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Bisphenol A Induces Sox2 in ER+ Breast Cancer Stem-Like Cells

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Abstract

Bisphenol A (BPA) is an endocrine disrupting compound used in food and beverage plastic containers, and has been shown to increase breast cancer cellular proliferation. However, the concentrations of BPA used in these experiments is far higher than the physiological levels of BPA detected in the human body. We observed *in vitro* that exposure of MCF-7 cells to physiological concentrations of BPA failed to increase cell proliferation or to induce canonical estrogen-responsive genes (pS2 and progesterone receptor, PR), in contrast to 17 β -estradiol (E2) treatment. However, MCF-7 cells treated with 10 nM BPA induced *ALDH1* expression, a marker of human mammary stem cells. When treated with 10 nM BPA, mammospheres derived either from MCF-7 cells, a patient-derived xenograft or the normal mouse mammary gland, exhibited increased size; however, these effects were not observed in MDA-MB-231 mammospheres. Mechanistically, BPA induced *SOX2* mRNA and protein in MCF-7 mammospheres, resulting from enhanced CREB phosphorylation, and subsequent binding of pCREB to a *SOX2* downstream enhancer. These findings suggest that physiological levels of BPA increase ER⁺ breast cancer tumor maintenance through enhanced cancer stem-like cell activity *via* direct regulation of *SOX2* transcription.

Keywords

BPA; mammospheres; SOX2; endocrine disruptors; breast cancer

INTRODUCTION

Endocrine disruptors (EDCs) are exogenous chemicals that interfere with an aspect of hormone action (1). Bisphenol A (BPA) is an EDC that has been shown to have effects in

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multiple tissues at multiple times during development (2–4). BPA is present in food and drink packaging and other consumer products (4, 5), and as a result, BPA is present at a low dose (approximately 1 ng/mL, which is equivalent to 4.38 nM) in most human tissues and fluids, including blood, breast milk and urine (4, 6, 7). BPA has estrogenic activity and its ability to compete with estradiol for binding to the estrogen receptors (ERs) has been shown (8–10). Given the role of estrogens in human breast cancer etiology, endocrine-active chemicals that interfere with or modulate estrogen signaling are potential factors contributing to the high incidence of breast cancer.

Fetal exposure to BPA in mice and monkeys results in altered mammary gland development, such that mammary gland stroma and epithelium are transformed (11–14). In murine models, intraductal hyperplasia and ductal carcinoma *in situ* (DCIS) were observed after BPA exposure (14–20). Besides the effects of BPA on fetal mammary gland development, BPA also effects ER-positive (ER⁺) breast cancer cell lines, by increasing proliferation, inhibiting apoptosis, and inducing chemoresistance (21–29).

It has been demonstrated that cells with cancer stem-like activity, including self-renewal potential, sustain the growth of breast tumors (30–32) and that MCF-7 mammospheres are enriched with cancer stem cells (33, 34). The transcription factors OCT4, SOX2 and NANOG, which are master regulators of pluripotency in embryonic stem cells (35–37), are also present in breast stem cells and function to maintain an undifferentiated state and to prevent differentiation (37, 38). Of these factors, SOX2 is the only one detectable by immunohistochemical staining of breast tumors or mammospheres (39). The objective of this study was to compare the effects of BPA and estrogen treatment on SOX2 expression in MCF-7 breast cancer stem cells.

RESULTS

Exposure of MCF-7 cells to physiological levels of BPA does not induce cell proliferation or the expression canonical estrogen-responsive genes

It has been previously shown that treatment of MCF-7 cells with 1 μ M BPA increases cell proliferation in an ER-dependent mechanism by 96 hours (21). However, this magnitude of BPA exposure is approximately 1000-fold higher than the physiological levels of BPA present in most human tissues and fluids (6). Therefore, we tested whether exposure of cells to a range of BPA concentrations (1 nM to 10 μ M) would increase MCF-7 cell number, using 10 nM E2 as a control. Whereas exposure to E2 increased the cell number three-fold after 72 hours, and exposure to 1 μ M or 10 μ M BPA increase proliferation >2-fold, neither 1 nM nor 10 nM of BPA treatment increased proliferation as compared to vehicle (Figure 1A). Longer exposure of cells to BPA did not increase cell proliferation, since 10 nM of BPA also failed to increase proliferation after a 1-week treatment (Supplementary Figure 1). We next tested whether “low” levels of BPA treatment would be sufficient to induce expression of canonical estrogen-responsive genes. Whereas E2 treatment induced expression of pS2 (*TFF1*) and progesterone receptor (PR) mRNA, 10 nM BPA was not sufficient to induce expression above levels observed with vehicle alone (Figure 1B).

