Arsenic-induced **BRCA1** CpG promoter methylation is associated with the downregulation of ERα and resistance to tamoxifen in MCF7 breast cancer cells and mouse mammary tumor xenografts

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**Abstract.** A significant percentage (~30%) of estrogen receptor-α (ERα)-positive tumors become refractory to endocrine therapies; however, the mechanisms responsible for this resistance remain largely unknown. Chronic exposure to arsenic through foods and contaminated water has been linked to an increased incidence of several tumors and long-term health complications. Preclinical and population studies have indicated that arsenic exposure may interfere with endocrine regulation and increase the risk of breast tumorigenesis. In this study, we examined the effects of sodium arsenite (NaAs\textsuperscript{III}) exposure in ERα-positive breast cancer cells in vitro and in mammmary tumor xenografts. The results revealed that acute (within 4 days) and long-term (10 days to 7 weeks) in vitro exposure to environmentally relevant doses reduced breast cancer 1 (BRCA1) and ERα expression associated with the gain of cyclin D1 (CCND1) and folate receptor 1 (FOLR1), and the loss of methylenetetrahydrofolate reductase (MTHFR) expression. Furthermore, long-term exposure to NaAs\textsuperscript{III} induced the proliferation and compromised the response of MCF7 cells to tamoxifen (TAM). The in vitro exposure to NaAs\textsuperscript{III} induced **BRCA1** CpG methylation associated with the increased recruitment of DNA methyltransferase 1 (DNMT1) and the loss of RNA polymerase II (PolII) at the **BRCA1** gene. Xenografts of NaAs\textsuperscript{III}-preconditioned MCF7 cells (MCF7NaAs\textsuperscript{III}) into the mammary fat pads of nude mice produced a larger tumor volume compared to tumors from control MCF7 cells and were more refractory to TAM in association with the reduced expression of **BRCA1** and ERα, CpG hypermethylation of estrogen receptor 1 (ESR1) and **BRCA1** and the increased expression of **FOLR1**. These cumulative data support the hypothesis that exposure to As\textsuperscript{III} may contribute to reducing the efficacy of endocrine therapy against ERα-positive breast tumors by hampering the expression of ERα and **BRCA1** via CpG methylation, respectively of **ESR1** and **BRCA1**.

**Introduction**

Inorganic arsenic is ubiquitously found in foods (i.e., rice and grains) (1,2) and drinking water (3-5). Chronic arsenic exposure through contaminated water has been linked to an increased incidence of several tumors (6,7) and long-term health complications at levels of exposure below safety limits (10 ppb) (8). Common human exposures to arsenic include inorganic trivalent arsenite (As\textsuperscript{III}) and pentavalent arsenate (As\textsuperscript{V}). The As\textsuperscript{III} form has potent estrogen-disrupting activities in connection with its affinity for the ligand-binding domain of the estrogen receptor-α (ERα). It also stimulates cell growth and the expression of the progesterone receptor (PR) (9). As the As\textsuperscript{III} form is enzymatically converted to As\textsuperscript{V}, it provides a reservoir for ERα-binding metabolites (10) that may disrupt estrogen signaling and response to endocrine therapies based on antagonists of the ERα (11-13).

Approximately 70-80% of diagnosed breast tumors are ER-positive and they are treated with anti-estrogens, including tamoxifen (TAM). However, over time, a significant percentage (~30%) of these tumors become resistant to treatment with anti-estrogens (14,15). The reasons for this acquired resistance...
remain largely unknown. However, the loss of ERα expression has been linked to a poor response to endocrine therapy (16–18). The deregulation of ERα signaling associated with the drinking of water contaminated with arsenic has been reported both in men and women (19). Arsenic-induced genomic instability via the Fanconi anemia (FA)/breast cancer (BRCA) pathway disruption has been shown to directly contribute to arsenic carcinogenic effects (20). A previous study using rodent models (e.g., Sprague-Dawley rats) demonstrated that the in utero exposure to AsIII induced an increase in the number of mammosphere-forming cells, the branching of epithelial cells and density in the mammary gland of pubertal offspring, and that these changes persisted into adulthood (21). Other studies using rodent models concluded that AsIII was a ‘complete’ transplacental carcinogen promoting the maternal dose-dependent induction of tumors in endocrine-related tissues (adrenal gland, ovary and uterus) in offspring (22,23).

