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

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Abbreviations: DTT, dithiothreitol; DEPC, diethylpyrocarbonate; EDTA, ethylenediamine

tetraacetic acid; EGF, epidermal growth factor; ER α , 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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Abstract

Early life exposure to estrogens and estrogen like contaminants in the environment are thought to contribute to the early onset of puberty and consequently increase 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 ($ER\alpha$) that was due to the increased and altered response of the $ER\alpha$ 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.

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 towards 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 phytoestrogens 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.

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 offspring 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 to 7.0 µg/kg bw/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/kg bw/day and from drinking water ranges from 0.2 to 0.539 µg/kg bw/day (35) but may be as high as 12.5 µg/kg bw/day in some areas (36). The estimated daily exposure from inhaled arsenic is also significant and 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 (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 µg/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 increase 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 ER α 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.

Materials and Methods

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 μ g/kgbw of arsenite dissolved in water by intraperitoneal (i.p.) injection on days 12 and 17 of gestation or with 50

134 $\mu\text{g/kgbw}$ of ethinyl estradiol dissolved in corn oil by daily oral gavage starting on day 12 of
135 gestation until birth. To avoid metabolism in the liver, ethinyl estradiol was given instead of
136 estradiol. Arsenite is methylated primarily in the liver (28;41). When given orally, arsenite is
137 absorbed from the gastrointestinal tract and transported to the liver where it is methylated
138 (28;42;43). When arsenite is inhaled or given by intratracheal instillation, it is absorbed
139 through the naso-pharynx, tracheobronchial, and pulmonary compartments and transported in
140 the plasma to multiple organs in the body (28;42;43). Similar to inhalation, when arsenite is
141 given by intravenous or intraperitoneal injection, it is transported to multiple organs (28;43;44).
142 As arsenite is the form of arsenic that activates $\text{ER}\alpha$ (27), arsenite was administered by i.p.
143 injection to increase its bioavailability. The control animals were treated with water by i.p.
144 injection. In this study, there were ten control dams, nine arsenite exposed dams, and four
145 ethinyl estradiol exposed dams. At 21 days of age, the pups were weaned and continued on
146 the purified diet. For the vaginal opening and postpubertal studies, pups were randomly
147 fostered with dams from the same treatment group and all of the pups were included in the
148 analyses. For the time course study, the pups were randomly fostered with dams from the
149 same treatment group. For the time points, three to four pups were then randomly selected
150 from different dams. To monitor normal development, eye lid opening and weekly weights
151 were determined. Vaginal opening was monitored daily from postnatal day 25 to 40.

153 ***Morphological analysis of the mammary gland***

154 For morphological analysis, the mammary glands were excised and processed as whole
155 mounts. The glands were fixed in Carnoy's fixative for 4 hrs 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.

Mammosphere culture

Whole mammary glands from 5 day old animals were minced, suspended in DMEM/F12 medium containing collagenase and hyaluronidase (Stemcell Technologies), and incubated for 7 hrs in a 5% CO₂ incubator (45). Epithelial organoids were isolated by centrifuged at 80xg for 30 seconds. Five ml of pre-warmed trypsin-EDTA (Stemcell Technologies) was added to the pellet and the pellet resuspended by pipetting. Ten ml of cold Hanks' Balanced Salt Solution Modified (Stemcell Technologies) supplemented with 2% FBS (HF) was added and the solution centrifuged at 350xg for 5 min. Two ml of pre-warmed 5 mg/ml Dispase and 200 ul of 1 mg/ml DNase I was added and the pellet was resuspended by pipetting. To obtain a single cell suspension, the cell sample was diluted with 10 ml of cold HF and filtered through a 40-µm cell strainer. The sample was then centrifuged at 350xg for 5 min and resuspended in 1-2 ml of serum-free mammary epithelial growth medium (MEGM, Lonza) supplemented with B-27 supplement minus vitamin A (2x; Invitrogen), 20 ng/ml recombinant rat EGF (PeproTech), 20

ng/ml recombinant rat bFGF (PeproTech), and 4 µg/ml heparin (Sigma). The cells were counted and visualized using a hemocytometer. In all experiments, the number of single cells was greater than 99%. Cells were plated in ultralow attachment 6 well plates (Corning) at a density of 40,000 cells in 2 ml of the supplemented MEGM per well and incubated in 5% CO₂ incubator at 37°C for 7-10 days. Although cell aggregates were present, only mammospheres larger than 60 µm in size (may have solid or hollow morphology) were counted and photo-documented. For mammosphere subcultures, the mammospheres were collected by centrifugation at 350xg for 5 min and dissociated with 0.5-1 ml of pre-warmed trypsin-EDTA. Mammospheres were triturated using a P-1000 pipette for 1.5-2 min. Five ml of cold HF was added and the suspension was centrifuged at 350xg for 5 min. Supernatant was aspirated and the pellet resuspended in 2 ml of supplemented MEGM. Single cells were plated at a density of 10,000 cells in 2 ml in ultralow attachment 6 well plates and incubated for 7-10 days as described above.

Real time reverse transcriptase-polymerase chain reaction

For RNA extraction, frozen tissue was pulverized in liquid nitrogen using Spectrum Bessman Tissue Pulverizers. One ml 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 12,000rpm at 4°C, the lipid layer was removed, and the supernatant was 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 12,000rpm 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 hrs to overnight and centrifuged for 15 min at 12,000rpm at 4°C. Following centrifugation, the pellet washed with 1 ml of 75% ethanol, centrifuged for 5 min at 7,000rpm 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 10x buffer (200mM Tris-HCl pH 8.4, 20mM MgCl₂, 500mM KCl; Invitrogen) was added to 2 µg of RNA and incubated at room temperature for 15 min. Then 2 µl of EDTA (25mM; Invitrogen) was added and the sample incubated for 15 min at 65°C. Each 70 µl RT reaction contained 7µl of 10x Taqman RT Buffer (500mM KCl, 100mM Tris-HCl pH 8.3), 15.4 µl of 25mM MgCl₂, 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 20x 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).