BPA increases phosphorylated CREB in MCF-7 cells

In addition to the classical E2 pathway, a growing body of evidence suggests that estrogens act through alternative pathways involving non-classical, membrane-associated estrogen receptors (mER) (40). Indeed, it has been demonstrated that CREB is phosphorylated and activated (phospho-CREB, pCREB) by low doses of E2 and BPA in pancreatic islet cells involving a non-classical mER (41). The expression of total CREB and pCREB was measured in MCF-7 cells by immunoblotting. Treatment with either 10 nM E2 or 10 nM BPA induced phosphorylation of CREB (Figure 1C). Stimulation of pCREB by E2 or BPA was not observed in human MDA-MB-231 triple negative breast cancer cells (Supplementary Figure 2).

BPA induces ALDH1 expression in MCF-7 cells and increases mammosphere size

Breast cancer stem cells were first identified as a CD24^{-low}/CD44⁺ population that have the ability to give rise to tumor growth when xenografted into immunocompromised mice (42). Subsequent studies identified several other markers of cells with stem-like features, including aldehyde dehydrogenase 1 (ALDH1). ALDH1⁺ cells are found in several breast cancer cell lines that have enhanced tumor initiation ability when xenografted into immunocompromised mice (43). MCF-7 cells treated with 10 nM BPA induced the expression of *ALDH1* mRNA (Figure 1D) (43) and ALDH1 protein expression (Figure 1E). In order to determine the effect of BPA treatment on MCF-7 breast cancer stem cell activity, MCF-7 cells were plated as single cells in stem cell culture conditions to form mammospheres. MCF-7 mammospheres are enriched in stem cells compared with adherent cells since mammospheres cells high express stem cell markers (NANOG SOX2 and OCT4) (Supplementary Figure 3A) and have self-renewal ability (Supplementary Figure 3B). Mammosphere cultures were treated on days 4 and 7 with either ethanol (EtOH, control), 10 nM E2 or 10 nM BPA during mammosphere growth. After ten days in culture, MCF-7 mammospheres treated with either E2 or 10 nM BPA were larger than mammospheres treated with vehicle (Figure 2A). Additional images are shown in Supplementary Figure 4. The largest mammospheres (150 μ m in diameter) are found in cultures treated with E2 or BPA, but not in control cultures (Figure 2B). The increased mammosphere size was observed for in cells isolated from an ER⁺ breast tumor patient-derived xenograft (PDX). PDX mammospheres treated with 10 nM BPA were larger than mammospheres exposed to EtOH (Figure 2C).

Not only does BPA increase the size of tumor-derived mammospheres, but it increases the size of normal mammary gland mammospheres. 10 nM BPA led to bigger size spheres than those treated with EtOH (Figure 2D). E2-treated mammospheres are intermediate of those treated with EtOH and BPA.

BPA induces SOX2 stem cell marker expression in MCF-7 mammospheres, but not in MDA-MB-231 mammospheres

SOX2, a transcription factor essential for maintaining self-renewal and pluripotency, is expressed in MCF-7 mammospheres and promotes sphere formation (39). Therefore, the effect of BPA treatment on *SOX2* expression in MCF-7 cancer stem cells was analyzed. MCF-7 mammospheres were treated with either 1 nM or 10 nM of E2 or BPA for 10 days.

Only 10 nM BPA was able to significantly induce *SOX2* mRNA and protein expression while 1 nM BPA and 1 or 10 nM E2 were not (Figure 3A–3B).

Human MDA-MB-231 triple negative breast cancer cells (44) also have the ability to be propagated *in vitro* as non-adherent mammospheres (33). Unlike MCF-7 mammospheres, MDA-MB-231 mammospheres treated with either 10 nM E2 or 10 nM BPA for ten days in culture were similar in size as control-treated mammospheres (EtOH) (Figure 4A). Furthermore, neither E2 nor BPA exposure increased *SOX2* expression in MDA-MB-231 mammospheres (Figure 4B).

