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Arsenic-induced cancer cell phenotype in human breast epithelia is estrogen receptor-independent but involves aromatase activation

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Abstract

Accumulating data suggest arsenic may be an endocrine disruptor, and tentatively linked to breast cancer by some studies. Therefore, we tested the effects of chronic inorganic arsenic exposure on the normal, estrogen receptor (ER)-negative breast epithelial cell line, MCF-10A. Cells were chronically exposed to a low-level arsenite (500 nM) for up to 24 weeks. Markers of cancer cell phenotype and expression of critical genes relevant to breast cancer or stem cells (SCs) were examined. After 24 weeks, chronic arsenic-exposed breast epithelial (CABE) cells showed increases in secreted MMP activity, colony formation, invasion and proliferation rate, indicating an acquired cancer cell phenotype. These CABE cells presented with basal-like breast cancer characteristics, including ER- α , HER-2 and progesterone receptor negativity, and overexpression of *K5* and *p63*. Putative CD44⁺/CD24^{-/low} breast SCs were increased to 80% over control in CABE cells. CABE cells also formed multilayer cell mounds, indicative of loss of contact inhibition. These mounds showed high levels of *K5* and *p63* indicating the potential presence of CSCs. Epithelial-to-mesenchymal transition occurred during arsenic exposure. Overexpression of *aromatase*, a key rate-limiting enzyme in estrogen synthesis, occurred with arsenic starting early on in exposure. Levels of 17 β -estradiol increased in CABE cells and their conditioned medium. The aromatase inhibitor, letrozole abolished arsenic-induced increases of 17 β -estradiol production, and reversed cancer cell phenotype. Thus, chronic arsenic exposure drive human breast epithelia into a cancer cell phenotype with an apparent overabundance of putative CSCs. Arsenic appears to transform breast epithelia through overexpression of *aromatase*, thereby activating oncogenic processes independent of ER.

Keywords

Arsenic; aromatase; breast cancer; estrogen; stem cells

Introduction

Breast cancer is one of the most frequently diagnosed cancers in the world and the second leading cause of cancer death among women. The etiology of breast cancer is not fully understood, though genetic, environmental, lifestyle, physiological and pharmaceutical factors all appear to contribute to the cumulative risk for this disease (Sasco 2001). Estrogen is closely related to breast cancer risk (Hilakivi-Clarke et al. 2013). It has been noted that exposure to classic endogenous or exogenous estrogenic compounds can't fully explain all

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breast cancer cases, and the level of cancer risk associated with other environmental exposures remains undefined (Brody et al. 2007).

Arsenic is ubiquitously present in the environment and also released by human activities (Erbanova et al. 2008). The largest source of human exposure to this metalloid is as a naturally occurring element via drinking water due to geophysical deposition which can lead to heavy exposure and is a major environmental concern worldwide. Arsenic is recognized as a human carcinogen, with the lung, liver, skin as cancer target sites (IARC 2012). In addition, chronic exposure to arsenic has been associated with diabetes, cardiovascular disease, neurological defects, and reproductive and developmental issues (Bolt 2012). There may be other health effects related to arsenic exposure that have yet to be fully realized.