Nuclear run-on assay

219 For the preparation of nuclei, mammary glands were frozen, pulverized in liquid
220 nitrogen, and resuspended in nuclei isolation buffer (15mM HEPES, 1.5M sucrose, 60mM KCl,
221 15mM NaCl, 0.15mM spermine, 0.5mM spermidine, 2mM EDTA, 0.5mM EGTA, 0.1mM PMSF*,
222 2mM DTT*; *added fresh) plus 0.1% Triton-X (Promega). Twelve ml ice-cold nuclei isolation
223 buffer were added to approximately 500 mg of frozen pulverized tissue and the tissue was
224 homogenized in a Dounce homogenizer. The homogenate was filtered through four-six layers
225 of Miracloth (EMD Chemicals). The nuclei were collected by centrifugation at 250xg for 10 min
226 at 4°C, washed with nuclear isolation buffer, resuspended in 100 µl of freezing buffer (50mM
227 HEPES, 0.1mM EDTA, 5mM MgCl₂, 40% glycerol), and stored at -80°C.

228 For the run-on assay, nuclei were thawed on ice in one volume of transcription buffer 2x
229 (20mM HEPES, 200mM KCl, 5mM MgCl₂, 200mM sucrose, 4mM ATP, 4mM CTP, 4mM GTP, 20%
230 glycerol, 4mM DTT-added fresh). Eight µl of biotin-16-UTP (Roche Diagnostics) were added and
231 the nuclei were incubated for 1 hr at 26°C. The reaction was stopped by adding 6 µl of 250mM
232 CaCl₂. The nuclei were collected by centrifugation for 20 min at 4°C at 12,000rpm. The RNA
233 was isolated using Trizol and resuspended in 50 µl of DEPC treated water. Dynabeads, M-280
234 Streptavidin (Invitrogen), were used to purify the nascent mRNA following the manufacturer's
235 protocol. Briefly, the Dynabeads were added to the mRNA samples and incubated for 20 min at
236 42°C and then 2 hrs at room temperature. The mRNA-bead complex was separated using a
237 Dynal magnet, washed, resuspended in 30 µl of DEPC water, and used to synthesize cDNA for a
238 qRT-PCR assay.

239

Statistical Analysis

Vaginal opening was determined by an investigator that was blinded to treatment and the data were analyzed by one-way ANOVA. All other data are presented as means $\pm$ SEM. Statistical analyses were performed only on groups that contained three or more animals using a two-tailed unpaired Student's test with unequal variance. Statistical significance was defined as $P < 0.05$.

Results

Effects of early life exposure to arsenite on vaginal opening in female offspring

To determine the effects of early life exposure to an environmentally relevant dose of arsenite on vaginal opening, pregnant animals were treated with 5 $\mu\text{g}/\text{kgbw}$ of arsenite by i.p. injection on day 12 and on day 17 of gestation or with ethinyl estradiol (50 $\mu\text{g}/\text{kgbw}$; positive control) by daily oral gavage starting on day 12 of gestation until birth (Figure 1a). Vaginal opening was monitored in female offspring beginning on postnatal day 25. There was a significant difference in the time of vaginal opening between control animals (postnatal day 34.76 ± 0.32) and animals exposed *in utero* to arsenite or ethinyl estradiol (postnatal day 33.45 ± 0.41 and 33.81 ± 0.32 , respectively, $p < 0.05$) that was not due to an increase in body weight (data not shown). As vaginal opening is controlled by the reactivation of the GnRH pulse generator (46), the amount of GnRH mRNA in the hypothalamus was measured on postnatal days 5, 10, 15, 20 and 25 (Figure 1b) to rule out a nonspecific effect of arsenite. In non-treated Sprague-Dawley rats, GnRH mRNA levels generally increase between postnatal day 28 and 34

(47;48). In the animals exposed *in utero* to arsenite, GnRH mRNA levels began to increase between postnatal days 20 and 25. As expected, there was no increase in the levels of GnRH mRNA in control animals. The earlier increase in GnRH mRNA in the exposed animals suggests that *in utero* exposure to arsenite alters GnRH expression and consequently the time of vaginal opening.

Figure 1. Effects of early life exposure to arsenite on puberty onset in female offspring.

Pregnant female rats were treated with arsenite (5 µg/kg bw) by i.p. injection on days 12 and 17 of gestation or ethinyl estradiol (EE, positive control, 50 µg/kg bw) by daily oral gavage beginning on day 12 until birth and the female offspring were examined.

(a) Effect of early life exposure to arsenite on the time of vaginal opening. Pregnant female rats were treated with arsenite or ethinyl estradiol and the female offspring were monitored for vaginal opening. Data are plotted as percent animals with vaginal opening (n=11-34/group).

(b) Effect of early life exposure to arsenite on the expression of GnRH in the hypothalamus. Pregnant rats were treated with arsenite and the expression of GnRH in the female offspring was determined. The amount of GnRH mRNA was measured on postnatal days 0, 5, 10, 15, 20 and 25 by a qRT-PCR assay and normalized to the amount of GAPDH mRNA (mean $\pm$ SEM; n=2-3/group; * p<0.05).

