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Methylparaben stimulates tumor initiating cells in ER+ breast cancer models

Maria Angeles Lillo Osuna[1,6], **Cydney Nichols**[2], **Chanel Perry**[1,6], **Stephanie Runke**[3], **Raisa Krutilina**[4,6], **Tiffany N. Seagroves**[4,6], **Gustavo A. Miranda-Carboni**[5,6], and **Susan A. Krum**[*,1,6]

[1]Department of Orthopaedic Surgery and Biomedical Engineering, University of Tennessee Health Science Center, Memphis, TN

[2]Department of Microbiology, Immunology and Molecular Genetics, UCLA

[3]Department of Obstetrics and Gynecology, UCLA

[4]Department of Pathology, University of Tennessee Health Science Center, Memphis, TN

[5]Department of Medicine, University of Tennessee Health Science Center, Memphis, TN

[6]Center for Cancer Research, University of Tennessee Health Science Center, Memphis, TN

Abstract

A body of epidemiological evidence implicates exposure to endocrine disrupting chemicals (EDCs) with increased susceptibility to breast cancer. To evaluate the physiological effects of a suspected EDC *in vivo,* we exposed MCF-7 breast cancer cells and a patient-derived xenograft (PDX, estrogen receptor positive) to physiological levels of methylparaben (mePB), which is commonly used in personal care products as a preservative. mePB pellets (4.4 μg/day) led to increased tumor size of MCF-7 xenografts and ER^+ PDX tumors. mePB has been thought to be a xenoestrogen; however, *in vitro* exposure of 10 nM mePB failed to increase MCF-7 cell proliferation or induction of canonical estrogen-responsive genes (*pS2* and progesterone receptor), in contrast to 17β-estradiol (E2) treatment. MCF-7 and PDX-derived mammospheres exhibited increased size and up-regulation of canonical stem cell markers *ALDH1, NANOG, OCT4* and *SOX2* when exposed to mePB; these effects were not observed for MDA-MB-231 (ER^-) mammospheres. Since tumor initiating cells (TICs) are also believed to be responsible for chemoresistance, mammospheres were treated with either tamoxifen or the pure anti-estrogen fulvestrant in the presence of mePB. Blocking the estrogenic response was not sufficient to block *NANOG* expression in mammospheres, pointing to a non-classical estrogen response or an ER-independent mechanism of mePB promotion of mammosphere activity. Overall, these results suggest that mePB increases breast cancer tumor proliferation through enhanced TIC activity, in part *via* regulation of *NANOG,* and that mePB may play a direct role in chemoresistance by modulating stem cell activity.

[*]Correspondence: Susan A. Krum, University of Tennessee Health Science Center, 19. S. Manassas St., Memphis, TN 38163, Smirand5@uthsc.edu, Phone: 901-448-1136.

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Keywords

methylparaben; parabens; mammospheres; Nanog; endocrine disruptor; breast cancer

INTRODUCTION

Endocrine disruptors (EDCs) are exogenous chemicals, or mixture of chemicals, that interfere with an aspect of hormone action (Zoeller, et al. 2012). Esters of p-hydroxybenzoic acid (parabens), which are used as preservatives in thousands of consumer products (Andersen 2008; Loretz, et al. 2006; Yazar, et al. 2011), have come under recent scrutiny. Parabens from personal care products have the ability to be absorbed systemically from dermal application, are accumulated into underlying tissues and are measurable in blood after as little as one hour from dermal application (Janjua, et al. 2007). There is a positive correlation between the amount of one or more personal care products used and levels of parabens measured in blood (Sandanger, et al. 2011) or urine (Braun, et al. 2014). In 2004, parabens were detected in human breast tumor tissue (Darbre, et al. 2004), and it is thought that their ability to stimulate growth of human breast cancer cells is through their estrogenic properties (Byford, et al. 2002; Darbre, et al. 2003; Pugazhendhi, et al. 2005), although the amount of parabens needed *in vitro* to stimulate growth of human breast cancer cells was higher than physiological levels measured in breast tissue. However, higher concentrations of parabens in human breast tissues have recently been measured (Barr, et al. 2012). Furthermore, mixtures of different parabens together in lower concentrations can stimulate human breast cancer cell proliferation (Charles and Darbre 2013).

The estrogenic activity of parabens and their presence in human breast tissue and tumors, combined with the known influence of estrogen on the development of breast cancer and on the proliferation of some breast cancers (ER+), suggest that parabens might play a functional role in influencing breast cancer behavior. The objective of this study was to determine if methylparaben (mePB), which is one of the highest paraben found in breast (Barr et al. 2012), affects breast cancer tumor initiating cells *in vitro* and tumor proliferation *in vivo*.

MATERIALS AND METHODS

Reagents and antibodies

Hormone- or placebo-containing pellets were purchased from Innovative Research of America, Sarasota, FL, USA. E2 pellets contained 0.72 mg to be released over 90 days. Methylparaben (mePB) pellets contained released an equivalent molarity as E2 over 90 days (0.4 mg/90 days). 17β-estradiol (E2) and mePB were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). (Z)-4-Hydroxytamoxifen (Tam) and ICI 182,780 (fulvestrant) were purchased from Tocris Bioscience (Bristol, UK). NANOG (D73G4, #4903) and β-Actin (8H10D10, #12262) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from (Thermo Fisher Scientific, Waltham, MA, USA).

