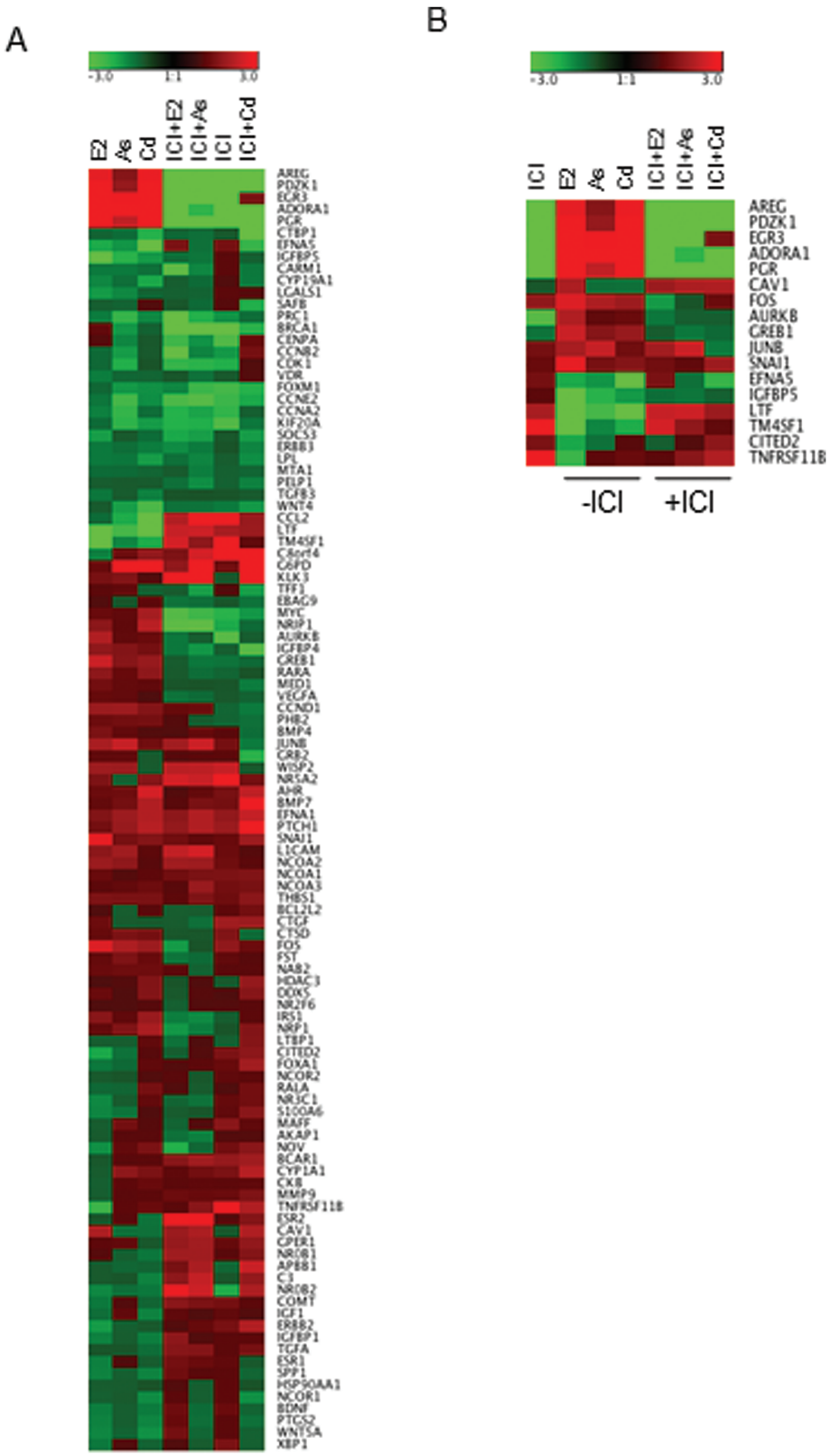

Figure 2. Gene expression profiles of selected ERa-responsive genes. MCF-7 cells were treated with estradiol, arsenite, or cadmium in the absence or presence of ICI-182,780 as described in Figure 1. (**A**) Two-dimensional clustering of 108 ER-responsive genes. (**B**) Heatmap of the 17 genes with the greatest fold change.