Effects of early life exposure to arsenite on the morphology of the mammary gland of female offspring

Early puberty onset may alter the development and/or morphology of the mammary gland due to an earlier increase in the synthesis of estrogens in the ovaries. To ask whether *in utero* exposure to arsenite alters mammary gland morphology and development prior to the onset of puberty, pregnant animals were again treated with arsenite and the female offspring examined. The fourth abdominal mammary glands were dissected and processed as whole mounts, and examined on postnatal days 0, 5, 15, and 20. On postnatal day 0, an abnormal pattern of lateral budding (49) was observed along the ducts of the mammary glands of animals exposed while *in utero* to arsenite, suggesting that the *in utero* exposure to the metalloid alters branching in the mammary gland (Figure 2a). To determine whether exposure to the metalloid alters branching in the mammary gland, the number of branch points along the ducts was counted on postnatal day 5 (Figure 2b). There was a significant increase in the number of branch points in the mammary glands of animals exposed to arsenite (2.99 ± 0.65 branch points/unit length) compared to the number of branch points in the mammary gland of control animals ($2.08 \pm$

0.26 branch points/unit length) suggesting that *in utero* exposure to arsenite increases the branching of the mammary gland. To ask whether the increase in branching is associated with an increase in epithelial cells, the cells were isolated and counted (Figure 2c). There was a significant 1.7-fold increase ($p < 0.05$) in the number of cells in the mammary glands of animals exposed to arsenite compared to control animals. To determine whether the increase in epithelial cells continues throughout the prepubertal growth of the gland, mammary gland density was measured on postnatal days 15 and 20 (Figures 2d-e). In animals exposed to arsenite, there was a significant increase ($p < 0.05$) in mammary gland density (43% and 38%, respectively) compared to controls (35% and 27%, respectively). Taken together, the results demonstrate a consistent increase in the number of branch points, number of epithelial cells, and density in the mammary glands of animals exposed to arsenite while *in utero*. Since the morphological and developmental effects of arsenite were observed in animals exposed while *in utero* and occurred prior to the onset of puberty, the results suggest a direct effect of early life exposure to arsenite on the mammary gland.

A

Control (Day 0)

Arsenite (Day 0)

B

C

D

Control (Day 15)

Arsenite (Day15)

E

Figure 2. Effects of early life exposure to arsenite on the morphology of the mammary gland of female offspring.

Pregnant female rats were treated with arsenite or ethinyl estradiol as described in Figure 1. For morphological analyses, the fourth abdominal mammary glands were excised.

(a) *In utero* effects on mammary gland morphology. Digital image on postnatal day 0 from a control animal (representative image, n=2) or an animal exposed to arsenite (representative image, n=3). Arrows indicate abnormal lateral budding.

(b) *In utero* effects on mammary gland branching. The number of branch points was quantified on postnatal day 5 as branches per unit length (mean±SEM; n=3/group; * p<0.05).

(c) *In utero* effects on the number of epithelial cells in the mammary gland. Both fourth abdominal mammary glands were excised on postnatal day 5. Epithelial cells were isolated and counted (mean±SEM; n=4/control group, n=3/arsenite group, n=2/ethinyl estradiol group; * p<0.05 for control vs arsenite).

(d-e) *In utero* effects on mammary gland density on postnatal days 15, and 20. (d) Digital image from 15 day old control animal (representative image, n=3) or animal exposed to arsenite (representative image, n=3). (e) Mammary gland density is expressed as the percent of epithelium in the fat pad (mean±SEM; n=3/group; * p<0.05).

Effects of early life exposure to arsenite on mammosphere-forming cells in the glands of female offspring

The increase in the number of branch points suggests that *in utero* exposure to arsenite increases the number of mammary stem and/or progenitor cells (9). Mammary stem cells form mammospheres when grown in nonadherent cell culture conditions. To ask whether there was an increase in the number of mammosphere-forming cells, mammospheres (50;51) were grown from cells isolated from the glands of 5 day old offspring (Figure 3a). Compared to control animals, there was a 3.1-fold increase in the number of mammospheres derived from the mammary glands of animals exposed *in utero* to arsenite and a 2-fold increase in animals exposed *in utero* to ethinyl estradiol. To ask whether the mammospheres were derived from a stem-like cell, the potential for self-renewal was tested. When subcultured every 10 days, the primary generation mammospheres produced secondary and tertiary generation mammospheres with an efficiency of approximately 2/1000 cells (Figure 3b). The mammospheres expressed aldehyde dehydrogenase 1 (Aldh1) (52), CD24 (53), epithelial cell adhesion molecule (EpCAM) (53) (data not shown), and estrogen receptor-alpha (ER α) (54-56) (Figure 3c). There was no significant difference in the expression of ER α mRNA in mammospheres isolated from control or exposed animals (Figure 3c). When the second generation mammospheres were treated with 17 β -estradiol, there was an increase in the number of mammospheres but no difference in the size (data not shown) or fold increase in the number of mammospheres derived from control or arsenite exposed animals (1.53- and 1.64-fold increase, respectively; Figure 3b) consistent with the expression of ER α . To ask whether *in utero* exposure to the metalloid alters the stem/progenitor like cells in the adult gland, pregnant animals were treated with arsenite or ethinyl estradiol and the expression of Aldh1 was measured in virgin animals (Figure 3d). To avoid the effects of estrogens on gene

expression, the animals were ovariectomized on postnatal day 45 and examined on postnatal day 60. There was a 2.43- and 2.49-fold increase in Aldh1 mRNA in animals exposed *in utero* to arsenite or estradiol, respectively. The increase in the number of mammospheres together with the increase in mammary gland Aldh1 suggests that *in utero* exposure to arsenite results in the expansion of the mammary stem/progenitor cell population that persists through development.

Figure 3. Effects of early life exposure to arsenite on mammosphere-forming cells in the mammary gland of female offspring.

Pregnant female rats were treated with arsenite or ethinyl estradiol as described in Figure 1.

(a) *In utero* effects on the number of mammospheres obtained from female offspring on postnatal day 5 (mean±SEM; n=4 animals/group; * p<0.05).

(b) *In utero* effects on estradiol induced proliferation of mammospheres. First generation mammospheres from control animals and arsenite exposed animals were digested with trypsin, stem/progenitor cells were selected in serum-free media under non-adherent conditions in the presence or absence of estradiol (1 nM), and the second generation mammospheres were counted (mean±SEM; n= 2/group).

(c) *In utero* effects on ERα expression in mammospheres obtained from female offspring on postnatal day 5. ERα mRNA was determined by a qRT-PCR assay and normalized to Arbp mRNA (mean±SEM; n=2/group).