Arsenic appears to act as an endocrine disruptor. *In vitro* inorganic arsenic has biphasic dose-response on transcription of genes regulated by receptors for glucocorticoids, androgens, mineralocorticoids, and progestins (Bodwell et al. 2006; Bodwell et al. 2004). While lower inorganic arsenic concentrations (0.05-1.0 μ M) stimulate hormone-mediated gene response in rat EDR3 hepatoma cells, slightly higher but still non-toxic arsenic concentrations (1.0-3.0 μ M) suppress these same responses (Bodwell et al. 2006; Bodwell et al. 2004). Arsenic may also have estrogen-like activity and/or alter the expression of the estrogen receptor (ER). Our previous studies find that mice transplacentally exposed to arsenic show cancers or proliferative lesions in consistent targets, such as the liver, ovary, adrenal, uterus, and oviduct (Waalkes et al. 2004; Waalkes et al. 2007). All these tissues can also be targets of carcinogenic estrogens (Castagnetta et al. 2003; Watanabe and Kobayashi 1993). Transplacental or whole-life exposure to arsenic in mice induces tumors with over-expression of estrogen-regulated genes and estrogen responsiveness, as well as specific increased *ER- α* expression (Shen et al. 2007; Tokar et al. 2011; Waalkes et al. 2004). All these results indicate that arsenic induces an abnormal estrogen signaling response during the carcinogenic process in some tissues. In this regard, in ER-negative breast cancer cells, arsenic can restore *ER- α* expression and sensitize cells to endocrine therapy probably by demethylation of relevant DNA (Du et al. 2012). However, arsenic is also reported to inhibit *ER* expression in breast cancer MCF-7 cells which may be caused by the high background expression of *ER* in these cancerous cells (Davey et al. 2007). In addition, arsenic may activate ER- α through an interaction with the hormone-binding domain of the receptor instead by an enhancement of *ER- α* expression in MCF-7 cells (Stoica et al. 2000). Thus the interaction of arsenic with ER- α is complex and incompletely defined.

None-the-less, some studies have shown arsenic has a potential role as an environmental endocrine disruptor that can act as an estrogen mimic to activate ER or to stimulate its production. The ability to activate quiescent *ER* genes could be a very important factor in this action and creates a possibility that arsenic could contribute to the etiology of breast cancer. Up to now, there are no definitive epidemiological data linking arsenic exposure and breast cancer risk. However, recent work has found women with dermatofibromas are much more likely to have breast cancer and that dermatofibromas from breast cancer patients have 2.4-fold more tissue arsenic than those from control in a small study (Dantzig 2009). Breast cancer patients have significantly higher arsenic levels in hair compared with controls in two other studies (Benderli Cihan et al. 2011; Joo et al. 2009). In a small case-control study, Polish women with a *BRCA1* mutation (cases) had a significantly higher risk for breast cancer based on higher serum arsenic levels (Muszynska et al. 2012). All these studies have significant flaws, such as small sample size or lack of the appropriate control group, making any relationship between breast cancer and arsenic exposure tenuous and further research is needed.

Thus, in this study, we exposed non-tumorigenic human breast epithelial cells, MCF-10A, to an environmental-relevant concentration of arsenic. We intentionally selected an ER negative cell, since there are reports that arsenic can restore quiescent ER- α expression in breast cells (Du et al. 2012) or cause ER- α overexpression in tumor target tissue *in vivo* (Waalkes et al. 2004), both through gene methylation changes, which may be a major mechanism of arsenic carcinogenesis. We found that arsenic could induce a cancer cell phenotype in the human breast epithelia, with an apparent overabundance of breast cancer stem cells (CSCs) but by an ER-independent pathway. Our evidence indicates that overexpression of aromatase may be a key factor in this acquisition of a cancer cell phenotype.

Materials and methods

Cell culture

MCF-10A, an immortal (spontaneous) and non-tumorigenic human breast epithelial cell line, was obtained from ATCC (Manassas, VA) and maintained in base mammary epithelial cell growth medium (MEGM) kit (Lonza, Walkersville, MD). Cultures were incubated at 37 °C in 5% CO₂ and subcultured once per week. Treated cells were continuously exposed to 500 nM of sodium arsenite (Sigma, St. Louis, MO) for up to 24 weeks, when cells were considered to be transformed into a cancer cell phenotype (see results). Unexposed time-matched cells were used as controls. In aromatase inhibition experiments, chronic arsenic-exposed breast epithelial (CABE) cells and corresponding time-matched controls were treated with letrozole (50 nM) (Sigma, St. Louis, MO) for another 3 weeks. MCF-7 cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's Medium (Invitrogen, Grand Island, NY) with 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY), and used as positive control for some assays as indicated.