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Tumor Xenografts

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*.

Six-week old ovariectomized female nu/nu mice were purchased from Charles River Laboratories. Two weeks after ovariectomy, 90-day slow release hormone- or placebo-containing pellets (Innovative Research of America, Sarasota, FL) were implanted subcutaneously into the lateral side of the neck between the ear and shoulder with a trochar, while the mice were under isoflurane anesthesia. The pellets contained 0.72 mg of 17β-estradiol (E2) or 0.4 mg of methylparaben. The placebo pellets contain the identical slow-release matrix as the hormone-containing pellets (Innovative Research of America). Three days after pellet implantation 5×10^6 MCF-7 cells were mixed with 200 μl phenol-red free Matrigel (Corning) and injected subcutaneously into the hind flank of each mouse. Mice were monitored twice a week for tumor size with digital calipers and mice were euthanized when the tumor diameter reached 1.5 cm or 90 days after the injection. None of the mice showed signs of morbidity besides the tumor formation. For the placebo-treated mice n=13 mice, for the E2-treated mice n=16 mice, and for the mePB-treated mice n=13 mice.

The patient-derived xenograft (PDX) breast tumor line (HCI-7; ER+) 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 generated from pleural effusion cells from a human patient with an ER+ breast tumor and transplanted by serial passage into NOD/SCID immunocompromised female mice as described in (DeRose, et al. 2011). Upon receipt at UTHSC, cryopreserved tumor fragments were thawed, washed and re-implanted into NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ, (NSG, The Jackson Laboratory) female recipients by orthotopic implantation into the inguinal mammary fat. Tumors were then serially re-passaged into additional NSG female hosts. HCI-7 tumors were pooled, digested to prepare small organoids and then transduced in stem cell culture conditions with pEIL-puro (Addgene) using protocols in (DeRose, et al. 2013) to generate a Luciferase2(Luc2)-labeled sub-line of HCI-7 (HCI-7-Luc2).

Six week old female NSG recipient mice were purchased from The Jackson Laboratory. mePB- or placebo- containing pellets were implanted subcutaneously into recipients three days prior to the implantation of 2 mm^2 HCI-7 tumor fragments into the cleared mammary fat pad (mammary gland pair six). Primary tumor size was monitored twice a week using digital calipers. After 90 days new mePB containing pellets were implanted. Five mice for each treatment were injected bilaterally with tumor fragments and the largest tumor volume for each mouse was quantified.

Cell culture and proliferation assays

MCF-7 human breast cancer cells and MDA-MB-231 cells were obtained from ATCC and grown according to standard protocols. The cell lines were verified each year by STR

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profiling. All experiments were performed in DMEM media without phenol red (Corning, Corning, NY, USA) and 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 vehicle control (0.1% ethanol), E2 (10 nM) or mePB (10 nM). On day 7 and 9 the media was refreshed, and on day 12 triplicate wells were counted in a hemocytometer.

Mammosphere Assays

To establish mammospheres, 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-MD-231 cells, the concentration was 250,000 cells/ml. On day 4 and 7 the mammospheres were treated with vehicle control (0.1% ethanol), E2 (10 nM), mePB (10 nM), tamoxifen (1 μM) or fulvestrant (100 nM). Treatments were performed in triplicate. The entire well was imaged on day 10 of hormone treatments.

HCI-7-Luc2 PDX tumors are collected and were mechanically dissociated and digested (4 hours, 37°C, 225 rpm) with 1.5 U/gr collagenase A (Roche Diagnostics, Indianapolis, IN, USA) in 1.8 ml mammosphere media per gram of tissue. The resulting cell suspension was washed three times with 1X PBS, 5 mM EDTA (Sigma) and 1% BSA (Santa Cruz Santa Cruz, CA, USA) and centrifuged (800g, 5 min). Red blood cells were lysed with NH_4Cl-solution (Stemcell Technologies, Vancouver, Canada), and then 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 conditions as MCF-7 cells.

Normal mammospheres were obtained from the mammary glands of 8 week old FVB mice (Charles River) as described in (Wend, et al. 2013).

RNA and Quantitative PCR (qPCR)

Adherent cells were hormone-deprived by culture for three days in phenol red-free medium (Corning) supplemented with 5% CDT-FBS. These cells were treated with vehicle control (0.1% ethanol), E2 (10 nM) or mePB (10 nM) for 24 hours and RNA was isolated. Mammospheres were grown for 8 days and then treated with hormones for 24 hours. 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 Primer3 (Rozen and Skaletsky 2000) and the sequences are: *ACTB*: F, GGACTTCGAGCAAGAGATGG, R, AGCACTGTGTTGGCGTACAG; *ALDH1*: F,

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GTTAGCTGATGCCGACTTGG, R, CCCACTCTCAATGAGGTCAAG; *ERα*: F, GAATCTGCCAAGGAGACTCGC, R, ACTGGTTGGTGGCTGGACAC; *NANOG*: F, CAAAGGCAAACAACCCACTT, R, TCTGCTGGAGGCTGAGGTAT; *OCT-4*: F, GGGCTCACCCTGGGGGTTCT, R, CTGCTGGGCGATGTGGCTGA; *PR*: F, AAATCATTGCCAGGTTTTCG, R, TGCCACATGGTAAGGCATAA; *PS2*: F, TTGTGGTTTTCCTGGTGTCA, R, CCGAGCTCTGGGACTAATCA; *SOX2*: F, GGGGAAAGTAGTTTGCTGCC, R, CGCCGCCGATGATTGTTATT.