(d) *In utero* effects on Aldh1 expression in the adult gland. Female offspring were ovariectomized on postnatal day 45, and on postnatal day 60, the amount of Aldh1 mRNA was measured by a qRT-PCR assay, normalized to Arbp mRNA, and presented as fold difference (mean±SEM; n=4-11/group; * p<0.05).

Effects of early life exposure to arsenite on gene expression in the mammary glands of female offspring

In utero exposure to endocrine disrupting chemicals has been shown to alter expression of ER α in the exposed offspring, e.g., in utero exposure to genistein or TCDD increases the expression of ER α in the mammary gland and in utero exposure to dioxin increase expression in the testis while in utero exposure to BPA decreases expression of ER α in the vagina of exposed offspring (14;57-59). To determine whether *in utero* exposure to arsenite alters ER α expression in the mammary gland, ER α mRNA was measured on postnatal days 0, 5, 10, 15, 20, and 25 and on postnatal day 60. To avoid the effects of estradiol on gene expression, the latter animals were ovariectomized on postnatal day 45 (Figures 4a-b). In control animals, the expression of ER α mRNA increased from postnatal day 0 to day 20 and decreased on postnatal day 25. In animals exposed *in utero* to arsenite, the expression of ER α mRNA increased from postnatal day 0 to day 20 but remained at the same level on postnatal day 25. In the ovariectomized animals (postnatal day 60), basal expression of ER α mRNA remained elevated with a 2.5- and 3.5-fold increase ($p < 0.05$) in ER α mRNA in animals exposed *in utero* to arsenite or ethinyl estradiol, respectively (Figure 4b) suggesting that in utero exposure to arsenite leads to the overexpression of ER α that persists into adulthood.

To ask whether *in utero* exposure to arsenite alters the expression of genes that are regulated by estradiol, candidate genes were measured in the mammary gland of the ovariectomized animals (Figure 4c-e). *In utero* exposure to arsenite increased the expression of GREB1 (60) and amphiregulin (61) mRNA but had no effect on progesterone receptor (PgR) mRNA. Although *in utero* exposure to arsenite increased the expression of amphiregulin mRNA, *in utero* exposure to ethinyl estradiol had no effect on its expression.

Figure 4. Effects of early life exposure to arsenite on gene expression in the mammary gland of female offspring.

Pregnant female rats were treated with arsenite or ethinyl estradiol as described in Figure 1 and the female offspring were examined.

(a) *In utero* effects on ER α expression in the prepubertal gland. The amount of ER α mRNA was measured on postnatal days 0, 5, 10, 15, 20 and 25 by qRT-PCR assay and normalized to Arbp mRNA (mean $\pm$ SEM; n=3-11/group; * p<0.05).

(b) *In utero* effects on ER α expression in the adult gland. Female offspring were ovariectomized on postnatal day 45. On postnatal day 60, the amount of ER α mRNA was measured by a qRT-PCR assay and normalized to Arbp mRNA. Data are presented as fold difference (mean $\pm$ SEM; n=4-11/group; * p<0.05).

(c-e) *In utero* effects on gene expression in the adult gland. Female offspring were ovariectomized on postnatal day 45. On postnatal day 60, the amount of Greb1 (c), amphiregulin (d), and PgR (e) mRNA was measured by a qRT-PCR assay, normalized to Arbp mRNA, and presented as fold difference (mean $\pm$ SEM; n=4-11/group; * p<0.05).

Effects of early life exposure to arsenite on the expression of ER α transcripts in the mammary gland of female offspring

The rat ER α gene has five promoters (OS, ON, O, OT, and E1) (62-65) that confer tissue specific expression (63;65) and result in ER α transcripts with different 5' untranslated regions

495 (5' UTR),. To determine which promoter is utilized in the mammary gland, the 5'UTRs of the
496 ER α transcript were measured using primers for exons OS, ON, O, OT, and E1 (Figure 5). In
497 control animals (postnatal day 15 to 25), the 5'UTR of ER α mRNA was derived predominantly
498 from exons O and OT with expression of exon O approximately 2- to 4.5-fold greater than exon
499 OT (Figure 5a). In the ovariectomized animals (postnatal day 60), the 5'UTR was derived from
500 exon O followed by exons E1, OT and OS suggesting that throughout development, the ER α
501 transcript derived from exon O is the predominant transcript in the mammary gland. To
502 determine whether *in utero* exposure to arsenite alters expression of the ER α transcripts, the
503 5'UTRs of ER α mRNA were again measured on postnatal days 15 to day 25 (Figure 5a), and in
504 the ovariectomized adult animals (Figure 5b). Compared to control animals, the transcript
505 derived from exon O increased from postnatal day 5 to 20 but there appeared to a delay in the
506 increase in expression. In the exposed animals, the exon O transcript was significantly less on
507 postnatal day 15 (2.75-fold, $p < 0.05$), similar on postnatal day 20, and significantly higher on
508 postnatal day 25 (2.28-fold; $p < 0.05$). In contrast to the transcript derived from exon O, the
509 transcript derived from exon OT did not significantly change from postnatal day 5 to 25. In the
510 exposed adult gland, expression of the transcripts derived from exons O and OT was increased
511 (2.08- and 2.37-fold; $p < 0.05$, respectively). The increase in ER α mRNA may be due to an
512 increase in basal transcription of the ER α gene, however, a nuclear run on assay showed that
513 there was no difference in transcription of the ER α gene between control animals and animals
514 exposed *in utero* arsenite or ethinyl estradiol (Figure 5c). To ask whether *in utero* exposure
515 alters the regulation of the O and OT transcripts, the ovariectomized animals were treated with
516 17 β -estradiol (Figures 5d-e). Treatment with estradiol had no effect on the expression of the O

and OT transcripts in control animals but decreased the expression of the O and OT transcripts in animals exposed to arsenite while *in utero*. Taken together, the data show that *in utero* exposure to arsenite increases the expression of the O and OT transcripts and alters their regulation by estradiol. The data also suggest that overexpression of ER α mRNA may be due, in part, to alternate promoter usage and/or posttranscriptional regulation.