Secreted metalloproteinase activity

MMP-9 and MMP-2 activity was measured by zymographic gels in conditioned medium as described (Tokar et al. 2010b), quantitated by ImageJ software (National Institutes of Health, Bethesda, MD), and then adjusted by cell number. Three independent samples were assessed for each group.

Colony formation in soft agar

Colony-forming ability, a typical characteristic of cancer cells, was assessed by growth in soft agar as described (Tokar et al. 2010b). Six independent dishes were prepared for each group.

Invasion assay

A modified Boyden chamber assay was conducted as described (Tokar et al. 2010b) to assess invasive ability, another typical characteristic of cancer cells. Six independent samples were assessed for each group.

Cell proliferation

Treated and control cells were plated at the same concentration and grown for seven days under normal culture conditions. At the point of assessment, cells were lifted with trypsin and EDTA, dyed with trypan blue and then counted by TC10 cell counter (Bio-Rad, Singapore). Proliferation was defined as number of cell doublings that occur per unit of time. Quantitation was based on control cell rate being set at 100%. Three independent samples were assessed for each group.

Flow cytometry

Fluorescence-activated cell sorting (FACS) was performed on single-cell suspension with CD44 and CD24 antibodies. Anti-CD44 and anti-CD24 antibodies, as well as their corresponding IgG isotype controls were purchased from BD Biosciences (San Jose, CA). Briefly, cells were grown to 70-80% confluence, washed with phosphate-buffered saline (PBS) and harvested with 0.05% trypsin and 0.025% EDTA. Detached cells were incubated with the appropriate antibodies or IgG isotype control at 4 °C in the dark for 30 mins. The labeled cells were washed with PBS containing 0.1% sodium azide, fixed with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and then analyzed using a the Becton Dickinson LSR II Flow Cytometer (BD Biosciences, San Jose, CA). This experiment was repeated 4 times with 3 independent samples each time.

Real-time PCR

Transcript levels of interested genes were examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) as described previously (Tokar et al. 2010b). Briefly, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), purified with RNeasy mini kit columns (Qiagen, Valencia, CA), and reverse transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primers. Primers of target genes were designed with Primer-BLAST online and obtained from Sigma (St. Louis, MO). The ABsolute SYBR Green ROX Mix (ABgene, Rockford, IL) was used for cDNA amplifications. Data were analyzed using the delta-delta cycle time (Ct) method in which Ct of target gene was first normalized to that of the average of the house keeping genes [β -actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S (RPS 18)], then to time-point matched controls. Primer sequences are listed in supplemental material.

Western blot

Cells were lysed in M-PER Protein Extraction Reagent (Pierce, Rockford, IL), containing 0.1 mM phenylmethyl sul-fonyl fluoride (Sigma-Aldrich, St. Louis, MO) and 1% Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). 8 μ g of total protein was resolved on a 4%-12% Bis-Tris gel with MOPS Running Buffer (Invitrogen, Grand Island, NY) and transferred to polyvinylidene fluoride membranes. The membranes were then probed with appropriate primary antibodies overnight at 4 °C and secondary antibodies for 1 hr at room temperature. Protein bands were detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

17 β -estradiol determination

Cells were seeded in 6-well plates at a density of 5×10^5 per well, cultured in phenol red-free MEGM medium (Lonza, Walkersville, MD). After 48 hrs, the medium and cells were collected and stored at -80 °C before further analysis. The level of 17 β -estradiol, a major form of mammalian estrogen, was determined with 17 β -estradiol ELISA Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. The sensitivity of this assay was 28.5 pg/ml (linear range 29.3-30,000 pg/ml). The absorbance values were measured at a wavelength of 405 nm with correction at 595 nm using an microplate reader (Bio-Rad, iMark) .