In a spontaneous mammary-tumor model (C3H/St mice), arsenic exposure was shown to abolish the anticancer effects of selenium and increase tumor growth rates and multiplicity (24). At the cellular level, in vitro studies have indicated that chronic exposure to low levels of arsenic induced the transformation of normal breast epithelial cells, and accelerated the growth of ERα-positive breast cancer cells (25,26). Exposure to AsIII has been shown to inhibit DNA mismatch repair, leading to genomic instability (27,28). In endocrine-responsive tissue (e.g., prostate), exposure to AsIII has been reported to induce the transition to a steroid receptor-independent tumor phenotype (29). These cumulative observations have raised the question of whether or not endocrine disruption associated with AsIII exposure contributes to breast carcinogenesis.

Epigenetics refers to changes in DNA methylation, histone post-translational modifications and the expression of non-coding RNAs (30). Maternal exposure to arsenic has been shown to alter DNA methylation in placental tissue (31), and to increase DNA methylation in children (32). Moreover, preclinical (33,34) and human (35) studies have demonstrated that arsenic causes the hypermethylation of tumor suppressor genes (i.e., p16INK4a and RASSF1) and a decrease in telomere length associated with genomic instability (36). Finally, exposure to AsIII has been found to induce cancer stem cell-like properties involving the epigenetic silencing of the let-7c via Ras/NF-κB pathways (37). Based on these observations, the main objective of this study was to investigate the effects of AsIII on BRCA1 and ESR1 (ERα) expression and CpG methylation, and response to TAM in cultured and xenografted MCF7 breast cancer cells.

**Materials and methods**

**Cells and cell culture.** Authenticated breast cancer MCF7 cells (Batch #62349993) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C with 5% CO2 in Dulbecco's modified Eagle's/F12 medium (DMEM) from Corning Cellgro (Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories Inc., Logan UT, USA) as previously described (38). Sodium arsenite (NaAsIII), genistein (GEN) and 17β-estradiol (E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TAM and E2 were solubilized in stock solutions with ethanol, which was added to DMEM/F12 as the vehicle control. For cell proliferation experiments, the MCF7 cells (passage nos. 3-15) were seeded in 6-well plates at a density of 5x103 cells/well in triplicate overnight, and then switched to phenol-free media containing 10% charcoal-stripped FCS (HyClone Laboratories Inc.) for 3 days before the start of each treatment. For proliferation measurements, the cells were washed with ice-cold PBS and counted by trypan blue exclusion.

**Promoter CpG methylation.** Quantitative polymerase chain reaction (qPCR) analysis of human **BRCA1** and **ESR1** promoter CpG methylation was performed as previously described (38) with genomic DNA (DNasey blood and tissue kit; Qiagen, Hilden, Germany) and bisulfonated with the Epitect bisulfite kit (Qiagen) using the following unmethylated (U)- and methylated (M)-specific primers (Sigma-Aldrich): **BRCA1** U-sense, 5'-TTGTTTATTAGTTAATGTTGTAATGGAAGTGTA-3' and U-antisense, 5'-CAAAAAATCTCACAACAAACTCACACCA-3'; **BRCA1** M-sense, 5'-TGGTAAACGGAAAAGCG-3' and M-antisense, 5'-ATCTCAACGAACTCACGC-3'; **ESR1** U-sense, 5'-GGGTTTATGTGTTTGTGTTT-3' and U-antisense, 5'-TGGTAAACGGAAAAGCG-3'; **ESR1** M-sense, 5'-ATCTCAACGAACTCACGC-3'. The qPCR was carried out in a volume of 10 μl consisting of the following master mix: 5 μl of SYBER-Green mix (Thermo Fisher Scientific), 1 μl each of forward and reverse primers, 2 μl nuclease-free water, and 1 μl of bisulfonated genomic DNA. Data from qPCR of bisulfonated DNA were presented as the fold-change compared to the control of CpG M/U, as previously described (38).
prepared for DNA enzymatic digestion. Aliquots of digested chromatin were immunoprecipitated using antibodies against DNMT1 (Abcam Inc, Cambridge, MA, USA) and PolIII (Thermo Fisher Scientific). qPCR was performed on aliquots of DNA obtained after the reversal of DNA-protein cross-links and purification through spin-filtration columns. Briefly, PCR amplification reactions were done at a final volume of 25 µl consisting of the following: 12.5 µl of SYBR-Green buffer, 1 µl each forward (5'-CTCCCATCCTGTAGTTGTTGAT-3') and reverse (5'-CAGGAGTCTCAGGCTCAC-3') oligonucleotides flanking exon-1a in the BRCA1 gene (39); 8.5 µl nuclease free water, and 2 µl DNA purified from the ChIP assay.