Immunohistochemistry

After 10 days mammospheres were collected and fixed in 75% cold ethanol. They were resuspended in HistoGel (Thermo Fisher Scientific) and processed according to the manufacturer's recommendations. MCF-7 tumors xenografts were formalin-fixed and paraffin-embedded. The slides were processed through standard deparaffinization protocols and the samples were then incubated in blocking buffer (5% normal goat serum, 2.5% BSA in PBS at pH 7.5) for 30 min. Primary antibodies were incubated overnight at 4°C in a humidified chamber followed by the DAKO Envision + visualization system and counterstaining with hematoxylin.

Immunoblot Analysis

Protein extracts were prepared by homogenizing cells on ice in RIPA buffer containing protease inhibitors. Protein concentrations were measured using the Bradford method. 40 μg of each sample was 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 using a transfer apparatus according to the manufacturer's protocols (Bio-Rad Laboratories, Hercules, CA, USA). After incubation with 5% BSA in TBST for 60 min, the membrane was incubated with antibodies (NANOG (1:2,000) or β-Actin (1:5,000)) at 4°C overnight. Membranes were washed three times for 10 min and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for one hour at room temperature. Blots were washed with TBST three times and developed with the ECL system (Thermo Fisher Scientific) according to the manufacturer's protocols.

Statistical Analysis

The data were expressed as the mean plus or minus the standard error. Analyses were performed using Prism software and statistically significant values were determined by two-tailed unpaired t-test to be $p < 0.05$. For tumors, a two-tailed Mann-Whitney test was performed with statistically significant values $p < 0.05$.

RESULTS

mePB induces human xenograft tumor formation in mice

Subcutaneous transplantation of MCF-7 cells to the hind flank of ovariectomized nude mice with slow-release 17β-estradiol (E2) pellets led to larger tumors compared to mice with placebo pellets. mePB also led to a statistically significant increase in tumor formation compared to placebo-treated mice (Fig. 1A). To test the effect of mePB on an ER+ breast

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tumor PDX model (HCI-7), fresh tumor fragments were transplanted into the mammary fat pad of NSG mice, which were exposed to either slow-release mePB or placebo pellets (Fig. 1B). After 17 weeks the tumors that developed in mice with mePB pellets were bigger than those developed in mice with placebo pellets.

mePB does not cause MCF-7 cells to proliferate, nor do they increase canonical estrogen-responsive genes

To determine the effects on proliferation at physiological levels, MCF-7 cells were exposed to 10 nM E2, which increased the cell number seven-fold after one week, but 10 nM mePB failed to increase the proliferation over vehicle alone (Fig. 2A). This concentration of mePB also failed to increase expression of pS2 (*TFF1*) or progesterone receptor (*PGR,* also known as PR) mRNA, which are canonical estrogen-responsive genes (Fig. 2B–C).

mePB induces ALDH1 and increases mammosphere size

MCF-7 cells treated with 10 nM mePB increased *ALDH1* expression (Fig. 2D), which is a marker of human mammary stem cells. Therefore, MCF-7 mammospheres were treated with EtOH (control), 10 nM E2 or 10 nM mePB. After ten days in culture, MCF-7 mammospheres treated with hormones were bigger than mammospheres not treated (EtOH), and the biggest mammosphere sizes were observed with 10 nM mePB (Fig. 3A).

The same size effect was observed in mammospheres grown from an ER+ breast tumor PDX treated with hormones (Fig. 3B). PDX mammospheres treated with 10 nM mePB were bigger than vehicle-treated (EtOH) mammospheres (Fig. 3B).

Not only do mePB increase the size of tumor-derived mammospheres, but they increase the size of normal mammary gland mammospheres. 10 nM mePB induces bigger size spheres than those treated with EtOH (Fig. 3C). E2-treated mammospheres are intermediate of those treated with EtOH and mePB.

mePB increases stem cell marker expression in mammospheres and tumors

While E2 induced classical E2-responsive genes in MCF-7 mammospheres (pS2 and PR), mePB did not (Fig. 4A), similar to what was observed in adherent MCF-7 cells. In addition, E2 decreased ERα mRNA levels (Fig. 4A). mePB had no effect on ERα mRNA levels. However, mePB increased *NANOG*, *OCT4* and *ALDH1* (Fig. 4B), which are known stem cell markers. Furthermore, mePB had the same effect in mammospheres from a PDX tumor: mePB increased NANOG, OCT4 and SOX2 expression in PDX mammospheres (Fig. 4C).

In MCF-7 mammospheres, NANOG protein expression was up-regulated by mePB (Fig. 5A) and mammospheres treated with mePB expressed more NANOG protein than mammospheres treated with EtOH (Fig. 5A). Also, in mePB-induced xenograft tumors there was a small percentage of cells, suggestive of tumor-initiating cells, expressing NANOG protein. The tumors that developed in mice with placebo pellets did not express NANOG protein, as demonstrated by immunohistochemistry (IHC) (Fig. 5B).