Immunofluorescence

Immunofluorescence was conducted as described (Xu et al. 2012). Briefly, cells were fixed with acetone and methanol (1: 1, V:V), then blocked with normal horse serum, incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hr at room temperature, and finally stained with 4', 6'-diamidino-2-phenylindole dihydrochloride

(DAPI) (Invitrogen, Eugene, OR). Cell images were captured with an automated Olympus inverted fluorescence microscope and were processed by CellSens software (Olympus Corporation, Center Valley, PA).

Statistical analysis

All data are presented as mean $\pm$ standard error (SE). For single comparison between arsenic-treated cells with controls, A Student's *t*-test was used. For multiple comparison in aromatase inhibiting experiment, Tukey's post-hoc test after analysis of variance (ANOVA) test was applied. A $p < 0.05$ was considered significant in all cases.

Results

Acquisition of a cancer cell phenotype after chronic arsenic exposure

Colony-forming ability is a characteristic of cancer cells. After 24 weeks of arsenic exposure, the colonies formed by arsenic-exposed breast epithelia were approximately double that to control (Figure 1A). Invasiveness of arsenicexposed cells also doubled compared to control (Figure 1B). MMPs are able to degrade extracellular matrix proteins and assist local cancer cell invasion and distant metastases. After chronic arsenic exposure for 24 weeks, cells showed robust increases of both secreted MMP-9 (400%) and MMP-2 activity (290%) (Figure 1C). We have previously seen that cells chronically treated with carcinogenic metals with elevated levels of secreted MMP in this range (i.e. 2.5-4.0 fold) consistently produce xenograft tumors in immunodeficient mice (Achanzar et al. 2002; Tokar et al. 2010b), including the cells used in the present study (MCF-10A; Benbrahim-Tallaa et al., 2009). The proliferation rate over one week in arsenic-exposed breast epithelia ($120 \pm 3\%$) was significantly higher than control ($100 \pm 2\%$) at week 24. In addition, expression of the tumor suppressor genes, *p53* and *PTEN*, were suppressed by arsenic exposure, particularly by 24 weeks (Figure 2). The above data indicate that after 24 weeks of chronic arsenic exposure, the human breast epithelia had developed to a cancer cell phenotype. Thus, these cells are henceforth called chronic arsenic-exposed breast epithelial (CABE) cells.

CABE cells have a higher portion of likely CSCs

CD44⁺/CD24^{-/low} cells are thought to be SC subpopulation in human breast cancers (Al-Hajj et al. 2003). In CABE cells (24 weeks of arsenic exposure), a significant increase of CD44⁺/CD24^{-/low} CSC-like population occurred (Figure 3A). *CD24* transcript in CABE cells decreased dramatically after 16 weeks of arsenic exposure to almost undetectable levels at 24 weeks (Figure 3B). *K5* and *p63* are markers for both breast CSCs and basal-like breast carcinoma (Ribeiro-Silva et al. 2005). After 24 weeks of arsenic exposure, the transcript levels of *K5* and *p63*, as well as *K14* (another basal-like breast carcinoma marker, see supplemental material), became dramatically elevated in CABE cells (Figure 3C). CABE cells formed more and larger multi-layer cell mounds in culture, which indicates the loss of cell-to-cell contact inhibition, a common trait of cancer cells. Such mounds were very rare in control cells. Moreover, CABE cells showed much higher protein expression of *K5* and *p63* in these mounds compared with control (Figure 3D). All above data suggest a proportional increase in CSCs in CABE cells.

EMT and arsenic exposure

During chronic exposure to arsenic, MCF-10A cells started to exhibit spindle-like morphology and tended to lose cell adhesion as early as week 8. Typical cell morphology at the approximate point of transformation (week 24) is shown in Figure 4A. The transcript levels of *SNAIL1*, a key EMT regulatory factor, was markedly increased at week16 of

arsenic exposure and thereafter. Transcript (from week 16, Figure 4B) and protein (from week 8, Figure 4C) levels of *E-CADHERIN*, an epithelial cell marker, decreased in arsenic-treated cells, while transcript (at week 24, Figure 4B) and protein (from week 8, Figure 4C and Figure 4D) levels of *VIMENTIN*, a mesenchymal cell marker, increased in arsenic-treated cells, typical for EMT. These data indicate EMT was initiated early and progressed with arsenic exposure, and help verify the acquisition of a cancer cell phenotype.