2.8-, and 1.5-fold increase in C3, c-fos, and cyclin D1 mRNA in the arsenite treated animals. The ability of arsenite to increase the expression of C3, c-fos, and cyclin D1 mRNA was blocked by the antiestrogen suggesting that the response to arsenite in the uterus was also mediated by the estrogen receptor. Taken together, the data suggest that an environmentally relevant dose

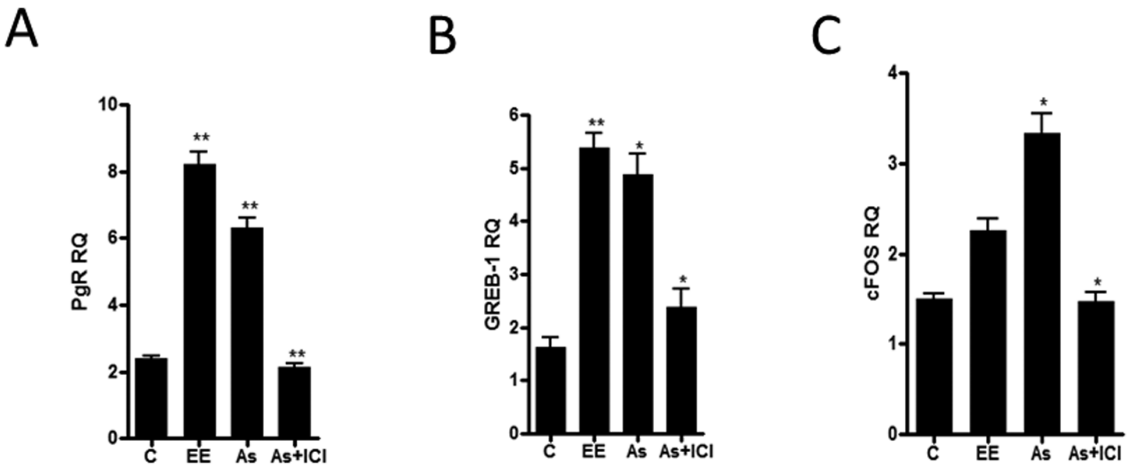

Figure 3. Effects of arenite on the expression of ERα regulated genes in the mammary gland. Virgin female Sprague-Dawley rats were ovariectomized at 28 days of age and allowed to recover for 2 to 3 weeks prior to treatment for 4 days with ethinyl estradiol (EE; 50 μg/kg bw/day by oral gavage) or arsenite (As; 5 μg/kg bw/day by i.p.) in the presence and absence of the antiestrogen ICI-182,780 (ICI; 500 μg/kg bw/day by s.c.). The third mammary gland was removed, RNA was extracted, and mRNA was measured by qRT-PCR. Data are presented as relative expression of progesterone receptor (PgR; A), GREB-1 (B), and c-fos (C) mRNA normalized to GAPDH mRNA (RQ). For each gene, separate comparisons and T-tests were conducted for ethinyl estradiol (EE) to control (C), arsenite (As) to C, and arsenite plus the antiestrogen ICI-182,780 (As+ICI) to As. (mean ± SD; n = 13–15 animals/group; *P = 0.05; **P = 0.005).