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mePB does not affect MDA-MB-231 mammosphere size

Unlike MCF-7 mammospheres, MDA-MB-231 mammospheres treated with 10 nM E2 or 10 nM mePB during ten days in culture were similar in size as control (EtOH)-treated mammospheres (Fig. 6A), and mePB did not increase *NANOG* expression in MDA-MB-231 mammospheres (Fig. 6B). While MDA-MB-231 cells do not express ERα, MCF-7 cells have a high expression of ERα. Moreover, MCF-7 mammospheres express ERα (Fig. 6C).

The ER antagonists tamoxifen and fulvestrant do not block the effects of mePB in MCF-7 mammospheres

Estrogens promote the growth of cancers that are estrogen receptor alpha-positive. Most types of hormone therapy for breast cancer either lower the levels of estrogens or stop estrogens from acting on breast cancer cells. Tamoxifen (Tam) is a first-line therapy used for all stages of ER-positive breast cancer and it binds to ERα in the same pocket as estrogens, blocking recruitment of coactivators. Tamoxifen blocks E2-mediated proliferation of MCF-7 cells (Supplemental Fig. S1). However, tamoxifen does not block the increased size in MCF-7 mammospheres generated by mePB, since MCF-7 mammospheres treated with tamoxifen and 10 nM mePB were bigger than mammospheres treated only with tamoxifen (Fig. 7A). Tamoxifen also does not block the increase in NANOG protein expression in MCF-7 mammospheres increased by mePB. MCF-7 mammospheres only treated with tamoxifen presented an increase in NANOG protein expression, and were bigger than MCF-7 mammospheres treated with 10 nM mePB (Fig. 7C).

As with tamoxifen, fulvestrant does not block the increased size of MCF-7 mammospheres by mePB (Fig. 7B), nor does it inhibit the increase in NANOG protein expression by mePB. Actually, MCF-7 mammospheres only treated with fulvestrant have an increase in NANOG protein that is greater than MCF-7 mammospheres treated with 10 nM mePB (Fig. 7C).

Discussion

Epidemiological, clinical and experimental studies over more than a century confirm that estrogens are associated with the development of breast cancer. This conclusion is based on the observations that several estrogen-related events such as early menarche, age at menopause, plasma estrogen levels, BMI, and menopausal hormone therapy are all associated with an increased risk of breast cancer (Simpson and Santen 2015). Besides endogenous estrogens synthesized by the body, synthetic compounds—endocrine disruptors that are able to mimic hormone action—can be detected in the body and in the mammary gland in particular (Sajiki, et al. 1999; Takeuchi and Tsutsumi 2002) (Sandanger et al. 2011). Parabens are considered to be EDCs and they are detected in the mammary gland (Darbre et al. 2004). However, epidemiological studies evaluating parabens and breast tumorigenesis are conflicting (Sasseville, et al. 2015) and parabens have demonstrated safety in classical toxicity tests (Society 2015).

In 1998, Routledge, *et al.*, showed that high concentrations of parabens possessed estrogenic activity when assessed *in vitro*: these esters could bind to rodent uterine estrogen receptor and increase estrogen-regulated gene expression in yeast cells (Routledge, et al. 1998). The estrogenic activity of parabens were also demonstrated by increased expression of an

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estrogen reporter gene in human cells, proliferation of ER+ breast cancer cells, and estrogen-regulated gene expression in ER+ breast cancer cells (Byford et al. 2002; Miyakoshi, et al. 2009; Pugazhendhi et al. 2005). Furthermore, parabens (methylparaben, n-propylparaben and n-butylparaben) have been shown to enable growth of MCF-10A cells in suspension (Khanna and Darbre 2013). However, in all of these studies the concentration of mePB used was a thousand times greater (i.e. 1 μM) than the concentration of mePB measured in human blood (60 nM) (Sandanger et al. 2011).

Previously, mePB has not been studied in mouse models of breast cancer. This is the first evidence demonstrating mePB leads to MCF-7 xenograft tumor formation. Furthermore, mePB increases the size of an ER+ breast patient-derived tumor xenograft (PDX). The results of these studies suggest that mePB is likely to have significant effects on breast cancer. Further studies should be done using similar assays to those done here to test other parabens that are commonly found in cosmetics and food.

Normal mammary stem cells from humans and mice are highly responsive to estrogen signaling, although they usually show a receptor negative phenotype for ERα and PR (Asselin-Labat, et al. 2006; Lim, et al. 2009). A paracrine signaling model was proposed to explain the effects of estrogen signaling in mammary stem/progenitor cells (Fillmore, et al. 2010; Mallepell, et al. 2006). However, MCF-7 mammospheres have ERα gene expression and E2 positively regulates ER-positive breast cancer stem/progenitor cells (Deng, et al. 2014). MDA-MB-231 mammospheres do not express ERα and mePB has no effect in MDA-MB-231 mammosphere size or stem cell markers gene expression. This suggests that mePB may act in MCF-7 mammospheres by ERα. However, in MCF-7 mammospheres treated with mePB, classical E2-responsive genes are not expressed. Moreover, neither tamoxifen nor fulvestrant not block the effects of mePB in MCF-7 mammospheres, suggesting a non-classical estrogen signaling response or an ER-independent effect of mePB. Expression array studies in MCF-7 cells showed only a 21% overlap between E2 and methylparaben (Pugazhendhi, et al. 2007), confirming a partial ER-independent effect of mePB.