Figure 5. Effects of early life exposure to arsenite on the expression of ER α transcripts in the mammary gland of female offspring.

Pregnant female rats were treated with arsenite (5 μ g/kg bw) or ethinyl estradiol (50 μ g/kg bw) as described in Figure 1 and the female offspring were examined.

(a) *In utero* effects on ER α transcripts in the prepubertal gland. The amount of 5'UTR ER α mRNA was measured on postnatal days 15, 20 and 25 by qRT-PCR and normalized to Arbp mRNA (mean $\pm$ SEM; n=2-3/group; * p<0.05).

(b) *In utero* effects on ER α transcripts in the adult gland. Female offspring were ovariectomized on postnatal day 45. On postnatal day 60, the amount of ER α 5'UTR transcript was measured by a qRT-PCR assay and normalized to Arbp mRNA (mean $\pm$ SEM; n=4-12/group; * p<0.05).

(c) *In utero* effects on ER α gene transcription in the adult gland. Female offspring were ovariectomized on postnatal day 45, nuclei were isolated on postnatal day 60, and a nuclear run on assay was performed. The amount of the nascent ER α transcript was measured by qRT-PCR, normalized to Arbp transcript, and presented as fold difference (n=4-10).

(d-e) *In utero* effects on the regulation of ER α transcripts in the adult gland. Female offspring were ovariectomized on postnatal day 45 and treated for four days with estradiol beginning on postnatal day 56. On postnatal day 60, the amount of O and OT transcripts was measured by a qRT-PCR assay, normalized to Arbp mRNA, and presented as fold difference (mean $\pm$ SEM; n=4-11/group; * p<0.05).

Discussion

560 Previous studies have shown that the metalloid arsenite has estrogen like activity *in*
561 *vitro* that is due to a high affinity interaction with the ligand binding domain of ER α (27)
562 suggesting that arsenite may be a potent endocrine disruptor. The results of this study show
563 that *in utero* exposure to an environmentally relevant dose of the metalloid mimics many of the
564 effects of *in utero* exposure to ethinyl estradiol in female offspring. Similar to ethinyl estradiol,
565 *in utero* exposure to arsenite advanced vaginal opening, increased the number of
566 mammosphere-forming cells and epithelial density in the prepubertal mammary gland, and
567 increased the expression of genes associated with stem- and progenitor-like cells as well as the
568 expression of ER α and estrogen regulated genes in the postpubertal gland. In contrast to
569 ethinyl estradiol, *in utero* exposure to arsenite also increase the number of epithelial cells and
570 the expression of specific ER α transcripts suggesting that not all of the effects of arsenite are
571 mediated by ER α . However, as many of the developmental changes in the mammary gland
572 mimic the effects of ethinyl estradiol and occurred prior to the increase in GnRH, the results
573 suggest a direct estrogen like effect of arsenite on the mammary gland that altered its pre- and
574 postpubertal development. In contrast to these results, a recently published study showed that
575 prepubertal exposure to arsenic delayed vaginal opening and suppressed the development of
576 the mammary gland (66). The reason for the differences between the two studies is not clear
577 but may be due to differences in the dose and timing of exposure to the metalloid. There is
578 increasing evidence that the dose-response curves of some endocrine disruptors are nonlinear
579 (67) and that the timing of exposure, i.e., exposure during sensitive stages of development,
580 alters later development (68). In the mammary gland, the periods of development that are
581 most sensitive to endocrine disruptors are the prenatal development of the epithelial sprout,

ductal elongation during puberty, and lobuloalveolar expansion during pregnancy (68). In the present study, animals were exposed to a low dose of arsenite while *in utero* (5 ug/ kg bw on gestation days 12 and 17) whereas in the previously published study, animals were exposed to a higher dose prior to the onset of puberty (10 mg/kg bw by daily gavage beginning on postnatal day 12) which may affect the response of the mammary gland to the metalloid. Although the reasons for the different results between the studies remain to be determined, the results from the present study further support for the hypothesis that, during early development, the mammary gland is particularly susceptible to exogenous hormone like substances which affect its development (69;70).

During development, mammary stem cells generate progenitor cells that proliferate and differentiate into luminal and basal cells resulting in the elongation and branching of the ducts. The increase in the number of mammosphere-forming cells in the neonatal gland followed by the increase in the number of branches, epithelial cells, and epithelial density in the prepubertal gland and the increase in the expression of genes associated with stem- and progenitor-like cells in the nonpregnant adult gland suggests that *in utero* exposure to arsenite altered mammary gland development through an expansion of the stem/progenitor cell population in the neonatal gland that persisted in the adult gland.

In addition to altering the morphological development of the mammary gland, *in utero* exposure to arsenite increased the expression of ER α and estrogen regulated genes in the adult gland. In the pubertal and adult gland, there was an increase in the expression of ER α transcripts derived from exons O and OT and a change in their regulation by estradiol. Interestingly, the overexpression and altered regulation of exons O and OT transcripts are

similar to the changes in the expression of the ER α transcripts in human breast cancer. The human ER α gene has eight promoters resulting in transcripts with 5' UTRs derived from exons A, B, C(2), D, T1/T2, E1/E2, and F (71). In normal human breast tissue, the ER α transcripts are derived predominantly from exons A and C with the expression of the transcript derived from exon A greater than the transcript derived from exon C. In human breast tumors and breast cancer cells, ER α is overexpressed due to an increase in the transcripts derived from exons A, B, and C with the expression of the transcript derived from exon C greater than the transcript derived from exon A (72-74). Exons O and OT in the rat are homologous to exons C and B in the human, respectively, suggesting that *in utero* exposure to arsenite alters ER α expression similar to the alterations observed in breast cancer. In addition to the overexpression of specific transcripts, *in utero* exposure altered the regulation of ER α by estradiol similar to the regulation of ER α by estradiol in breast cancer cells (75). As stem/progenitor cells are thought to be the targets of malignant transformation in the breast and overexpression of ER α is thought to be the initial event in the development of ER positive tumors, the results of this study suggest that early life exposure to arsenite may increase the risk of developing breast cancer.