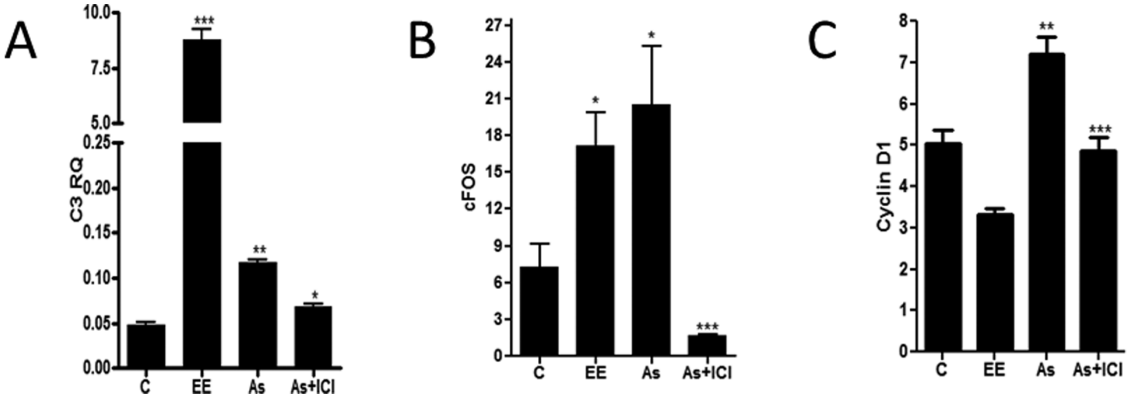

Figure 4. Effects of arsenite on the expression of ERα regulated genes in the uterus. Virgin female Sprague-Dawley rats were ovariectomized at 28 days of age and allowed to recover for 2 to 3 weeks prior to treatment for 4 days with ethinyl estradiol (EE; 50 μg/kg bw/day by oral gavage) or arsenite (5 μg/kg bw/day by i.p.) in the presence and absence of the antiestrogen ICI-182,780 (ICI; 500 μg/kg bw/day by s.c.). The uterus was removed, RNA was extracted, and mRNA was measured by qRT-PCR. Data are presented as relative expression of complement C3 (C3; A), c-fos (B), and cyclin D1 (C) mRNA normalized to GAPDH mRNA (RQ). For each gene, separate comparisons and T-tests were conducted for ethinyl estradiol (EE) to control (C), arsenite (As) to C, and arsenite plus the antiestrogen ICI-182,780 (As+ICI) to As. (mean ± SD; n = 13–15 animals/group; *P = 0.05; **P = 0.005; ***P = 0.0005).

of arsenite induces an estrogen-like response in the mammary gland and uterus through ERα pathways.

Effects of arsenite and cadmium on mammary tumorigenesis

To determine whether the ability of arsenite and cadmium to activate the estrogen receptor *in vivo* promotes mammary tumorigenesis, the DMBA-induced rat mammary tumor model was employed. In this model, administration of estradiol to intact animals treated with the chemical carcinogen enhances the development and growth of mammary tumors (reviewed in [43]). To ask whether arsenite or cadmium mimic the effects of estradiol on the development of mammary tumors, virgin female Sprague-Dawley rats at 25 days of age were placed on a control diet or the control diet supplemented with either the metalloid or metal. To mimic environmental exposures (approximately 10 μg/kg bw/day), the diets contained 200 μg of either arsenite or cadmium/kg diet. At 49 days of age, the animals were challenged with DMBA. Mammary tumor development was monitored beginning 11 weeks after treatment with the carcinogen. Compared to the control diet, the diets supplemented with either the metalloid or metal had a significant effect on the incidence and latency of mammary tumors. There was an approximately 2-fold increase in mammary tumor incidence in animals fed either the arsenite or cadmium containing diet (Figure 5A; 80%; 16/20 and 70%; 14/20, respectively; $P < 0.015$, Kruskal–Wallis, ANOVA) compared to animals fed the control diet (40%; 8/20). There was also a significant decrease in the time to tumor onset in the animals fed the arsenite or cadmium containing diets (Figure 5B and C; $P = 0.001$ and $P = 0.044$, respectively; log-rank test). The median time to tumor onset was 15 weeks in animals fed the metal supplemented diets but undefined in animals fed the control diet. In contrast to tumor incidence and latency, there was no difference in total tumor volume (Figure 5D), total number of tumors (Figure 5E; week 22; $P = 0.090$ and $P = 0.176$; Kruskal–Wallis), or tumor multiplicity (median