Although tamoxifen lowers the risk of recurrence for several years, late recurrences remain a major clinical challenge. Among women treated with a recommended 5-year tamoxifen regimen, one-third of them experienced recurrent disease within 15 years (Early Breast Cancer Trialists' Collaborative 2005). Breast cancer-derived tumor initiating cells exhibit higher resistance to radiation and chemotherapy (Fillmore and Kuperwasser 2008; Li, et al. 2008; Phillips, et al. 2006), and greater tumorigenic potential (Cariati, et al. 2008; Grimshaw, et al. 2008). However, it has not been reported whether these cells are responsive to anti-hormonal treatment.

Tamoxifen and fulvestrant may attenuate MCF-7 mammosphere growth (Ao, et al. 2011). However, when treatments were stopped, the sphere formation frequency and tumorigenic potential of these cells remained unchanged and that mammospheres retained the same capability of sphere formation *in vitro* and tumorgenicity *in vivo*. Here, we observe that tamoxifen and fulvestrant increase the expression of *NANOG* protein in MCF-7 mammospheres, suggesting that anti-estrogen treatments that are effective in ER-positive

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breast cancer cells proliferation control may not be effective in targeting of cancer stem cells/tumor initiating cells, which are able to sustain tumor growth, metastasis and recurrence (Kakarala and Wicha 2008; Reya, et al. 2001; Visvader and Lindeman 2008).

Together, this data, for the first time, demonstrates that mePB affects breast cancer tumor-initiating cells, and directly regulates stem cell genes, including *NANOG*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Andersen. Final amended report on the safety assessment of Methylparaben, Ethylparaben, Propylparaben, Isopropylparaben, Butylparaben, Isobutylparaben, and Benzylparaben as used in cosmetic products. Int J Toxicol. 2008; 27(Suppl 4):1–82. DOI: 10.1080/10915810802548359

Ao A, Morrison BJ, Wang H, Lopez JA, Reynolds BA, Lu J. Response of estrogen receptor-positive breast cancer tumorspheres to antiestrogen treatments. PLoS One. 2011; 6:e18810.doi: 10.1371/journal.pone.0018810 [PubMed: 21533195]

Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ. Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst. 2006; 98:1011–1014. DOI: 10.1093/jnci/djj267 [PubMed: 16849684]

Barr L, Metaxas G, Harbach CA, Savoy LA, Darbre PD. Measurement of paraben concentrations in human breast tissue at serial locations across the breast from axilla to sternum. J Appl Toxicol. 2012; 32:219–232. DOI: 10.1002/jat.1786 [PubMed: 22237600]

Braun JM, Just AC, Williams PL, Smith KW, Calafat AM, Hauser R. Personal care product use and urinary phthalate metabolite and paraben concentrations during pregnancy among women from a fertility clinic. J Expo Sci Environ Epidemiol. 2014; 24:459–466. DOI: 10.1038/jes.2013.69 [PubMed: 24149971]

Byford JR, Shaw LE, Drew MG, Pope GS, Sauer MJ, Darbre PD. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol. 2002; 80:49–60. [PubMed: 11867263]

Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C, Purushotham AD. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. Int J Cancer. 2008; 122:298–304. DOI: 10.1002/ijc.23103 [PubMed: 17935134]

Charles AK, Darbre PD. Combinations of parabens at concentrations measured in human breast tissue can increase proliferation of MCF-7 human breast cancer cells. J Appl Toxicol. 2013; 33:390–398. DOI: 10.1002/jat.2850 [PubMed: 23364952]

Darbre PD, Aljarrah A, Miller WR, Coldham NG, Sauer MJ, Pope GS. Concentrations of parabens in human breast tumours. J Appl Toxicol. 2004; 24:5–13. DOI: 10.1002/jat.958 [PubMed: 14745841]

Darbre PD, Byford JR, Shaw LE, Hall S, Coldham NG, Pope GS, Sauer MJ. Oestrogenic activity of benzylparaben. J Appl Toxicol. 2003; 23:43–51. DOI: 10.1002/jat.886 [PubMed: 12518336]

Deng H, Zhang XT, Wang ML, Zheng HY, Liu LJ, Wang ZY. ER-alpha36-mediated rapid estrogen signaling positively regulates ER-positive breast cancer stem/progenitor cells. PLoS One. 2014; 9:e88034.doi: 10.1371/journal.pone.0088034 [PubMed: 24558373]

DeRose YS, Gligorich KM, Wang G, Georgelas A, Bowman P, Courdy SJ, Welm AL, Welm BE. Patient-derived models of human breast cancer: protocols for in vitro and in vivo applications in

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tumor biology and translational medicine. Curr Protoc Pharmacol. 2013; Chapter 14(Unit14): 23.doi: 10.1002/0471141755.ph1423s60

DeRose YS, Wang G, Lin YC, Bernard PS, Buys SS, Ebbert MT, Factor R, Matsen C, Milash BA, Nelson E, et al. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. Nat Med. 2011; 17:1514–1520. DOI: 10.1038/nm.2454 [PubMed: 22019887]

Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005; 365:1687–1717. DOI: 10.1016/S0140-6736(05)66544-0 [PubMed: 15894097]

Fillmore CM, Gupta PB, Rudnick JA, Caballero S, Keller PJ, Lander ES, Kuperwasser C. Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. Proc Natl Acad Sci U S A. 2010; 107:21737–21742. DOI: 10.1073/pnas.1007863107 [PubMed: 21098263]

Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res. 2008; 10:R25.doi: 10.1186/bcr1982 [PubMed: 18366788]

Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, Taylor-Papadimitriou J, Burchell JM. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res. 2008; 10:R52.doi: 10.1186/bcr2106 [PubMed: 18541018]

Janjua NR, Mortensen GK, Andersson AM, Kongshoj B, Skakkebaek NE, Wulf HC. Systemic uptake of diethyl phthalate, dibutyl phthalate, and butyl paraben following whole-body topical application and reproductive and thyroid hormone levels in humans. Environ Sci Technol. 2007; 41:5564–5570. [PubMed: 17822133]

Kakarala M, Wicha MS. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol. 2008; 26:2813–2820. DOI: 10.1200/JCO.2008.16.3931 [PubMed: 18539959]

Khanna S, Darbre PD. Parabens enable suspension growth of MCF-10A immortalized, non-transformed human breast epithelial cells. J Appl Toxicol. 2013; 33:378–382. DOI: 10.1002/jat.2753 [PubMed: 22744862]

Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst. 2008; 100:672–679. DOI: 10.1093/jnci/djn123 [PubMed: 18445819]

Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med. 2009; 15:907–913. DOI: 10.1038/nm.2000 [PubMed: 19648928]

Loretz L, Api AM, Barraj L, Burdick J, Davis de A, Dressler W, Gilberti E, Jarrett G, Mann S, Laurie Pan YH, et al. Exposure data for personal care products: hairspray, spray perfume, liquid foundation, shampoo, body wash, and solid antiperspirant. Food Chem Toxicol. 2006; 44:2008–2018. DOI: 10.1016/j.fct.2006.06.029 [PubMed: 16920244]

Mallepell S, Krust A, Chambon P, Brisken C. Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. Proc Natl Acad Sci U S A. 2006; 103:2196–2201. DOI: 10.1073/pnas.0510974103 [PubMed: 16452162]

Miyakoshi T, Miyajima K, Takekoshi S, Osamura RY. The influence of endocrine disrupting chemicals on the proliferation of ERalpha knockdown-human breast cancer cell line MCF-7; new attempts by RNAi technology. Acta Histochem Cytochem. 2009; 42:23–28. DOI: 10.1267/ahc.08036 [PubMed: 19492024]

Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst. 2006; 98:1777–1785. DOI: 10.1093/jnci/djj495 [PubMed: 17179479]

Pugazhendhi D, Pope GS, Darbre PD. Oestrogenic activity of p-hydroxybenzoic acid (common metabolite of paraben esters) and methylparaben in human breast cancer cell lines. J Appl Toxicol. 2005; 25:301–309. DOI: 10.1002/jat.1066 [PubMed: 16021681]

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Pugazhendhi D, Sadler AJ, Darbre PD. Comparison of the global gene expression profiles produced by methylparaben, n-butylparaben and 17beta-oestradiol in MCF7 human breast cancer cells. J Appl Toxicol. 2007; 27:67–77. DOI: 10.1002/jat.1200 [PubMed: 17121429]

Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001; 414:105–111. DOI: 10.1038/35102167 [PubMed: 11689955]

Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicol Appl Pharmacol. 1998; 153:12–19. DOI: 10.1006/taap.1998.8544 [PubMed: 9875295]

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000; 132:365–386. [PubMed: 10547847]

Sajiki J, Takahashi K, Yonekubo J. Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1999; 736:255–261. [PubMed: 10677006]

Sandanger TM, Huber S, Moe MK, Braathen T, Leknes H, Lund E. Plasma concentrations of parabens in postmenopausal women and self-reported use of personal care products: the NOWAC postgenome study. J Expo Sci Environ Epidemiol. 2011; 21:595–600. DOI: 10.1038/jes.2011.22 [PubMed: 21610754]

Sasseville D, Alfalah M, Lacroix JP. "Parabenoia" Debunked, or "Who's Afraid of Parabens?". Dermatitis. 2015; 26:254–259. DOI: 10.1097/DER.0000000000000147 [PubMed: 26551603]

Simpson E, Santen RJ. Celebrating 75 years of oestradiol. J Mol Endocrinol. 2015; 55:T1–T20. DOI: 10.1530/JME-15-0128 [PubMed: 26438567]

Society AC. Antiperspirants and breast cancer risk. 2015. Avaiable at: http:/http://www.who.int/ipcs/publications/jecfa/reports/trs940.pdf

Takeuchi T, Tsutsumi O. Serum bisphenol a concentrations showed gender differences, possibly linked to androgen levels. Biochem Biophys Res Commun. 2002; 291:76–78. DOI: 10.1006/bbrc.2002.6407 [PubMed: 11829464]

Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer. 2008; 8:755–768. DOI: 10.1038/nrc2499 [PubMed: 18784658]