Involvement of aromatase in arsenic-induced cancer cell phenotype

MCF-10A cells are ER- α , HER-2, and progesterone receptor (PR) negative, but can be converted by transformation with chemical agents that activate ER production (Zhang et al. 2005). During arsenic exposure, ER- α (both full length 66 kDa and truncated 46 kDa), HER-2 and PR all remained negative (Figure 5A). ER- β expression was low in MCF-10A cells and was not changed by arsenic exposure (not shown). Interestingly, arsenic-treated cells showed a significant increase of *aromatase* expression, a key rate-limiting enzyme in estrogen synthesis. *Aromatase* overexpression has been reported to be a key to ER-independent malignant transformation of the MCF-10A cell line that was used in this study (Wang et al. 2012). The increase of *aromatase* expression started early (week 4) for transcript (Figure 5B) and by week 8 for protein (Figure 5C). The arsenic-induced overexpression of *aromatase* was most pronounced at week 24 (Figure 5B, 5C), when cells are considered to have a cancer cell phenotype. Immunofluorescence at week 24 showed that the *aromatase* was heterogeneously overexpressed in the CAGE cells (Figure 5C).

To further test the role of aromatase in arsenic-induced malignancy, the levels of 17 β -estradiol, a major mammalian estrogen, were determined. The levels of 17 β -estradiol in CAGE cells and their conditioned medium were significantly higher than those in controls (Figure 6A). In addition, CAGE cells were treated with letrozole, an aromatase inhibitor. Letrozole prevents the aromatase activity by competitive binding to the heme of its cytochrome P450 unit. In the present study, we observed a dramatic transcriptional suppression of aromatase by letrozole treatment in CAGE cells (Figure 6B). After 3 weeks of letrozole treatment, the arsenic-induced increases in 17 β -estradiol were abolished (Figure 6A), while secreted MMP activity (Figure 6C) and colony forming capacity (Figure 6D) were both returned to normal, indicating reversal of cancer cell phenotype.

Discussion

Breast cancer is one of the most common cancers in the United States. Both genetic and environmental factors contribute to the development of breast cancer (Lichtenstein et al. 2000). With regards to environmental factors, studies have suggested the association of exposure to some inorganics with the initiation and progression of breast cancer (Benbrahim-Tallaa et al. 2009; Julin et al. 2012; Martin et al. 2003), especially metals with a capacity to disrupt estrogen functions. Arsenic is a common environmental contaminant and human exposure occurs from both natural sources and human activities. Recent studies indicate that arsenic may act as an endocrine disruptor (Bodwell et al. 2006; Bodwell et al. 2004), interfering with the estrogen signaling (Tokar et al. 2010a; Waalkes et al. 2004; Waalkes et al. 2007) or even the expression of ER (Davey et al. 2007; Du et al. 2012; Shen et al. 2007; Waalkes et al. 2004). Some studies implicate a potential contribution of this metalloid to breast cancer development (Benderli Cihan et al. 2011; Dantzig 2009; Joo et al. 2009). However, there are no adequate epidemiological studies showing a firm association between arsenic exposure and an elevated risk of breast cancer. The potential role of arsenic in human breast cancer should receive further study. Cellular models, such as the one developed in the present work, can be very helpful in defining plausibility of carcinogenic potential.