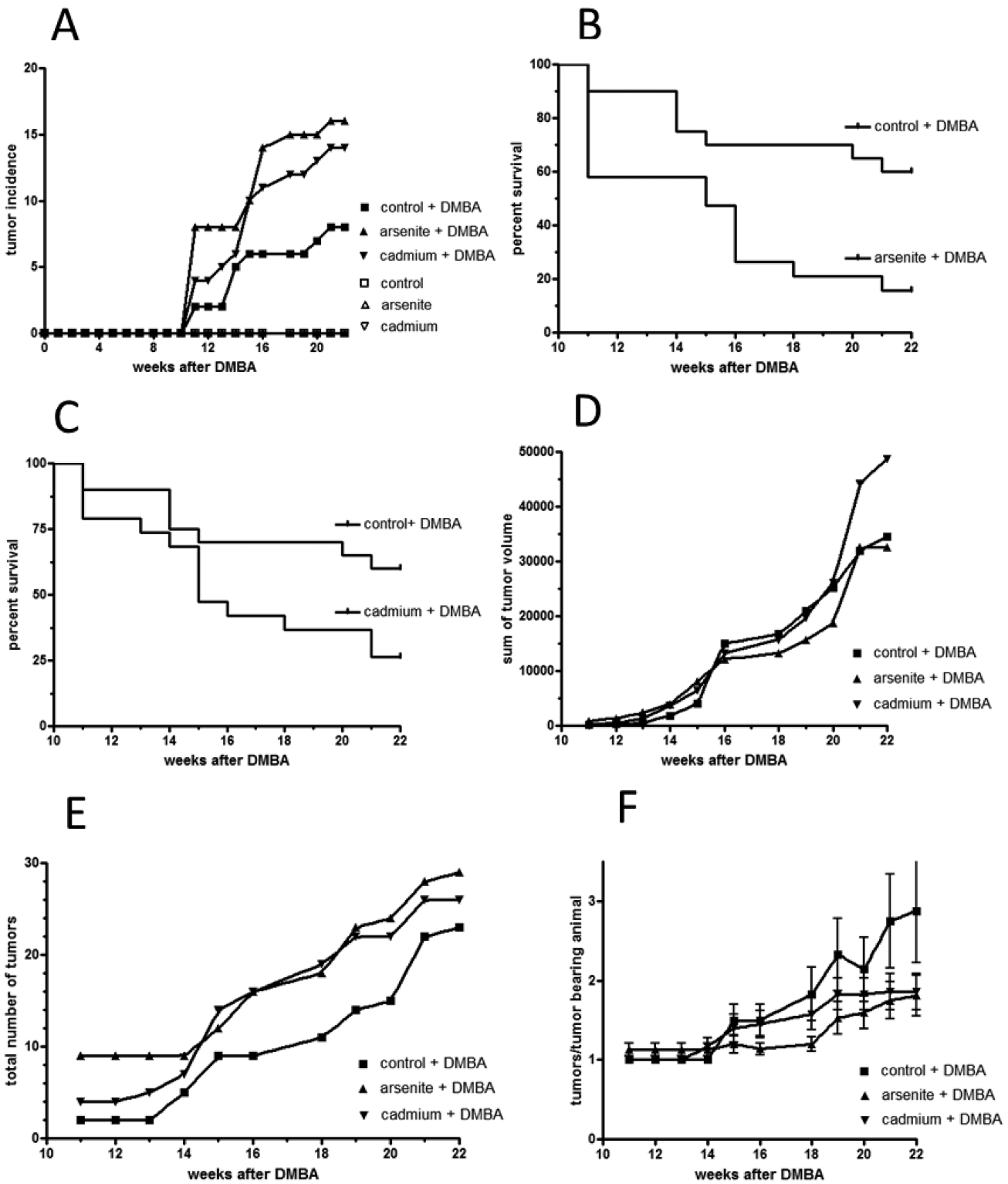

Figure 5. Effects of arsenite and cadmium on mammary tumorigenesis. Virgin female Sprague-Dawley rats at 25 days of age were placed on the control diet or the control diet containing 0.2 mg/kg diet of arsenite or cadmium to mimic dietary exposure the metalloid and metal (approximately 10 μg/kg bw/day). At 49 days of age, the animals were challenged with DMBA (50 mg/kg bw by oral gavage). Tumor development was monitored beginning 11 weeks after treatment with the carcinogen. (**A**) tumor incidence in animals fed the metalloid or metal containing diet and challenged with DMBA (closed symbols; *n* = 20 animals/group) and in animals fed the metalloid or metal containing diet but not challenged with DMBA (open symbols; *n* = 10 animals/group); (**B**) time to tumor onset in animals fed the arsenite containing diet and treated with DMBA (*n* = 20 animals/group); (**C**) time to tumor onset in animals fed the cadmium containing diet and treated with DMBA (*n* = 20 animals/group); (**D**) total tumor volume (*n* = 20 animals/group); (**E**) total number of tumors (*n* = 20 animals/group); (**F**) tumor multiplicity (*n* = 20 animals/group).

number of tumors/tumor bearing animals) (Figure 5F; week 22; $P = 0.247$ and $P = 0.416$; Kruskal–Wallis) between animals fed the control diet and animals fed the diets supplemented with arsenite or cadmium, respectively. There was also no difference in the overall distribution ($P = 0.201$; Kruskal–Wallis) or median number of tumors (week 22; $P = 0.788$; Kruskal–Wallis) between the animals fed the control and supplemented diets. The ability of arsenite and cadmium to increase the incidence and decrease the latency of tumors but inability to increase the total number of tumors or tumor multiplicity suggests that the metalloid and metal promote, but do not initiate, mammary tumors. To ask whether arsenite and cadmium initiate mammary tumors, the animals were fed the control or supplemented diets beginning at 25 days of age but were not treated with DMBA. The development of tumors was then monitored as described above (Figure 5A). As expected, there were no mammary tumors in animals fed the control diet (0/10). There were also no tumors in the non-DMBA treated animals fed either the arsenite or cadmium containing diet (0/10; 0/10) suggesting that the metalloid and metal do not initiate tumors. The ability of arsenite and cadmium to increase the incidence and decrease the latency but inability to initiate tumors suggests that the metalloid and metal are promoters of mammary tumorigenesis in a manner qualitatively similar to that of estrogens.