RNA analyses. Total RNA was purified using RNeasy Mini kit as per the manufacturer's instructions (Qiagen) (38). The concentrations and quality of RNA were verified using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). Equal amounts of total RNA (500 ng) were transcribed into cDNA using ISCRIPT supermix kit (Bio-Rad Laboratories, Hercules, CA, USA). Next, cDNA aliquots were analyzed by qPCR using the SYBR-Green PCR Reagents kit (Life Sciences, Little Chalfont, UK). Immunocomplexes were amplified for DNA-protein cross linkage and purified through spin-filtration columns. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000). qPCR was performed on aliquots of the transcript levels.

Mouse mammary xenografts. All in vivo mouse xenograft experiments were performed under the #07-029 protocol approved by the University of Arizona Institutional Animal Care and Use Committee approved on 02/22/2016. All procedures were performed in compliance with the standard operating procedures and relevant guidelines of the University of Arizona Animal Care. MCF7 cells (7.5-10^6 cells in 50 µl of Matrigel resuspension) were injected into the left number 4 mammary fat pad of 4-week-old (19-22 g) ovariectomized (OVX) athymic rTac:NCr- Foxn1 nude female mice (Taconic Biosciences, Rensselaer, NY, USA) implanted with an estradiol pellet (0.72 mg, 60 days release; Innovative Research of America, Sarasota, FL, USA). Mice were run at a final volume of 25 µl consisting of the following master mix: 12.5 µl of SYBR-Green mix, 1 µl each of forward and reverse primers, 9.5 µl nuclease-free water and 1 µl cDNA. The primer (Sigma-Aldrich) sequences were: ERα sense, 5'-CAAGCCCGCTTCAATGATCAG-3' and antisense, 5'-CTGATCATGGAGGGTCAAATCCAC-3'; BRCA1 sense, 5'-AGCTCGCTGAGACTTCCTGGA-3' and antisense, 5'-CAATTCAATGTAGACAGACGT-3'; cyclin D1 (CCND1) sense, 5'-ACAAACAGATCATCAGGCCAAAAAC-3' and antisense, 5'-TGTTGGGCTCTTCAGGTTTC-3'; folate receptor 1 (FOLR1) sense, 5'-ATTCCTTGGTGCCACCTGACC-3' and antisense, 5'-ATAGACCTCCTGCACCTCTCCT-3'; methyltetrahydrofolate reductase (MTHFR) sense, 5'-AGGACCCCTGCCCTGATG-3' and antisense, 5'-AGGACCCCTGCTGATG-3'; and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-ACCACAATCTGCACACCCTT-3' and antisense, 5'-CTCTGTGTCTTCTGGTGGG-3'. Amplification of GAPDH mRNA was used for the normalization of the transcript levels.

Western blot analysis. Western blot analysis was performed as previously described (38). Protein lysates were obtained from cells scraped in triplicates from 6-well plates and using Pierce RIPA buffer (Thermo Fisher Scientific), with 1% proteinase inhibitors. The protein concentration was calculated using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). Immunoblotting was carried out with antibodies against BRCA1 (cat. no. 9010); GAPDH (cat. no. 2118) (both from Cell Signaling Technology, Beverly, MA, USA); and ERα (cat. no. sc-542) (Santa Cruz Biotechnology, Dallas, TX, USA). Immunocomplexes were detected using enhanced chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK). Immunocomplexes for GAPDH were used as an internal control for the normalization of protein expression. Western blot analyses were carried out at least twice for each experiment. The quantification of immunocomplexes was carried out by densitometry performed using Kodak ID Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA).