Taken together, the results of this study support the growing evidence that the developing organism is particularly susceptible to small changes in its environment that can induce phenotypic changes which effect tissue function and response in later life (69;70). Although several laboratories have studied the impact of phytoestrogens, pesticides, fungicides, plasticizers, and industrial chemicals on puberty onset, mammary gland development, and breast cancer (11), there is limited research on metals and metalloids with estrogen like activity. Thus, it is important to understand the impact of environmental

exposure to metalloestrogens on puberty onset, mammary gland development, and the risk of developing breast cancer.

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References

1. DeMuinck Keizer-Schrama SMPF, Mul D 2001 Trends in pubertal development in Europe. Human Reproduction Update 7:287-291
2. Herman-Giddens ME, Slora EJ, Wasserman RC, Bourdony CJ, Bhapkar MV, Koch GG, Hasemeier CM 1997 Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. Pediatrics 99:505-512
3. Hamilton AS, Mack TM 2003 Puberty and genetic susceptibility to breast cancer in a case-control study in twins. N Engl J Med 348:2313-2322
4. Feigelson HS, Henderson BE 1996 Estrogens and breast cancer. Carcinogenesis 17:2279-2284
5. Ibanez L, Dimartino-Nardi J, Potau N, Saenger P 2000 Premature adrenarche--normal variant or forerunner of adult disease? Endocr Rev 21:671-696

- 646 6. Hennighausen L, Robinson GW 2001 Signaling pathways in mammary gland development.
647 Dev Cell 1:467-475
- 648 7. Russo IH, Russo J 1996 Mammary gland neoplasia in long-term rodent studies. Environ
649 Health Perspect 104:938-967
- 650 8. Daniel CW, Silberstein GB 1987 Postnatal development of the rodent mammary gland. In:
651 Neville MC, Daniel CW, eds. The mammary gland. New York: Plenum Press; 3-36
- 652 9. Kenney NJ, Smith GH, Lawrence E, Barrett JC, Salomon DS 2001 Identification of Stem Cell
653 Units in the Terminal End Bud and Duct of the Mouse Mammary Gland. J Biomed
654 Biotechnol 1:133-143
- 655 10. Colborn T, von Saal FS, Soto AM 1993 Developmental effects of endocrine-disrupting
656 chemicals in wildlife and humans. Environ Health Perspect 101:378-384
- 657 11. Fenton SE, Reed C, Newbold RR 2012 Perinatal environmental exposures affect mammary
658 development, function, and cancer risk in adulthood. Annu Rev Pharmacol Toxicol 52:455-
659 479
- 660 12. Freni-Titulaer LW, Cordero JF, Haddock L, Lebron G, Martinez R, Mills JL 1986 Premature
661 thelarche in Puerto Rico. A search for environmental factors. Am J Dis Child 140:1263-
662 1267

- 663 13. Blanck HM, Marcus M, Tolbert PE, Rubin C, Henderson AK, Hertzberg VS, Zhang RH,
664 Cameron L 2000 Age at menarche and tanner stage in girls exposed in utero and
665 postnatally to polybrominated biphenyl. *Epidemiology* 11:641-647
- 666 14. Hilakivi-Clarke L, Cho E, Onojafe I, Raygada M, Clarke R 1999 Maternal exposure to
667 genistein during pregnancy increases carcinogen- induced mammary tumorigenesis in
668 female rat offspring. *Oncol Rep* 6:1089-1095
- 669 15. Hilakivi-Clarke L, Cho E, Clarke R 1998 Maternal genistein exposure mimics the effects of
670 estrogen on mammary gland development in female mouse offspring. *Oncol Rep* 5:609-
671 616
- 672 16. Kampert JB, Whittemore AS, Paffenbarger RSJr 1988 Combined effect of child-bearing,
673 menstrual events, and body size on age-specific breast cancer risk. *Am J Epidemiol*
674 128:962-979
- 675 17. Butler LM, Potischman NA, Newman B, Millikan RC, Brogan D, Gammon MD, Swanson CA,
676 Brinton LA 2000 Menstrual risk factors and early-onset breast cancer. *Cancer Causes*
677 *Control* 11:451-458
- 678 18. Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, Barbieri RL,
679 Speizer FE 1998 Plasma sex steroid hormone levels and risk of breast cancer in
680 postmenopausal women. *J Natl Cancer Inst* 90:1292-1299

- 681 19. Gerhard I, Vollmar B, Runnebaum B, Kubli F 1987 Weight percentile at birth. I. Clinical
682 data of pregnancy and relevance for early childhood development. Eur J Obstet Gynecol
683 Reprod Biol 26:303-311
- 684 20. Hsieh C, Pavia M, Lambe M, Lan SJ, Colditz GA, Ekbom A, Adami HO, Trichopoulos D,
685 Willett WC 1994 Dual effect of parity on breast cancer risk. Eur J Cancer 30A:969-973
- 686 21. Ekbom A, Trichopoulos D, Adami HO, Hsieh CC, Lan SJ 1992 Evidence of prenatal
687 influences on breast cancer risk. Lancet 340:1015-1018
- 688 22. Michels KB, Trichopoulos D, Robins JM, Rosner BA, Manson JE, Hunter DJ, Colditz GA,
689 Hankinson SE, Speizer FE, Willett WC 1996 Birthweight as a risk factor for breast cancer.
690 Lancet 348:1542-1546
- 691 23. Sanderson M, Williams MA, Malone KE, Stanford JL, Emanuel I, White E, Daling JR 1996
692 Perinatal factors and risk of breast cancer. Epidemiology 7:34-37
- 693 24. Hilakivi-Clarke L, Cho E, Cabanes A, DeAssis S, Olivo S, Helferich W, Lippman ME, Clarke R
694 2002 Dietary Modulation of Pregnancy Estrogen Levels and Breast Cancer Risk among
695 Female Rat Offspring. Clin Cancer Res 8:3601-3610
- 696 25. Boylan ES, Calhoon RE 1979 Mammary tumorigenesis in the rat following prenatal
697 exposure to diethylstilbestrol and postnatal treatment with 7,12-
698 dimethylbenz[a]anthracene. J Toxicol Environ Health 5:1059-1071