Wend P, Runke S, Wend K, Anchondo B, Yesayan M, Jardon M, Hardie N, Loddenkemper C, Ulasov I, Lesniak MS, et al. WNT10B/-catenin signalling induces HMGA2 and proliferation in metastatic triple-negative breast cancer. Embo Molecular Medicine. 2013; 5:264–279. DOI: 10.1002/emmm.201201320 [PubMed: 23307470]

Yazar K, Johnsson S, Lind ML, Boman A, Liden C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. Contact Dermatitis. 2011; 64:265–272. DOI: 10.1111/j.1600-0536.2010.01828.x [PubMed: 21138445]

Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, Woodruff TJ, Vom Saal FS. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. Endocrinology. 2012; 153:4097–4110. DOI: 10.1210/en.2012-1422 [PubMed: 22733974]

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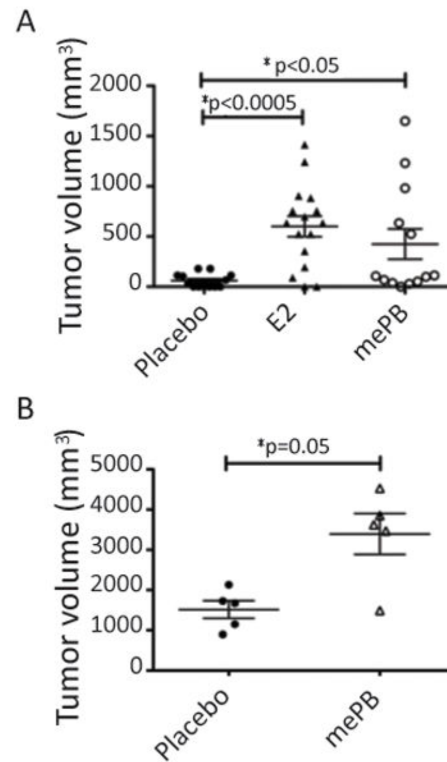

Figure 1.
mePB increases ER+ breast tumor size *in vivo*. A, Mice treated with mePB pellets develop bigger tumors than mice treated with placebo pellets when MCF-7 cells (ER+ human breast cancer) are xenografted into nude mice. B, HCI-7-Luc2 PDX tumor growth is increased when NSG mice are exposed to a mePB pellet relative to a placebo pellet.

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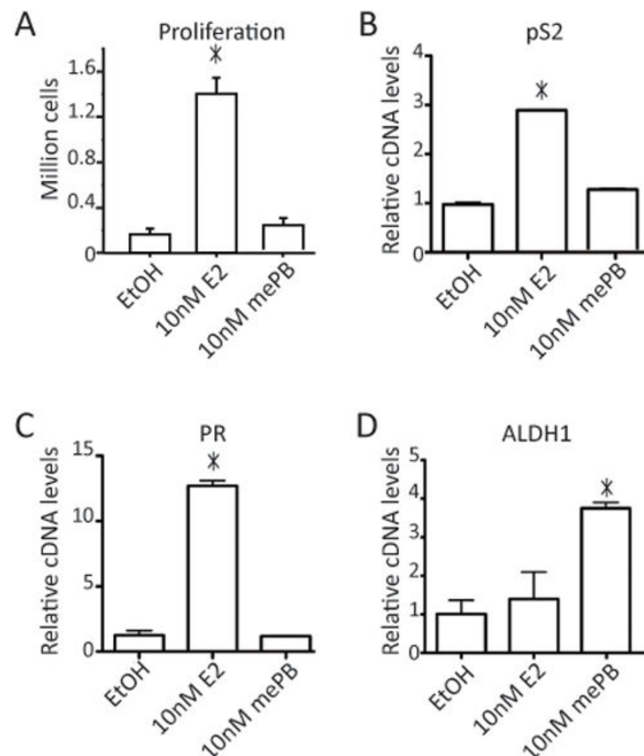

Figure 2.

Effect of hormone exposure in adherent MCF-7 cells. **A**, MCF-7 cells were treated for one week with 10 nM E2, mePB or control treatment (EtOH) and then counted. * = P<0.0001. Expression of classical ER target genes, **B** pS2, **C**, progesterone receptor (PR) and **D**, a marker of stem cell activity, ALDH1, were compared in MCF-7 cells treated with E2 or mePB by quantitative PCR; * = P<0.05.