In this present study, we found that a chronic arsenic exposure could transform the ER-negative human breast epithelial cells (MCF-10A) into a cancer cell phenotype. In addition, there was a loss of critical tumor suppressor genes, like *PTEN*. This provides *in vitro* evidence for a plausible association between arsenic exposure and the capacity to induce breast cancer characteristics in breast epithelial cells. The arsenic-transformed breast epithelia showed ER-, PR- and HER-2-negativity, as well as increased expression of *K5*, *p63* and *K14*, all typical characteristics of basal-like or triple negative breast cancer (Fadare and Tavassoli 2008), an aggressive, metastatic and chemotherapy-resistant sub-type of breast cancer with a poor patient survival rate (Fadare and Tavassoli 2008). Stimulation of ER- α expression is a key early event during carcinogenesis of estrogen-dependent breast cancer. ER- α can be activated during malignant transformation of these ER- α negative MCF-10A cells used in this study (Zhang et al. 2005). Based on the previous observed estrogen signaling interfering function of arsenic, one presumed mechanism for arsenic-induced breast epithelial transformation is that arsenic would act through ER- α and activate the downstream pathways. However, the arsenic-exposed MCF-10A cells remained ER- α negative during the acquisition of a cancer cell phenotype, negating an ER- α centered hypothesis. It is of interest that another inorganic carcinogen, cadmium, when transforming ER- α negative MCF-10A cells produces a basal-like (ER- α negative) phenotype as well (Benbrahim-Tallaa et al. 2009). So this may be common to the inorganic carcinogens.

Clinical and experimental studies suggest that *aromatase* expression can have a key role in breast cancer development (Chumsri et al. 2011; Diaz-Cruz et al. 2011; Kirma et al. 2001). Breast cancers have been shown to overexpress *aromatase* and produce higher levels of estrogens compared with control non-cancer breast tissues (Chumsri et al. 2011). *Aromatase* overexpression in transgenic mice induces preneoplastic alterations in mammary glands and activates biochemical pathways leading to mammary carcinogenesis (Kirma et al. 2001). In transgenic mouse models, *aromatase* overexpression induced more advanced mammary preneoplasia and carcinoma when compared with *ER- α* overexpression (Diaz-Cruz et al. 2011). Furthermore, only the *aromatase*-overexpressing mice showed invasive mammary adenocarcinomas (Diaz-Cruz et al. 2011). Although it is thought that in the mammary tissue fibroblasts are the primary cells accounting for expression of *aromatase*, breast epithelia also show *aromatase* expression (Brodie et al. 1997). In the present work, we observed a dramatic increase in *aromatase* expression after arsenic exposure in breast epithelial cells, which started early and lasted through the whole process of acquired cancer cell phenotype. *Aromatase* is the rate-limiting enzyme for estrogen synthesis. *Aromatase* overexpression increases local estrogen levels and enhances cell proliferation of mammary tissues (Kirma et al. 2001; Wang et al. 2012). The arsenic-exposed CAGE cells clearly showed elevated estrogen production based on 17β -estradiol levels, supporting the role of *aromatase* in estrogen synthesis. More importantly, the arsenic-induced increases in secreted MMP activity and in colony formation were completely abolished by *aromatase* inhibition. These results strongly suggest the involvement of *aromatase* activation in arsenic-induced acquisition of a cancer cell phenotype. At the same time, these cells remained ER-negative during this entire process. Estrogen is widely accepted to combined with ER- α , and then stimulate cell proliferation etc., as part of its carcinogenic activity. However, accumulating data support ER-independent mechanisms in estrogen-mediated breast cancer development (Yue et al. 2013). In ER-independent mechanisms, estrogen can be carcinogenic through production of fenotoxic estrogen metabolites (such as catechols and quinones), which can cause DNA damage and/or form DNA adducts (Yue et al. 2013). Consistent with our study, a recent study showed *aromatase* overexpression in MCF-10A cells elevated production of estrogens and thereby induced malignant changes in the absence of ER- α activation (Wang et al. 2012). Malignant transformation in this case was completely abolished by *aromatase* inhibitor (Wang et al. 2012). Estrogen metabolite genotoxicity or induction of a CSC population were considered as likely mechanisms for *aromatase*-based malignant

transformation of MCF-10A cells (Wang et al. 2012). The present results indicate a CSC population has likely been formed during arsenic-induced cancer cell phenotype. Thus, the observed arsenic-induced acquisition of a cancer cell phenotype in MCF-10A cells in the present study likely goes through an ER-independent mechanism with *aromatase* overexpression playing an important role, and meshes nicely with prior work providing plausible modes of action in general for *aromatase* overexpression and breast epithelia transformation.