The arsenite and cadmium supplemented diets were also well tolerated. In animals challenged with DMBA, there was no difference in body weights between animals fed the control or supplemented diets (Figure 6A; week 22; $P = 0.534$ and $P = 0.200$, respectively; ANOVA) or between animals fed the arsenite or cadmium supplemented diets (week 22; $P = 0.070$; ANOVA). There was also no interaction between treatment and body weight over time. In animals not challenged with DMBA, there was an overall difference in body weights of the animals fed the three different diets at the beginning of the study (Figure 6B; $P = 0.044$; ANOVA). At the beginning of the observation period, there was an increase in the body weights of the animals fed the arsenite or cadmium containing diets (week 11; $P = 0.07$ and $P = 0.017$, respectively; ANOVA). The difference in mean body weight between the animals fed the control and arsenite diets was 13.9 g (95% CI = –3.34 to +31.14) and the difference between animals fed the control and cadmium diet was 18.3 g (95% CI 1.52–35.08). The difference in body weights between animals fed the arsenite and cadmium supplemented diets was not significant ($P = 0.555$). At the end of the observation period, there was no overall difference in body weight between the animals fed the three diets (week 22; $P = 0.242$), between the animals fed the control and arsenite diet (week 22; $P = 0.652$), or between the animals fed the control and cadmium diet (week 22; $P = 0.105$).

Discussion

Our previous studies demonstrate that cadmium and arsenite activate estrogen receptor-alpha in breast cancer cells by forming a high-affinity complex with the ligand-binding domain of the receptor (16,22) and that environmentally relevant doses of cadmium have estrogen-like activity *in vivo* in ovariectomized animals (14). The present study shows that in estrogen-receptor-positive cells, arsenite and cadmium increase the global expression of estrogen-responsive genes and in ovariectomized animals, an environmentally relevant dose of arsenite has estrogen-like activity. Similar to estrogens, exposure of ovariectomized animals to arsenite induced the expression of genes that are regulated by ERα and the increase was blocked by the antiestrogen ICI-182,780. When virgin female animals were fed a diet, that mimics environmental exposure to either arsenite or cadmium, and challenged with a chemical carcinogen, there was an increase in the incidence of mammary tumors and a decrease in the time to tumor onset. Together with published results, these data show that environmentally relevant amounts of arsenite and cadmium have estrogen-like activity *in vivo* and promote mammary tumorigenesis.

