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Alteration of mammary gland development and gene expression by *in utero* exposure to arsenic

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ABSTRACT

Early life exposure to estrogens and estrogen like contaminants in the environment is thought to contribute to the early onset of puberty and consequently increases the risk of developing breast cancer in the exposed female. The results of this study show that *in utero* exposure to the metalloestrogen arsenite altered mammary gland development prior to its effect on puberty onset. In the prepubertal gland, *in utero* exposure resulted in an increase in the number of mammosphere-forming cells and an increase in branching, epithelial cells, and density. In the postpubertal gland, *in utero* exposure resulted in the overexpression of estrogen receptor-alpha (ERα) that was due to the increased and altered response of the ERα transcripts derived from exons O and OT to estradiol. These results suggest that, in addition to advancing puberty onset, *in utero* exposure to arsenite alters the pre- and postpubertal development of the mammary gland and possibly, the risk of developing breast cancer.

1. Introduction

It has been suggested that the high incidence of hormone related diseases in children and adults, such as early onset of puberty and breast cancer, may be due to exposure to environmental estrogens. Since the beginning of the 20th century, there has been a trend toward an earlier onset of puberty. Although the trend has ceased during the last several decades in many European countries [1], it continued in the United States, especially in African American girls [2]. Early puberty onset is associated with adverse social and clinical outcomes. Early thelarche (breast development) [3] and menarche (first menstrual period) [4] are risk factors for earlier development of breast cancer and early adrenarche (increase production of androgens) is associated with an increased risk of developing obesity, type 2 diabetes, and cardiovascular disease [5]. Thelarche is triggered by the activation of the hypothalamic–pituitary–gonadal axis and the increase in circulating ovarian and pituitary hormones that initiate and promote the growth and morphogenesis of the breast [6].

The mammary gland is unique in that it grows and develops throughout the lifetime of a female [7]. Development begins in fetal life and ends following the first full term pregnancy with the greatest growth occurring at puberty. In the immature gland, mammary stem cells are responsible for growth and development. Located in the terminal end buds [8] and along the ducts [9], these cells have the capacity to self renew and to generate progenitor cells that proliferate and differentiate into luminal and basal cells resulting in the elongation of the ducts and the development of branches. With the onset of puberty, elongation and branching occur in response to estrogens, progestins, and growth hormone. As estrogens play a central role in puberty onset and breast development, it has been suggested that exposure to environmental estrogens in early life, when the fetus and infant are susceptible to small hormonal changes [10,11], contributes to early puberty onset. In support of this hypothesis, epidemiological studies have linked exposure to the phythoestrogens in soy based formulas [12] and the xenoestrogen polybrominated biphenol [13] to early puberty onset and animal studies have shown that early life exposure to genistein [14] or estradiol [15] advances the onset of puberty.

Abbreviations: DTT, dithiothreitol; DEPC, diethylpyrocarbonate; EDTA, ethylenediamine tetraacetic acid; EGF, epidermal growth factor; ERα, estrogen receptor-alpha; FGF, fibroblast growth factor; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; i.p., intraperitoneal; kgbw, kilogram per body weight; PgR, progesterone receptor; qRT-PCR, quantitative real-time polymerase chain reaction.

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Breast cancer is also associated with exposure to estrogens and the mammary stem and/or progenitor cells are thought to be the targets of malignant transformation because of their ability to self renew and proliferate. The most prominent risk factors for developing breast cancer are associated with increased lifetime exposure to estrogens, either to endogenous estrogens (*e.g.*, early age at thelarche and menarche and late age at menopause) or exogenous estrogens (*e.g.*, combined oral contraceptives or hormone replacement therapy [16,17]). In addition to lifetime exposure, the timing of exposure to estrogens appears to be a critical determinant of risk. Increased exposure to estrogens during pregnancy is associated with an increased risk of developing breast cancer in both the mother and the daughter; while decreased exposure to estrogens in daughters, whose mothers suffered from preeclampsia, is associated with a lower risk of developing the disease [18–23]. Animal studies show that female offsprings exposed *in utero* to estradiol, diethylstilbestrol, or genistein have an earlier vaginal opening and an increased risk of developing mammary tumors [14,24–26] suggesting that early life exposure to estrogens and estrogen-like substances is also a risk factor of developing breast cancer.