CSCs are cells with SC-like properties and have been implicated in cancer initiation, progression and repopulation in general but more specifically in formation of basal-like breast cancers (Polyak and Weinberg 2009). Breast CSCs show a CD44⁺/CD24^{-/low} phenotype and are highly tumorigenic in immunodeficient mice (Al-Hajj et al. 2003). In this present study, chronic arsenic exposure caused a robust increase in CD44⁺/CD24^{-/low} potential breast CSCs. This is consistent with multiple previous studies from our laboratory that observe CSC increases during arsenic-induced tumor formation *in vivo* or cellular malignant transformation *in vitro* of other cell types (Tokar et al. 2010b; Tokar et al. 2011; Waalkes et al. 2008). Breast SCs are sensitive to steroid hormone signaling, even in the absence of ER and PR (Asselin-Labat et al. 2010). Wang et al. (Wang et al. 2012) found *aromatase* overexpression as the basis of a basal-like breast cancer cell model occurred concurrently with expansion of a likely CSC pool as a potential mechanism of malignant transformation (Wang et al. 2012). Aromatase inhibition *in vivo* can also result in a dramatic reduction in breast SC number in mice (Asselin-Labat et al. 2010). Thus, the arsenic-induced *aromatase* overexpression, which increases the estrogen synthesis, may also likely contribute to the enlarging of breast CSC pool in this basal breast cancer model, and produce a mechanism by which a silenced ER- α can be by-passed.

Additional factors may contribute to the arsenic-induced acquisition of a cancer cell phenotype in our study. During chronic exposure to arsenic, MCF-10A cells showed cell morphological and molecular changes typical for EMT, which started before the onset of a cancer cell phenotype. EMT is a dedifferentiation program that is often activated in cancer that aids in invasion and metastasis (Thiery 2002), and also involved in tumor initiation (Morel et al. 2012). In addition, the EMT program can itself generate CSC population (Mani et al. 2008; Morel et al. 2008; Santisteban et al. 2009). Mammary epithelia transfected with EMT inducer genes show increased CSC markers as well as typical CSC characteristics (Mani et al. 2008). On the other hand, CSCs displayed EMT characteristics (Mani et al. 2008). The generation of CD44⁺/CD24^{-/low} breast CSCs is accelerated by EMT in non-tumorigenic breast epithelia (Morel et al. 2008). EMT induced by CD8⁺ T cells led to breast tumors with CSC properties, such as CD44⁺/CD24^{-/low} phenotype, ability to reestablish an epithelial tumor, resistance to environmental insults, and capacity of forming spheres (Santisteban et al. 2009). Thus, the induction of EMT by chronic arsenic exposure may contribute to not only the acquisition of a cancer cell phenotype but the breast CSC increase in breast epithelia.

In summary, in the present study arsenic-induced the acquisition of a cancer cell phenotype in human breast epithelial cells resulting in a basal-like phenotype concurrently with *aromatase* overexpression and CSC increase. This provides evidence of the plausibility that arsenic exposure may contribute to the development of an important subset of breast cancers. Furthermore, it is possible that arsenic acts as an environmental endocrine disruptor through *aromatase* overexpression, thereby activating oncogenic process through ER-independent pathways, a process not previously considered. The enhanced CSC occurrence with arsenic-induced acquisition of a cancer cell phenotype in breast epithelia is consistent with multiple *in vivo* and *in vitro* models of arsenical carcinogenesis and appears to have mechanistic significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

MCF-10A breast epithelial cells acquire a cancer cell phenotype with arsenite exposure for 24 weeks. (A) Colony formation in soft agar. Quantative data (bottom) and representative image (top) for colonies (n = 6). (B) Invasive capacity (n = 6). (C) Secreted (metalloproteinase-9) MMP-9 and MMP-2 activity. Quantative data (bottom) and representative zymogram image (top) (n = 3). Numerical data are expressed as mean ± SE with control set to 100%. * $p < 0.05$ compared with control.