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Figure 1. AsIII reduces the expression of BRCA1 and ERα. (A) MCF7 cells were cultured for 72 h in control DMEM, or DMEM plus E2 (10 nM) alone or various concentrations of NaAsIII as described in the Materials and methods. In (B) MCF7 cells were co-treated for 72 h with E2 plus 2 µM NaAsIII and various concentrations (0.02, 0.2 and 2.0 µM) of GEN. Bands are representative immunocomplexes for BRCA1 and internal standard GAPDH from two (n=2) separate experiments performed in duplicate. (C) Bars represent the means ± SEM of ERα mRNA expression (fold-change of E2 Control) from 2 separate experiments (n=2) performed in triplicate. Different letters indicate statistically significant multiple comparison (a>b>c>d) differences (P<0.05). AsIII, trivalent arsenite; BRCA1, breast cancer 1; ERα, estrogen receptor-α; E2, 17β-estradiol; NaAsIII, sodium arsenite; GEN, genistein.
(10 animals/group x 4 experimental groups, 40 animals in total) were housed in conventional pathogen-free cages under a 12 h light/12 h dark cycle, at 20-22°C, and 50-55% humidity with free access to Teklad Global Rodent Diet (Harlan Laboratories, Madison, WI, USA) and tap water. The animals were sacrificed at 60 days after the start of TAM treatment. Tumor growth was measured once/week with a caliper until there were visible signs of tumor growth, then twice/week until the end of the study. Tumor volume was estimated using the following formula: \( [(\text{width})^2 \times \text{length}] / 2 \). Tumor tissue was snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

**Statistical analysis.** Data were analyzed by ANOVA as previously described (38). Post-hoc multiple comparisons among all means were conducted using Tukey’s Test after main effects and interactions were found to be significant at \( P \leq 0.05 \). Data are presented as the means ± SEM and statistical differences highlighted with different letters for multiple comparisons (a>b>c, etc.) or asterisks when compared to the control.

**Results**

\( \text{NaAs}^{III} \) reduces the expression of BRCA1 via CpG hypermethylation in ERα-positive breast cancer cells. Previously (38–40), we reported that the expression of BRCA1 was stimulated by E2 in ER-positive MCF7 breast cancer cells (38). In this study, using western blot analysis (Fig. 1A), we observed that E2-induced BRCA1 expression was antagonized by \( \text{NaAs}^{III} \), starting at the 1 µM concentration, and to a larger degree upon co-treatment with higher doses of \( \text{NaAs}^{III} \) (2 to 8 µM). As a control, we co-treated MCF7 cells with \( \text{NaAs}^{III} \) (2 µM) plus various doses (0.02, 0.2 and 2.0 µM) of the isoflavone GEN, which was found in our previous study to induce BRCA1 expression (38). Co-treatment with 0.2 and 2 µM GEN reversed the repressive effects of \( \text{NaAs}^{III} \) on BRCA1 expression (Fig. 1B).
mRNA. The treatment with NaAs reduced markedly (~50%) α for 6 weeks antagonized E2-induced BRCA1.

increased the resistance of MCF7 cells revealed that exposure to 1 µM NaAs by RT-qPCR (Fig. 3A) showed that 1 to 6 weeks exposure to 1 µM NaAs decreases the expression of MTHFR, an enzyme involved in one-carbon metabolism. Analysis of MTHFR expression by RT-qPCR (Fig. 3A) showed that 1 to 6 weeks exposure of MCF7 cells to 1 µM NaAs reduced markedly (~50%) MTHFR mRNA. The treatment with NaAs had a biphasic effect on expression of FOLR1 mRNA, which was reduced at 1 and 3 weeks, but induced at 6 weeks of exposure. FOLR1 participates in cellular uptake of 5-methyltetrahydrofolate into cells, and its overexpression has been linked to poor prognosis in particular in triple-negative breast cancers (TNBC) (46). As another control, we confirmed the repressive effects on E2-induced BRCA1 (Fig. 2B) and ERα (Fig. 2C), but induced the expression of CCND1.