In contrast to the naturally occurring phytoestrogens and synthetic xenoestrogens, less is known about the contribution of the metalloestrogens, such as the metalloid arsenite [27], to the high incidence of early puberty onset and breast cancer. Arsenic is a semi-metal having both metal and nonmetal properties. It is rarely found as a native element but occurs as an organic compound with carbon and hydrogen or as an inorganic compound with oxygen, chlorine, and sulfur. Arsenic is a prevalent environmental contaminant that has no known physiological function, is present in the body as a result of occupational and non-occupational exposures [28] and crosses the placental [29–31] and blood-brain barriers [32]. Although the Environmental Protection Agency set the Reference Dose for inorganic arsenic as 0.3 μg/kg body weight (bw)/day [33] and the Joint FAO/WHO Expert Committee on Food Additives set the health concern level as 2.0–7.0 ug/kgbw/day [34], some populations may exceed these exposures. In the general population, the estimated daily intake of arsenic from food ranges from 0.01 to 5.6 μg/kgbw/day and from drinking water ranges from 0.2 to 0.539 ug/kgbw/day [35] but may be as high as 12.5 ug/kgbw/day in some areas [36]. The estimated daily exposure from inhaled arsenic is also significant and ranges from 0.02 to 0.2 ug/kgbw/day in populations living in rural areas and from 0.4 to 0.6 ug/kgbw/day in populations living in cities [37] and may be higher in populations living near nonferrous smelters and power plants that burn coal high in arsenic. Cigarette smokers also inhale arsenic (0.7–6.0 ug/day) [38] and prior to the ban of arsenic in pesticides, smokers in the 1950s inhaled more than 100 μg of arsenic per day.

We have previously shown that arsenite, an oxyanionic form of arsenic, but not arsenate, has potent estrogen like activity *in vitro* due to a high affinity interaction with the ligand binding domain of estrogen receptor-alpha (ERα) [27]. Similar to estradiol, arsenite induces the growth and expression of estrogen regulated genes in breast cancer cells and activates ERα in transient transfection experiments [27]. Others have shown in a spontaneous mouse model that arsenite in drinking water increases the growth of mammary tumors [39] and that *in utero* exposure to arsenite influences the estrogen response pathways in both male and female offspring [29,30,40] suggesting that arsenite also has estrogen like activity *in vivo*. In this study, we show that, in female offspring, *in utero* exposure to an environmentally relevant dose of arsenite advances the timing of vaginal opening and alters the development of the mammary gland prior to its effect on the hypothalamic–pituitary–gonadal axis. *In utero* exposure to the metalloid also results in an expansion of the mammosphere-forming cell population in the prepubertal mammary gland and alters the expression and regulation of ERα in the postpubertal gland suggesting that *in utero* exposure to arsenite causes changes in the mammary gland which result in abnormal growth and potentially, increases the susceptibility of the gland to neoplasia later in life.

2. Materials and methods

2.1. Animals

All animal studies were conducted in accordance with the Georgetown University Animal Care and Use Committee. Pregnant Sprague-Dawley rats were delivered on day 7 of gestation from Harlan Breeding Facilities (Frederick, MD) and maintained on a purified phytoestrogen-free diet that contained no detectable arsenic and was not supplemented with copper, chromium, and selenium (Tekland Lab Animal Diets TD02373). The animals were provided with purified water. The amount of arsenic in the water was not measured but was expected to be negligible. Pregnant female rats were treated with 5 μg/kgbw of arsenite dissolved in water by intraperitoneal (i.p.) injection on days 12 and 17 of gestation or with 50 μg/kgbw of ethinyl estradiol dissolved in corn oil by daily oral gavage starting on day 12 of gestation until birth. To avoid metabolism in the liver, ethinyl estradiol was given instead of estradiol. Arsenite is methylated primarily in the liver [28,41]. When given orally, arsenite is absorbed from the gastrointestinal tract and transported to the liver where it is methylated [28,42,43]. When arsenite is inhaled or given by intratracheal instillation, it is absorbed through the naso-pharynx, tracheobronchial, and pulmonary compartments and transported in the plasma to multiple organs in the body [28,42,43]. Similar to inhalation, when arsenite is given by intravenous or intraperitoneal injection, it is transported to multiple organs [28,43,44]. As arsenite is the form of arsenic that activates ERα [27], arsenite was administered by i.p. injection to increase its bioavailability. The control animals were treated with water by i.p. injection. In this study, there were ten control dams, nine arsenite exposed dams, and four ethinyl estradiol exposed dams. At 21 days of age, the pups were weaned and continued on the purified diet. For the vaginal opening and postpubertal studies, pups were randomly fostered with dams from the same treatment group and all of the pups were included in the analyses. For the time course study, the pups were randomly fostered with dams from the same treatment group. For the time points, three to four pups were then randomly selected from different dams. To monitor normal development, eye lid opening and weekly weights were determined. Vaginal opening was monitored daily from postnatal days 25 to 40.

2.2. Morphological analysis of the mammary gland

For morphological analysis, the mammary glands were excised and processed as whole mounts. The glands were fixed in Carnoy's fixative for 4 h to overnight, defatted overnight in xylene, rehydrated, stained overnight with carmine alum (Sigma), dehydrated in a series of graded alcohols, and cleared in xylene. Digital images were obtained and analyzed using MetaMorph Microscopy Automation & Image Analysis Software (Sunnyvale, CA). To determine mammary gland density, the images were binarized and the density was calculated as the percentage of the epithelium compared to the fat pad. The mammary gland branching was calculated as the number of branching points per unit length of two branches from the nipple to the terminal end.

2.3. Mammosphere culture

Whole mammary glands from 5-day-old animals were minced, suspended in DMEM/F12 medium containing collagenase and

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