- 699 26. Nikaido Y, Yoshizawa K, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A 2004
700 Effects of maternal xenoestrogen exposure on development of the reproductive tract and
701 mammary gland in female CD-1 mouse offspring. *Reprod Toxicol* 18:803-811
- 702 27. Stoica A, Pentecost E, Martin MB 2000 Effect of arsenite on estrogen receptor- α
703 expression and activity in MCF-7 breast cancer cells. *Endocrinol* 141:3595-3602
- 704 28. Agency for Toxic Substances and Disease Registry 2007 Toxicological profile of arsenic.
705 Atlanta, GA: U.S. Department of Health and Human Services
- 706 29. Waalkes MP, Ward JM, Diwan BA 2004 Induction of tumors of the liver, lung, ovary and
707 adrenal in adult mice after brief maternal gestational exposure to inorganic arsenic:
708 promotional effects of postnatal phorbol ester exposure on hepatic and pulmonary, but
709 not dermal cancers. *Carcinogenesis* 25:133-141
- 710 30. Waalkes MP, Ward JM, Liu J, Diwan BA 2003 Transplacental carcinogenicity of inorganic
711 arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal
712 tumors in mice. *Toxicol Appl Pharmacol* 186:7-17
- 713 31. Devesa V, Adair BM, Liu J, Waalkes MP, Diwan BA, Styblo M, Thomas DJ 2006 Arsenicals in
714 maternal and fetal mouse tissues after gestational exposure to arsenite. *Toxicology*
715 224:147-155
- 716 32. Jin Y, Wang G, Zhao F, Liao Y, Sun D, Zhong Y, Yu X, Lv X, Li G, Sun G 2010 Distribution of
717 speciated arsenicals in mice exposed to arsenite at the early life. *Ecotoxicol Environ Saf*
718 73:1323-1326

- 719 33. Environmental Protection Agency 2012 Integrated Risk Information System: Arsenic,
720 inorganic. <http://www.epa.gov/iris/subst/0278.htm>
- 721 34. Joint FAO/WHO Expert Committee on Food Additives Seventy-second Meeting 2010 JECFA
- 722 35. Xue J, Zartarian V, Wang SW, Liu SV, Georgopoulos P 2010 Probabilistic Modeling of
723 Dietary Arsenic Exposure and Dose and Evaluation with 2003-2004 NHANES Data. Environ
724 Health Perspect 118:345-350
- 725 36. Fifty-fifth report of the joint FAO/WHO expert committee on food additives, Geneva, 6-15
726 June 2000. Evaluation of certain food additives and contaminants. 2001 Geneva: World
727 Health Organization
- 728 37. Agency for Toxic Substances and Disease Registry 1991 Toxicological profile of arsenic.
729 Atlanta, GA: U.S. Department of Health and Human Services
- 730 38. Agency for Toxic Substances and Disease Registry 1993 Toxicological profile for arsenic.
731 Atlanta, GA: U.S. Department of Health and Human Services
- 732 39. Schrauzer GN, White DA, McGinness JE, Schneider CJ, Bell LJ 1978 Arsenic and cancer:
733 effects of joint administration of arsenite and selenite on the genesis of mammary
734 adenocarcinoma in inbred female C3H/St mice. Bioinorg Chem 9:245-253
- 735 40. Waalkes MP, Liu J, Chen H, Xie Y, Achanzar WE, Zhou YS, Cheng ML, Diwan BA 2004
736 Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by
737 exposure to arsenic in utero. J Natl Cancer Inst 96:466-474

- 738 41. Styblo M, Delnomdedieu M, Thomas DJ 1996 Mono- and dimethylation of arsenic in rat
739 liver cytosol in vitro. *Chem Biol Interact* 99:147-164
- 740 42. Mann S, Droz PO, Vahter M 1996 A physiologically based pharmacokinetic model for
741 arsenic exposure. II. Validation and application in humans. *Toxicol Appl Pharmacol*
742 140:471-486
- 743 43. Mann S, Droz PO, Vahter M 1996 A physiologically based pharmacokinetic model for
744 arsenic exposure. I. Development in hamsters and rabbits. *Toxicol Appl Pharmacol* 137:8-
745 22
- 746 44. Hughes MF, Kenyon EM, Edwards BC, Mitchell CT, Thomas DJ 1999 Strain-dependent
747 disposition of inorganic arsenic in the mouse. *Toxicology* 137:95-108
- 748 45. Prater M, Shehata M, Watson CJ, Stingl J 2013 Enzymatic dissociation, flow cytometric
749 analysis, and culture of normal mouse mammary tissue. *Methods Mol Biol* 946:395-409
- 750 46. Banerjee I, Clayton P 2007 The genetic basis for the timing of human puberty. *J*
751 *Neuroendocrinol* 19:831-838
- 752 47. Gore AC, Wu TJ, Rosenberg JJ, Roberts JL 1996 Gonadotropin-releasing hormone and
753 NMDA receptor gene expression and colocalization change during puberty in female rats.
754 *J Neurosci* 16:5281-5289
- 755 48. Iwasa T, Matsuzaki T, Murakami M, Kinouchi R, Gereltsetseg G, Yamamoto S, Kuwahara A,
756 Yasui T, Irahara M 2011 Delayed puberty in prenatally glucocorticoid administered female

757 rats occurs independently of the hypothalamic Kiss1-Kiss1r-GnRH system. *Int J Dev*
758 *Neurosci* 29:183-188

759 49. Welm BE, Freeman KW, Chen M, Contreras A, Spencer DM, Rosen JM 2002 Inducible
760 dimerization of FGFR1: development of a mouse model to analyze progressive
761 transformation of the mammary gland. *J Cell Biol* 157:703-714