It has previously been documented (45) that AsIII treatment decreases the expression of MTHFR, an enzyme involved in one-carbon metabolism. Analysis of MTHFR expression by RT-qPCR (Fig. 3A) showed that 1 to 6 weeks exposure of MCF7 cells to 1 µM NaAs reduced markedly (~50%) MTHFR mRNA. The treatment with NaAs had a biphasic effect on expression of FOLR1 mRNA, which was reduced at 1 and 3 weeks, but induced at 6 weeks of exposure. FOLR1 participates in cellular uptake of 5-methyltetrahydrofolate into cells, and its overexpression has been linked to poor prognosis in particular in triple-negative breast cancers (TNBC) (46). As another control, we confirmed the repressive effects on BRCA1 mRNA expression by treatment of the MCF7 cells with NaAs by RT-qPCR. As another control, we also examined the expression of FOLR1 protein and found that exposure to NaAs reduced its expression within 3 days, although it had a stimulatory effect long-term (19 weeks) (Fig. 3B).

One mechanism through which NaAs may lower BRCA1 expression is epigenetic silencing involving DNA methylation. The analysis of bisulfonated genomic DNA prepared from the MCF7 cells revealed that exposure to 1 µM NaAs from 4 days to 10 weeks brought about an increase (2.5- to 5-fold) in BRCA1 CpG methylation (Fig. 4A), which was associated at 6 days post-treatment with a reduction in the recruitment of PolII to the BRCA1 promoter and increased occupancy by DNMT1 (Fig. 4B). These results suggested that the NaAs-dependent downregulation of BRCA1 was associated with the reduced transcription and recruitment of DNA-modifying enzymes (i.e., DNMT1) to the BRCA1 gene.

NaAsIII disrupts the response to TAM in MCF7 cells in culture and in mouse mammary tumor xenografts. The observed reduction in ERα expression depicted in Figs. 1 and 2 raised the question as to whether NaAsIII exposure influences E2-induced cell proliferation and response to TAM. The results presented in Fig. 5 indicated that treatment of the MCF7 cells with TAM for 72 h reduced E2-induced cell growth. Conversely, in the MCF7 cells pre-treated for 6 weeks with 1 µM NaAsIII, treatment with TAM increased cell proliferation (Fig. 5A). The results of western blot analysis indicated that pre-treatment with NaAsIII for 6 weeks antagonized E2-induced BRCA1 expression, while it reduced ERα expression, a known target for TAM (Fig. 5B). The analysis of cell cycle distribution by flow cytometry revealed that a larger percentage of cells co-treated for 6 weeks with NaAsIII plus TAM or E2 plus TAM were positioned in the S-phase of the cell cycle compared to the control MCF7 cells (Fig. 5C). These cumulative results suggested that long-term exposure to environmentally relevant doses (1 µM) of NaAsIII increased the resistance of MCF7 cells to TAM through the downregulation of ERα.

To further investigate the influence of NaAsIII exposure on tumor development, we injected control MCF7 cells or MCF7 cells pre-treated with 1 µM NaAsIII for 4 weeks (MCF7 NaAsIII) into the cleared mammary fat pad of 4-week-old OVX athymic rTac:NCr-Foxn1 nude female mice also implanted with an E2 pellet. We then monitored tumor growth for 24 days and noted a higher tumor volume for mice injected with MCF7 NaAsIII compared to mice xenografted with control MCF7 cells (Fig. 6A). Subsequently, the xenografted mice were
implanted with a TAM pellet and tumors were allowed to grow for an additional 45 days. Mammary tumors that originated from xenografted MCF7 NaAs\textsuperscript{III} cells were more refractory (~40%) to TAM treatment compared with tumors that developed from control MCF7 cells (Fig. 6B). The resilience of MCF7 NaAs\textsuperscript{III} tumors to TAM was coupled with the reduced expression of \textit{BRCA1} and \textit{ER\textalpha} mRNA (Fig. 7A), and increased CpG methylation of the respective genes (\textit{i.e., BRCA1} and \textit{ESR1}) (Fig. 7B). As a control, we measured the expression of \textit{FOLR1} mRNA (Fig. 8A) and \textit{FOLR1} protein (Fig. 8B), which were increased (~1.0-fold) in mammary tumors from xenografted MCF7 NaAs\textsuperscript{III} cells compared to tumors that developed from control MCF7 cells. Taken together, the results of the tumor xenograft experiments indicated that exposure to NaAs\textsuperscript{III} conferred the resistance of mammary tumors to TAM and that this resilience was associated with the hypermethylation of \textit{BRCA1} and \textit{ESR1}, the reduced expression of \textit{BRCA1} and \textit{ER\textalpha}, and increased levels of \textit{FOLR1} mRNA and tumor burden.