BPA induces binding of pCREB to a downstream enhancer of *SOX2*, but does not induce expression of canonical estrogen-responsive genes in MCF-7 cells

PR and pS2 are classical E2-induced target genes, and are known to be responsive to E2 in MCF-7 cells. Whereas E2 treatment induced pS2 and PR expression in MCF-7 cell derived mammospheres, treatment with 10 nM BPA did not (Figure 5), similar to our observations in adherent MCF-7 cells. Whereas, E2 decreased ER α mRNA levels (Figure 5A), as expected based on the known ER α transcriptional activation cycle (45), BPA treatment had no effect on ER α mRNA levels (Figure 5B).

Based on these results, potential non-genomic ER-dependent regulation of *SOX2* expression was explored by measuring activation of CREB (pCREB). To determine whether BPA-activated pCREB directly regulates *SOX2*, ChIP was performed after a 5-minute treatment with either E2 or BPA. pCREB was enriched at an enhancer region located 143 kilobases downstream of the *SOX2* coding region only after BPA treatment (Figure 6), which correlates with gene expression.

Discussion

Epidemiological, clinical and experimental studies over more than a century confirm that estrogen is associated with the development of breast cancer. Increased exposure to estrogen, caused by early menarche, late menopause, high plasma estrogen levels and menopausal hormone replacement therapy, is associated with an increased risk of breast cancer (46).

BPA is considered a xenoestrogen since many studies have shown that it binds to, and/or activates, estrogen receptors (47, 48). *In vitro*, BPA can bind to the estrogen receptor, increase estrogen-regulated gene expression, and increase cell growth in estrogen-sensitive human breast cancer cell lines (21). However, in these studies the concentration of BPA used was up to a thousand times greater (i.e. 1 μ M) than the concentration of BPA measured in human serum (1–7 nM) (49, 50). These experiments show clear differences between BPA and E2 in breast cancer cells (ALDH expression, *SOX2* regulation, ER α target genes).

Extensive studies *in vitro* and *in vivo* have demonstrated that BPA has an effect in mammary gland tumorigenesis, since fetal exposure to BPA significantly increases pre-neoplastic lesions in rodents mammary glands, which are similar phenotypes to those observed in human breast carcinogenesis (18, 29, 51–53). Clinical observations have shown that higher circulating serum BPA levels are also correlated with increased mammographic breast

density (29, 54). Taken together, the results of these studies suggest that BPA is likely to have significant effects on mammary epithelium and carcinogenesis. Therefore, studies focused on proliferation of already transformed and immortalized breast cancer cells do not adequately address the potential for malignant transformation from a benign state.

Breast tumors harbor a subset of cancer stem cells that have the ability to initiate and maintain tumorigenesis, as well as resist conventional anti-cancer therapies (55, 56). Similar to normal breast stem cells, breast cancer stem cells possess the ability to generate highly proliferative cells, as well as more differentiated cancer cells representing several lineages that constitute the bulk of heterogeneous tumors (55). Also, breast cancer stem cells are able to maintain stem-like properties (tumor initiation, self-renewal and limited differentiation potential) while growing as spheres (mammospheres) in *in vitro* assays (33, 57). It has been previously shown that 10 μ M BPA, which increases MCF-7 cell proliferation, also increases MCF-7 mammosphere size (58). Our studies are unique in that we are able to demonstrate that 10 nM BPA (1000 times less) does not induce MCF-7 cell proliferation, but is sufficient to increase mammosphere size.

The transcription factors OCT4, SOX2 and NANOG are required to maintain the pluripotency and self-renewal in embryonic stem cells (35, 36). Moreover, these pluripotency transcription factors are present in breast stem cells and help to maintain an undifferentiated state (38). However, in breast cancer stem cells pluripotency transcription factors are overexpressed (39, 55, 56), which suggests the hypothesis that “aberrant expression of OCT4, SOX2 and NANOG causes continuous activation of self-renewal leading to oncogenic transformation and tumor initiation and maintenance” (37). For example, SOX2 is overexpressed in gliomas and has an essential role in the initiation, maintenance and recurrence of tumors (37, 59, 60). Similarly, SOX2 is overexpressed in breast carcinomas (37, 61), and in particular, in breast cancer stem cells relative to bulk tumor cells (39). In this study, we showed that SOX2 expression can be induced in MCF-7 mammospheres treated with 10 nM BPA. These data suggest that SOX2 is a suitable marker to monitor the effect of BPA exposure in cancer stem cell phenotypes. In addition, SOX2 presents a novel therapeutic target for breast tumors. Ideally, therapeutic strategies to SOX2 would preferentially target cancer stem cells and could augment current clinically used frontline therapies. There is sufficient pre-clinical evidence that silencing of SOX2 gene expression in breast cancer cell lines reduces cell proliferation and colony formation, and significantly reduces primary tumor growth (62).