Although exposure to chemicals in the environment with estrogen-like activity is thought to increase the risk of developing breast cancer, the contribution of environmental estrogens to the etiology of the disease is still not clear (44). Phytoestrogens with mixed weak estrogen agonist and antagonist activity are thought to protect against breast cancer, whereas, xenoestrogens are thought to increase the risk of developing the disease. Bisphenol A is a xenoestrogen that is prevalent in the environment due to its wide use in the production of polycarbonate plastics, epoxy resins, dental implants, thermal and carbonless papers, and the inner lining of food containers. Although substantial attention has focused on BPA, it is considered a weak estrogen. The reported Kd's for BPA binding to ERα range from 195 nM to 32 μM whereas the Kd's for estradiol binding to ERα range from 0.05 to 15 nM (45–48).

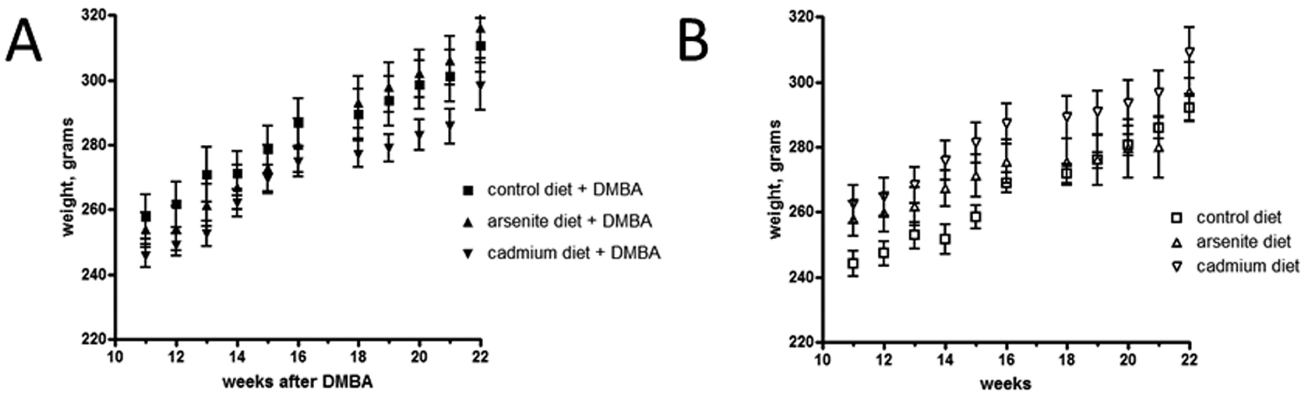

Figure 6. Effects of arsenite and cadmium on body weight. Virgin female Sprague-Dawley rats at 25 days of age were placed on the control diet or the control diet containing 0.2 mg/kg diet of arsenite or cadmium to mimic dietary exposure the metalloid and metal (approximately 10 μg/kg bw/day). At 49 days of age, the animals were either challenged or not with DMBA (50 mg/kg bw by oral gavage) as described above. Body weight was monitored beginning 11 weeks after treatment with the carcinogen. (**A**) Animals fed the arsenite or cadmium containing diets and challenged with DMBA (mean ± SEM; $n = 19$–20); (**B**) Animals fed the arsenite or cadmium containing diets (mean ± SEM; $n = 9$–10).

In ovariectomized rats, BPA induces estrogen-like effects in the uterus at doses that range from 37.5 to 150 mg/kg/day (47–49). In contrast to BPA, cadmium and arsenite are potent estrogens; the Kd for cadmium binding to ERα is approximately 0.5 nM (16) and the Ki for arsenite binding to the receptor is approximately 5 nM (18). In ovariectomized rats, cadmium induces estrogen-like effects in the mammary gland and uterus at a dose of 5 μg/kg (14) and as demonstrated in this study, arsenite also induces estrogen-like effects in the mammary gland and uterus at a dose of 5 μg/kg.