**Discussion**

The loss of \textit{ER\textalpha} expression has been linked to a poor response to endocrine therapy (16-18). Drinking water contaminated with arsenic has been linked to the disruption of \textit{ER\textalpha} signaling (19) and arsenic exposure has been shown to contribute to genomic instability through the disruption of \textit{BRCA1}-regulated DNA repair (20). Arsenic may accelerate cancer growth (24) and confer a steroid receptor-independent phenotype (29). These
cumulative observations suggest arsenic exposure may interfere with endocrine regulation and prompted our investigation into whether or not As\textsuperscript{III} contributes to resistance to TAM therapy through the silencing of BRCA1 and ESR1. In this study, we first examined the \textit{in vitro} effects of NaAs\textsuperscript{III} in ER\textalpha-positive breast cancer cells and found that acute (within 4 days) and long-term (10 days to 7 weeks) exposure to environmentally relevant doses of As\textsuperscript{III} reduced BRCA1 expression. Furthermore, NaAs\textsuperscript{III} compromised ER\textalpha expression and the \textit{in vitro} response of MCF7 cells to treatment with TAM. In normal breast epithelial cells, the BRCA1 and ESR1 (encoding for ER\textalpha) genes are regulated through a positive feedback loop in which ER\textalpha induces expression of BRCA1 in the presence of E2 (40). In turn, BRCA1 transcriptionally activates the ESR1 gene (47). This crosstalk between BRCA1 and ER\textalpha is thought to favor DNA repair controlled by BRCA1 before cells progress through division under the proliferative pressure of estrogens. Conversely, in BRCA1 mutation and sporadic breast tumors, the reduced expression of BRCA1, also termed ‘BRCAness’, is usually associated with the reduced expression of ER\textalpha and resistance to TAM (48). Our cell culture data suggested that exposure to NaAs\textsuperscript{III} may compromise BRCA1 expression and confer resistance to antagonists of the ER\textalpha such as TAM. The results of this study are in agreement with those of a previous study (49) showing that environmentally relevant doses of NaAs\textsuperscript{III} (~1-5 µM) reduced the expression of the ER\textalpha.

A mechanism that may contribute to the NaAs\textsuperscript{III}-dependent loss of BRCA1 is epigenetic silencing via CpG methylation, which has been documented in sporadic breast tumors, particularly in those that are more invasive (i.e., TNBC) compared to lobulo-alveolar breast cancers (50). In this study, we documented that in MCF7 cells both the short- (4 days) and long- (10 days to 10 weeks) \textit{in vitro} exposure to NaAs\textsuperscript{III} induced BRCA1 CpG methylation was associated with the increased recruitment of DNMT1 and the loss of PolII at the BRCA1 gene. These observations are in accordance with those

![Figure 7](image1.png)

**Figure 7.** As\textsuperscript{III} induces BRCA1 and ESR1 CpG methylation in MCF7 cell mammary tumor xenografts. Bars are from 5 animals/group from 2 separate experiments (n=10) and represent means (fold-change of MCF7 Control xenograft) ± SEM for (A) BRCA1 and ER\textalpha mRNA expression; (B) BRCA1 and ESR1 CpG methylation. Different letters represent statistically significant multiple comparison (a>b>c) differences (P<0.05). As\textsuperscript{III}, trivalent arsenite; BRCA1, breast cancer 1; ESR1, estrogen receptor 1; ER\textalpha, estrogen receptor-\alpha.

![Figure 8](image2.png)

**Figure 8.** As\textsuperscript{III} induces expression of FOLR1 in MCF7 cell mammary tumor xenografts. (A) FOLR1 mRNA expression in MCF7 and MCF7NaAs\textsuperscript{III} cell mammary tumor xenografts. Bars are from 5 animals/group from two separate experiments (n=10) and represent means (fold-change of MCF7 Control xenograft) ± SEM. Different letters represent statistically significant multiple comparison (a>b) differences (P<0.05). (B) Bands are representative immunocomplexes for FOLR1 and internal standard GAPDH from 2 (n=2) separate experiments performed in duplicate. As\textsuperscript{III}, trivalent arsenite; BRCA1, breast cancer 1; FOLR1, folate receptor 1.
of a previous study reporting promoter hypermethylation and silencing of other DNA repair (MLH1 and MSH2) genes in arsenic-exposed populations (51). The reprogramming of DNA methylation elicited by NaAsIII has been previously linked to increased growth rate (52). In keeping with these earlier reports, in this study, we noted that MCF7 treated for 6 weeks with NaAsIII displayed increased proliferative capacity and were refractory to TAM.