Despite the fact that normal mammary stem cells do not express ER α , it has been demonstrated that they respond to estrogen signaling (63, 64). However, the mechanism of estrogen signaling in mammary stem cells remains unclear. MCF-7 mammospheres express ER α and estrogen regulates the ER-positive breast cancer stem/progenitor cells (65).

It has also been proposed that BPA is an androgen receptor (AR) ligand that triggers anti-androgenic responses, although these data are controversial (66). MDA-MB-231 mammospheres do not express ER α nor AR, and BPA has no effect on MDA-MB-231 mammosphere size or SOX2 expression. These data suggest that BPA may act *via* ER α or

AR, or an independent pathway, to increase MCF-7 mammospheres size to regulate the phosphorylation of CREB and SOX2 expression.

Classical E2-responsive genes are not up-regulated in MCF-7 mammospheres treated with 10 nM BPA. However, E2 and BPA are able to activate a functional non-classical membrane estrogen receptor (mER) in the endocrine pancreas (67, 68). Indeed, it is demonstrated that a low dose of BPA (1 nM) activates CREB (pCREB) in pancreatic islets cells involving a non-classical membrane estrogen receptor (41). We demonstrated that 10 nM of BPA induced pCREB levels in MCF-7, but not in MDA-MB-231, cells. In addition, we showed that pCREB was recruited to an enhancer of SOX2 to induce SOX2 expression. These results point to a non-classical mER, or ER-independent signaling pathway, to mediate BPA activity in E2-dependent MCF-7 cells.

Overall, we have shown that low, physiological levels of BPA increase ER+ breast cancer tumor maintenance through enhanced cancer stem cell activity *via* regulation of SOX2 expression, which is activated by pCREB *via* mER. Based on this evidence, we predict that anti-estrogen treatments that are effective in ER-positive control of breast cancer cell proliferation may not be effective in blocking BPA-dependent proliferation of cancer stem cells, which activates a non-classical membrane estrogen receptor pathway. A therapy targeting SOX2⁺ breast cancer stem cells, which are able to sustain tumor growth, metastasis and recurrence (69–71), could be a novel approach to eradicate ER⁺ breast cancers.

MATERIALS AND METHODS

Reagents and antibodies

17 β -estradiol (E2) and bisphenol A (BPA) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). SOX2 (clone D6D9), CREB (clone 48H2), pCREB (clone 87G3) and β -Actin (#4967) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). ALDH-1 antibody (clone 44/ALDH) was purchased from BD Biosciences (San Jose, CA). β -Tubulin antibody was purchased from Santa Cruz Biotechnology (clone H-235, Dallas, TX). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture and proliferation assays

MCF-7 and MDA-MB-231 cells were obtained from ATCC and grown according to standard protocols, and cell lines were verified each year by STR profiling. All *in vitro* assays were performed using DMEM media without phenol red (Corning, Corning, NY, USA) supplemented with 5% charcoal dextran-treated fetal bovine serum (CDT-FBS) (Omega Scientific, Tarzana, CA, USA). MCF-7 cells were plated at 50,000 cells/ml in quadruplicate on day 1. On days 2 and 3 the media was changed to media without phenol red and with 5% CDT-FBS. On day 5 the cells were treated with either vehicle (0.1% ethanol, EtOH), E2 (10 nM) or BPA (1–10 nM). For proliferation experiments on day 7 and 9 the media was refreshed, and on day 12 triplicate wells were counted by a hemocytometer. To analyze protein expression, the cells were harvested 15 minutes after treatment on day 5, and for RNA, the cells were harvested 24 hours after treatment on day 5.

Mammosphere Assays

From cell lines—To establish MCF-7 mammospheres, on day 1 cells were seeded onto ultralow attachment plates at 50,000 cells/ml and cultured in mammosphere medium (phenol-red free DMEM (Corning) supplemented with B27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL bFGF (BD Biosciences, San Jose, CA, USA), and 4 µg/mL heparin (Sigma-Aldrich Corporation)). To establish mammospheres with MDA-MB-231 cells, cells were plated at a density of 250,000 cells/ml. On days 4 and 7, the mammospheres were treated with vehicle control (0.1% ethanol), E2 (10 nM) or BPA (10 nM). Treatments were performed in triplicate. The entire well was imaged on day 10 of hormone treatments and the mammospheres were then collected for RNA or protein extraction. Sphere size was performed by counting spheres over 50 µm in size from images of each well.