Figure 2.

Expression of the tumor suppressor genes *p53* and *PTEN* during arsenite-induced acquisition of a cancer cell phenotype. (A) *p53* transcript. (B) *PTEN* transcript. Data are expressed as mean $\pm$ SE with control set to 100% ($n = 3$). * $p < 0.05$ compared with time-matched controls.

Figure 3.

Chronic arsenite exposure induced overabundance of cancer stem cells (CSCs) during acquisition of a cancer cell phenotype. (A) Increased CD44⁺/CD24^{low} CSC-like population in chronic arsenite-exposed breast epithelial (CABE) cells determined by flow cytometry at week 24 (4 repeated assays with $n = 3$ each assay). (B) Decreased *CD24* transcript in CABE cells ($n = 3$). (C) Increased transcript of *K5* and *p63* in CABE cells ($n = 3$). (D) Cell mounding (indicated with white arrows) in control and CABE cells. CABE cells showed more cell mounds which overexpressed of the CSC markers, *K5* and *p63*, specifically in the mounds. Assessed at week 24 of arsenite exposure (bar = 20 μ M). Numerical data are expressed as mean $\pm$ SE with control set to 100%. * $p < 0.05$ compared with time-matched controls.

Figure 4. Epithelial-to-mesenchymal transition (EMT) as induced by chronic arsenite exposure. (A) Cells morphology at week 24 (bar = 100 μ M). Note: arrows indicating cells showing spindle-shape typical of EMT in CABE cells compared to rounded morphology of control cells. (B) Increased transcript of *SNAIL1* and *VIMENTIN*, and decreased transcript of *E-CADHERIN* during chronic arsenite exposure (n = 3). (C) Protein levels of *E-CADHERIN* and *VIMENTIN* during chronic arsenite exposure examined by western blot with representative image (top) and quantative data (bottom) at week 24 (n = 3). (D) Increased expression of *VIMENTIN* in CABE cells at week 24 examined by immunofluorescence (bar = 20 μ M). Note: wide-spread cytosolic localization in CABE cells. Numerical data are

expressed as mean $\pm$ SE with control set to 100%. * $p < 0.05$ compared with time-matched controls.

Figure 5.

Expression of *ER-α*, *HER2*, *PR* and *aromatase* in arsenite-exposed cells. (A) *ER-α*, *HER2* and *PR* by western blot. All three remained negative in arsenite-exposed cells during transformation. (B) *Aromatase* transcript during arsenite exposure ($n = 3$). (C) Protein levels of *aromatase* during arsenite exposure by western blot ($n = 3$). Top: western blot; bottom: quantitative data. (D) Increased *aromatase* protein expression in CAGE cells at week 24 examined by immunofluorescence (bar = 20 μ M). Green staining is indicative of *aromatase* protein; blue is DAPI, a general nuclear stain. Numerical data are expressed as mean $\pm$ SE with control set to 100%. * $p < 0.05$ compared with time-matched controls.

Figure 6. Effects of aromatase inhibition on estrogen synthesis and arsenite-induced cancer cell phenotype. CABC cells (24 weeks of arsenite exposure) and control cells were treated with 50 nM letrozole (LET) for 3 weeks. (A) Levels of 17β -estradiol in cells and medium. (B) Aromatase transcript. (C) Secreted MMP-9 and MMP-2 activity. (D) Colony forming capacity. Numerical data are expressed as mean $\pm$ SE ($n = 3$). Differing superscripts indicate a significant difference between groups.