The injection of NaAsIII-preconditioned MCF7 cells into the mammary fat pad of nude mice provided in vivo evidence that the prior exposure to NaAsIII may alter the behavior of ERα-positive breast cancer cells. Xenografted MCF7NaAs III cells produced a larger tumor volume compared to control MCF7 cells and were more refractory to treatment with TAM. We attributed this resilience of MCF7NaAs III to TAM, at least in part, to the reduced expression of ERα associated with the CpG hypermethylation of ESR1. The reduced expression of ERα in MCF7NaAs III tumors was paralleled by the lower expression and hypermethylation of BRCA1, further supporting the hypothesis that exposure to NaAsIII may contribute to breast tumorigenesis by hampering DNA repair capacity controlled by BRCA1 and altering the crosstalk between BRCA1 and ERα.

In agreement with previous findings (45), we noted that the expression of MTHFR in MCF7 cells treated in vitro with NaAsIII was markedly downregulated. Thus, exposure to inorganic arsenic may deplete the pool of methyl groups and interfere with folate metabolism with consequences on DNA synthesis and repair. The reduced expression of MTHFR has been previously associated with breast cancer development (53). Conversely, in this study, we noted in MCF7 cells in culture that exposure to NaAsIII had a biphasic effect on the expression of FOLR1, a membrane-bound protein involved in transport of folate into cells. Short-term exposure to NaAsIII reduced FOLR1 expression, whereas a stimulatory effect on FOLR1 levels was observed after long-term exposure. The upregulation of FOLR1 was confirmed in mammary tumors that developed from xenografted MCF7NaAsIII cells. The upregulation of FOLR1 has been interpreted as an adaptive response triggered by cellular depletion of methyl groups by metabolism of NaAsIII (45). Moreover, recent studies reported that the increased expression of FOLR1 was associated with a higher risk of recurrence in patients with TNBC (54), which were significantly enriched in FOLR1 compared to ERα- and human epidermal growth factor receptor 2-positive breast tumors (46). Whereas it remains unknown whether NaAsIII affects expression of MTHFR and FOLR1 through epigenetic mechanisms, a possible translational implication of our data is that breast cancer patients exposed to NaAsIII and undergoing treatment with TAM may benefit from combination therapy with anti-FOLR1 agents (54).

Taken together, the data of the present study provide novel in vitro and mammary tumor xenograft evidence that exposure to inorganic trivalent arsenic, such as NaAsIII, may increase resistance to endocrine therapy based on TAM through reduction in BRCA1 and ERα expression. Future studies with ERα-positive breast cancer patients residing in geographical regions at high risk of exposure to AsIII are warranted to investigate whether the dysregulation of CpG hypermethylation of BRCA1 and ESR1 causes persistent genomic instability (55), and variations in efficacy of therapies based on antagonists of the ERα. As DNA methylation changes are potentially reversible, they may offer a novel target for combination therapies of ER-positive breast tumors with epigenetic drugs.

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Availability of data and materials

All data generated during this study are included in this published article.

Authors’ contributions

OIS and DFR conceived the study and drafted the manuscript. OIS and MGD contributed to laboratory experiments, data analysis, and writing of the manuscript. OIS conducted cellular and molecular measurements with cell lines and tumor xenografts. BS and GDPM contributed to designing and performing the xenograft experiments and review of data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All mouse xenograft experiments were performed under the #07-029 protocol approved by the University of Arizona Institutional Animal Care and Use Committee approved on 02/22/2016. All procedures were performed in compliance with the standard operating procedures and relevant guidelines of the University of Arizona Animal Care.

Patient consent for publication

Not applicable

Competing interests

The authors declare they have no competing interests.

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