To assess the self-renewal of the stem-like cells, mammospheres were dissociated in trypsin 1:3 (v/v) in mammosphere medium, and cells from first generation of mammospheres were seeded onto ultralow attachment plates and cultured seven days in the mammosphere media to form second generation mammospheres.

From tumor or mammary gland—Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center. Animals were maintained in a specific pathogen free environment at 20–26°C with a relative humidity of 30–70% and a 12 h light/dark cycle. Commercial rodent chow (LM-485, Teklad, Madison, WI) and drinking water were available *ad libitum*. The patient-derived xenograft (PDX) breast tumor line (HCI-7; ERα+, Caucasian) was generously provided by the laboratory of Dr. Alana Welm and the Huntsman Cancer Institute (HCI) Tissue Resource and Applications Core. This line was originally established from pleural effusion cells collected from a patient diagnosed with ER+ infiltrating ductal carcinoma and intraductal lobular carcinoma, as described in (72). A sub-line of HCI-7 cells were labeled with luciferase 2 (Luc2) as previously described (73).

To generate HCI-7-Luc2 mammospheres, PDX tumors were collected and mechanically dissociated and digested (4 hours, 37°C, 225 rpm) with 1.5 U/g collagenase A (Roche Diagnostics, Indianapolis, IN, USA) in 1.8 ml/g mammosphere medium. The resulting cell suspension was washed 3 times with 1X PBS, 5 mM EDTA (Sigma) and 1% BSA (Santa Cruz Santa Cruz, CA, USA) and then centrifuged (800g, 5 min). Red blood cells were then lysed with NH₄Cl-solution (Stem cell Technologies, Vancouver, Canada), and samples were sieved through a 40-µm mesh. A single-cell suspension was obtained by passing the sample repeatedly through a 25-gauge needle. Mammospheres were grown using the same culture conditions as MCF-7 cells. Normal mammospheres were prepared from the mammary glands of 8 week old FVB mice as described in (74). On days 4 and 7, the mammospheres were treated with either vehicle control (0.1% ethanol, EtOH), E2 (10 nM) or BPA (10 nM). Each experiment was performed at least three independent times and treatments were performed in triplicate replicates. The entire well was imaged on day 10 of hormone treatments and the mammospheres were then collected for RNA or protein extraction.

RNA and Quantitative PCR (qPCR)

Total cellular RNA was extracted from cells by using TRIzol Reagent (Invitrogen). Each RNA sample was collected in triplicate and each PCR reaction was amplified in triplicate. Total RNA was converted to cDNA with Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was subjected to quantitative PCR using the Thermo Scientific Maxima SYBR Green qPCR Master Mix with ROX (Thermo Fisher Scientific). Gene expression levels were compared after normalization to endogenous β -actin (*ACTB*). Primers were selected using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and the sequences used are (5' to 3'): *ACTB*: F, GGACTTCGAGCAAGAGATGG, R, AGCACTGTGTGGCGTACAG; *ALDH1*: F, GTTAGCTGATGCCGACTTGG, R, CCCACTCTCAATGAGGTCAAG; *ER α* : F, GAATCTGCCAAGGAGACTCGC, R, ACTGGTTGGTGGCTGGACAC; *NANOG*: F, CAAAGGCAAACAACCCACTT, R, TCTGCTGGAGGCTGAGGTAT; *OCT-4*: F, GGGCTCACCTGGGGGTCT, R, CTGCTGGGCGATGTGGCTGA; *PR*: F, AAATCATTGCCAGGTTTTTCG, R, TGCCACATGGTAAGGCATAA; *PS2*: F, TTGTGGTTTTCTGGTGTCA, R, CCGAGCTCTGGGACTAATCA; *SOX2*: F, GGGGAAAGTAGTTTGCTGCC, R, CGCCGCCGATGATTGTTATT. *SOX2* enhancer: F, AAGGATCCTGGAAAGCAGGC, R, GTTTCCTTGGAGGTGGGAGG; *ACTB* promoter: F, CAGTGCCTAGGTACCCACT, R, AGAAGTCGCAGGACCACACT.