The ability of cadmium and arsenite to mimic the effects of estradiol suggests that the metalloid and metal may contribute to the development of breast cancer. In the case of cadmium, there is increasing epidemiological evidence that links exposure to the metal to an increased risk of developing breast cancer (reviewed in [36]). In a case–control study that examined death certificates, coded for occupation and industry, occupational exposure to cadmium was associated with an increased risk of developing the disease (50). In that study, Caucasian women had an approximate 8–20% increase in risk and African American women had an approximate 50–130% increase in risk (50). In two population-based case–control studies, nonoccupational exposure to cadmium was also associated with an increased risk of developing breast cancer. In these studies, women in the highest cadmium quartile had more than a 2-fold increased risk of developing the disease compared to women in the lowest quartile (51,52). A similar increase was found in a cross-sectional analysis of data from the National Health and Nutrition Examination Survey (NHANES 1999–2008), a probability sample of the US population. The multivariant adjusted model in NHANES found a significant trend ($P = 0.034$) in increased risk for developing breast cancer across the four quartiles of urinary cadmium (52). In a prospective population-based cohort study, long-term dietary intake of cadmium was associated with an increased risk of developing postmenopausal breast cancer (53). Although these studies show an association between exposure to cadmium and an increased risk of developing breast cancer, the studies do not establish a cause and effect relationship between the metal and the disease. Together with previous studies, the present study provides support for a causative role for cadmium in the etiology of breast cancer. The ability of environmentally relevant amounts of cadmium to activate the estrogen receptor *in vitro* and *in vivo* (14,15) induces the proliferation of breast cancer cells (15), and significantly increases the incidence and decreases the latency of mammary tumors in animals challenged with a chemical carcinogen supports a cause and effect relationship between cadmium and breast cancer.

Less well understood and studied is the contribution of arsenite to the etiology of breast cancer. A small number of epidemiological studies show that breast cancer patients have high concentrations of arsenic in their hair (54–56) and tumor tissue (57) but not in plasma (58) which may be due to the rapid clearance of arsenite from blood. An ecological study also suggests a link between residential exposure to arsenic and breast cancer (59). In addition to population studies, previous laboratory studies show that environmentally relevant amounts of arsenite have potent estrogen-like activity in breast cancer cells (18) and in female animals exposed while *in utero* (29). In breast cancer cells, arsenite activates estrogen receptor-alpha, increases the transcription of estrogen-regulated genes, and induces proliferation (18). In *in utero* exposed female animals, arsenite advances the onset of puberty and alters the development of the mammary gland (29). More importantly, the present study demonstrates that an environmentally relevant amount of arsenite activates the estrogen receptor in the mammary gland and uterus of ovariectomized animals and increases the incidence and decreases the latency of mammary tumors in animals challenged with a chemical carcinogen. In a spontaneous mouse mammary tumor model, a previously published study also shows that a low concentration of arsenite in drinking water stimulates the growth of mammary tumors (60). The ability of an environmentally relevant amount of arsenite to activate the estrogen receptor *in vitro* and *in vivo*, increase the growth of breast cancer cells, and promote mammary tumorigenesis in a spontaneous mouse model and in animals challenged with a chemical carcinogen also suggests a causative role for arsenite in the etiology of breast cancer.

In summary, the results of this study together with previously published studies support a role for arsenite and cadmium as causative agents in the development of breast cancer that may be due, in part, to their ability to activate ERα.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Supplementary Figure

Figure S1. Gene expression signatures in MCF-7 cells treated with estradiol, arsenite, and cadmium in the presence of ICI-182,780. Venn diagram of significantly perturbed genes in cells treated with E2, arsenite, or cadmium in the presence of ICI-182,780. 2,160 up-regulated genes and 239 down-regulated genes were common genes in the three treatments.

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