762 50. Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS 2005 A putative human
763 breast stem cell population is enriched for steroid receptor-positive cells. *Dev Biol*
764 277:443-456

765 51. Stingl J 2009 Detection and analysis of mammary gland stem cells. *J Pathol* 217:229-241

766 52. Ma I, Allan AL 2011 The role of human aldehyde dehydrogenase in normal and cancer
767 stem cells. *Stem Cell Rev* 7:292-306

768 53. Visvader JE 2009 Keeping abreast of the mammary epithelial hierarchy and breast
769 tumorigenesis. *Genes Dev* 23:2563-2577

770 54. Clarke RB 2005 Isolation and characterization of human mammary stem cells. *Cell Prolif*
771 38:375-386

772 55. Booth BW, Smith GH 2006 Estrogen receptor-alpha and progesterone receptor are
773 expressed in label-retaining mammary epithelial cells that divide asymmetrically and
774 retain their template DNA strands. *Breast Cancer Res* 8:R49

- 775 56. Stingl J 2011 Estrogen and progesterone in normal mammary gland development and in
776 cancer. *Horm Cancer* 2:85-90
- 777 57. Barthold JS, Kryger JV, Derusha AM, Duel BP, Jednak R, Skafar DF 1999 Effects of an
778 environmental endocrine disruptor on fetal development, estrogen receptor(alpha) and
779 epidermal growth factor receptor expression in the porcine male genital tract. *J Urol*
780 162:864-871
- 781 58. Lewis BC, Hudgins S, Lewis A, Schorr K, Sommer R, Peterson RE, Flaws JA, Furth PA 2001 In
782 utero and lactational treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin impairs
783 mammary gland differentiation but does not block the response to exogenous estrogen in
784 the postpubertal female rat. *Toxicol Sci* 62:46-53
- 785 59. Schonfelder G, Flick B, Mayr E, Talsness C, Paul M, Chahoud I 2002 In utero exposure to
786 low doses of bisphenol A lead to long-term deleterious effects in the vagina. *Neoplasia*
787 4:98-102
- 788 60. Ghosh MG, Thompson DA, Weigel RJ 2000 PDZK1 and GREB1 are estrogen-regulated
789 genes expressed in hormone-responsive breast cancer. *Cancer Res* 60:6367-6375
- 790 61. Kariagina A, Xie J, Leipprandt JR, Haslam SZ 2010 Amphiregulin mediates estrogen,
791 progesterone, and EGFR signaling in the normal rat mammary gland and in hormone-
792 dependent rat mammary cancers. *Horm Cancer* 1:229-244

- 793 62. Monje L, Varayoud J, Luque EH, Ramos JG 2007 Neonatal exposure to bisphenol A
794 modifies the abundance of estrogen receptor alpha transcripts with alternative 5'-
795 untranslated regions in the female rat preoptic area. J Endocrinol 194:201-212
- 796 63. Hirata S, Koh T, Yamada-Mouri N, Hoshi K, Kato J 1996 The untranslated first exon 'exon
797 0S' of the rat estrogen receptor (ER) gene. FEBS Lett 394:371-373
- 798 64. Hirata S, Koh T, Yamada-Mouri N, Kato J 1996 The novel untranslated first exon "exon 0N"
799 of the rat estrogen receptor gene. Biochem Biophys Res Commun 225:849-854
- 800 65. Osada N, Hirata S, Shoda T, Hoshi K 2001 The novel untranslated exon "exon 0T" encoded
801 between the exon 0 and exon 1 of the rat estrogen receptor alpha (ER alpha) gene. Endocr
802 J 48:465-472
- 803 66. Reilly MP, Saca JC, Hamilton A, Solano RF, Rivera JR, Whitehouse-Innis W, Parsons JG,
804 Dearth RK 2014 Prepubertal exposure to arsenic(III) suppresses circulating insulin-like
805 growth factor-1 (IGF-1) delaying sexual maturation in female rats. Reprod Toxicol 44:41-
806 49
- 807 67. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, Shioda T, Soto AM,
808 Vom Saal FS, Welshons WV, Zoeller RT, Myers JP 2012 Hormones and endocrine-
809 disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev
810 33:378-455
- 811 68. Fenton SE 2006 Endocrine-disrupting compounds and mammary gland development:
812 early exposure and later life consequences. Endocrinology 147:S18-S24

- 813 69. Bern HA 1992 The development of the role of hormones in development--a double
814 remembrance. *Endocrinology* 131:2037-2038
- 815 70. Soto AM, Brisken C, Schaeberle C, Sonnenschein C 2013 Does cancer start in the womb?
816 altered mammary gland development and predisposition to breast cancer due to in utero
817 exposure to endocrine disruptors. *J Mammary Gland Biol Neoplasia* 18:199-208
- 818 71. Kos M, Reid G, Denger S, Gannon F. 2001 Minireview: Genomic organization of the human
819 ERα gene promoter region. *Mol Endocrinol* 15:2057-2063
- 820 72. Weigel RJ, Crooks DL, Iglehart JD, deConinck EC 1995 Quantitative analysis of the
821 transcriptional start sites of estrogen receptor in breast cancer. *Cell Growth Differ* 6:707-
822 711
- 823 73. Tanimoto K, Eguchi H, Yoshida T, Hajiro-Nakanishi K, Hayashi S 1999 Regulation of
824 estrogen receptor alpha gene mediated by promoter B responsible for its enhanced
825 expression in human breast cancer. *Nucleic Acids Res* 27:903-909
- 826 74. Hayashi S, Imai K, Suga K, Kurihara T, Higashi Y, Nakachi K 1997 Two promoters in
827 expression of estrogen receptor messenger RNA in human breast cancer. *Carcinogenesis*
828 18:459-464
- 829 75. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitourngkol M, Puente M, Martin
830 MB 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Mol Endocrinol*
831 2:1157-1162