Immunoblot Analysis

For pCREB analysis, cells were cultured in serum free media for 6 hours, followed by a 15 minute treatment with 10 nM E2 or BPA. Protein extracts were prepared by homogenizing cells on ice in RIPA buffer containing protease inhibitors. Protein concentrations were measured using the Bradford method. Samples (40 μ g/lane) were boiled at 95°C for 10 minutes in sodium dodecyl sulfate (SDS) loading buffer (Boston BioProducts, Ashland, MA, USA), separated on 8% SDS-PAGE gels and transferred to a polyvinylidenedifluoride membrane according to manufacturers' protocols (Bio-Rad Laboratories, Hercules, CA, USA). After incubation with 5% BSA in TBST for 60 min, the membrane was incubated with primary antibodies at 4°C overnight. Membranes were washed three times for 10 minutes and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for 1 hour at room temperature. Blots were washed with TBST three times and developed with an ECL system (Thermo Fisher Scientific) according to the manufacturer's protocols.

Chromatin immunoprecipitation

Cells were hormone deprived by culture for 3 days in phenol red-free medium (Invitrogen) supplemented with 5% CDT-FBS. Cells were treated with 10 nM E2, 10 nM BPA or ethanol as a vehicle control for 5 minutes, and ChIP was performed as described (75, 76). Immunoprecipitated DNA was amplified by qPCR. Each ChIP was performed with triplicate biological replicates. Percent of inputs were normalized to percent of inputs for *ACTB* promoter.

Statistical Analysis

All data are expressed as the mean plus or minus the standard error of the mean. All analyses were performed using Prism software, and statistically significant values were determined by Student's *t*-test ($p < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of effects of MCF-7 cell exposure to E2 versus physiological levels of BPA in adherent culture

(A) MCF-7 cells were treated for 72 hours with 10 nM E2, increasing concentrations of BPA or vehicle (0.1% ethanol, EtOH) and cells counted by hemocytometer (** $p=0.007$, * $p=0.003$). (B) Expression of classical ER target genes *pS2* and *PR* was compared in MCF-7 cells treated with E2, BPA or EtOH by quantitative RT-PCR; * $p<0.05$. (C) Immunoblotting for total CREB, pCREB and β -tubulin (loading control) in protein extracts prepared from MCF-7 cells treated with 10 nM E2, BPA or EtOH for 15 minutes at day 5 in culture. Expression of *ALDH1* was compared in MCF-7 cells treated with E2, BPA or EtOH by (D) quantitative RT-PCR; * $p<0.05$ and by (E) immunoblot analysis. ALDH1 mRNA or protein expression was normalized to β -actin mRNA or protein.

Figure 2. Estrogen and BPA increase the size of mammospheres derived from ER⁺ MCF-7 cells, the HCI-7-Luc2 PDX model or the normal mammary gland

(A) MCF-7 mammospheres treated with vehicle or 10 nM hormones for 10 days. (B) MCF-7 mammosphere size (diameter) was quantified (% of mammospheres per well greater than 50 μm). (C) HCI-7-Luc2 PDX mammospheres treated with vehicle or 10 nM hormones for 10 days. (D) Normal mammary epithelial cells were grown under mammosphere conditions in the presence of hormones for 10 days. Magnification bar, 400 μm.

Figure 3. BPA treatment increases SOX2 expression in MCF-7 mammospheres

(A) Expression of SOX2 was compared in MCF-7 mammospheres treated with E2, increasing concentrations of BPA or vehicle (EtOH) by qPCR; * $p < 0.05$, ** $p < 0.005$ (B) Immunoblot analysis of SOX2 protein expression was normalized to β -Actin protein.

Figure 4. BPA treatment does not increase MDA-MB-231 mammosphere size or SOX2 expression

(A) MDA-MB-231 mammospheres treated with 10 nM E2 or BPA have a similar size as vehicle control (EtOH)-treated mammospheres. Magnification bar, 400 μ m. (B) *SOX2* levels were compared in MDA-MB-231 mammospheres treated with E2, BPA or EtOH by qPCR.

Figure 5. BPA does not induce expression of canonical estrogen-responsive genes in MCF-7 mammospheres

Classical E2-responsive genes: **(A)** pS2 and PR, and **(B)** ERα were analyzed in MCF-7 mammospheres treated with 10 nM hormones for 10 days by qPCR. * $p < 0.05$

Figure 6. BPA induces pCREB recruitment to a SOX2 enhancer in MCF-7 cells
(A) An enhancer is located downstream of the *SOX2* coding sequence. **(B)** MCF-7 cells were treated for 5 minutes with 10 nM E2, 10 nM BPA or vehicle control. ChIP was performed using an antibody to pCREB and primers flanking the enhancer.