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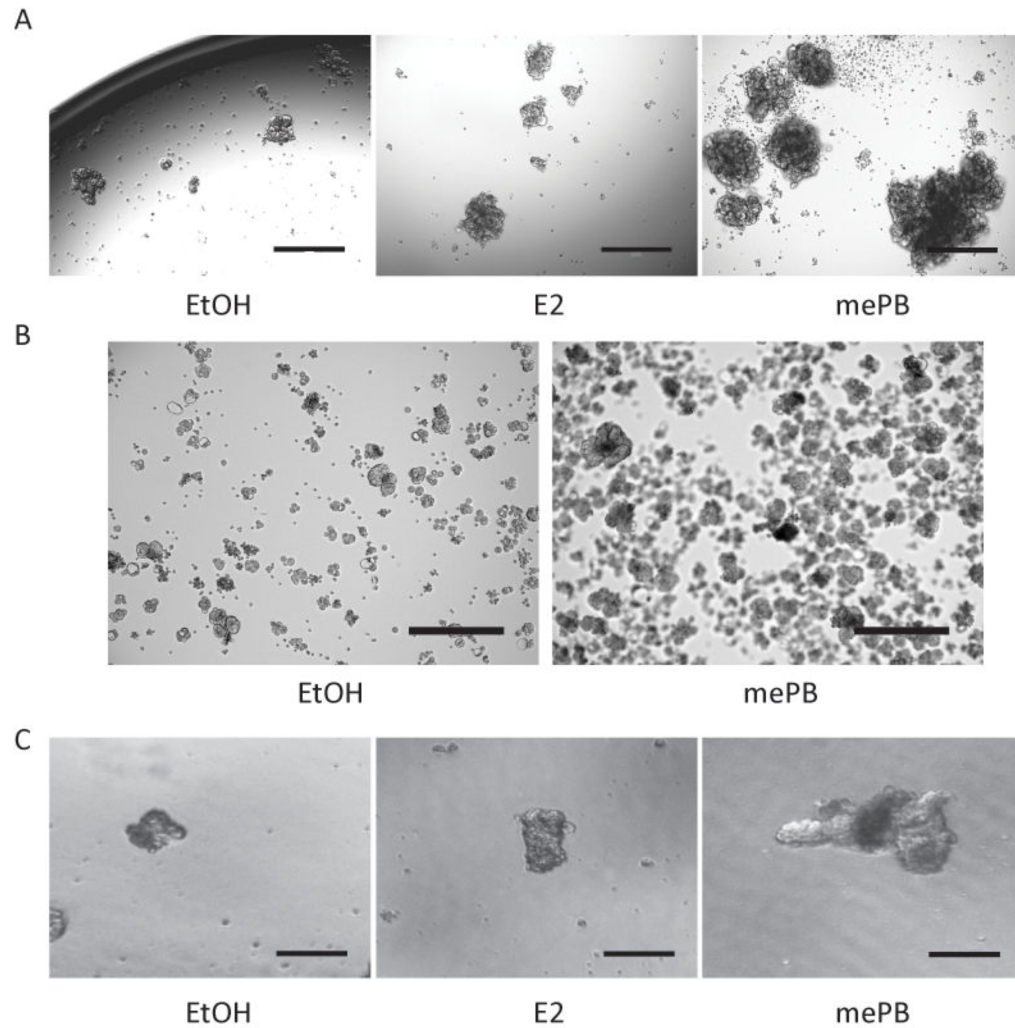

Figure 3.
E2 and mePB increase size of mammospheres derived from MCF-7 cells, ER+ HCI-7-Luc2 PDX breast cancer or the normal mammary gland. A, MCF-7 mammospheres were treated with 10 nM hormones for 10 days. B, HCI-7-Luc2 PDX mammospheres were treated with 10 nM hormones for 10 days. C, Normal mammary epithelial cells were grown under mammosphere conditions in the presence of hormones for 10 days. Magnification bar, 400 μm.

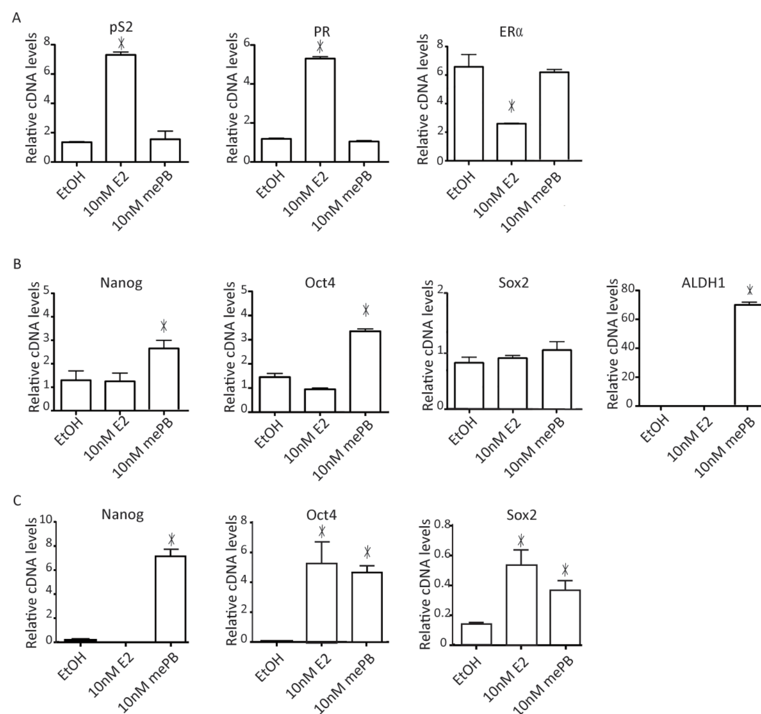

Figure 4.

mePB induces expression of stem cell markers in mammospheres. **A,** Classical E2-responsive genes and **B**, classical stem cells markers were analyzed in MCF-7 mammospheres. C, Classical stem cells markers were analyzed in PDX-derived mammospheres. Gene expression was analyzed by quantitative PCR in cDNAs isolated from mammospheres treated with each hormone. *, P<0.05.

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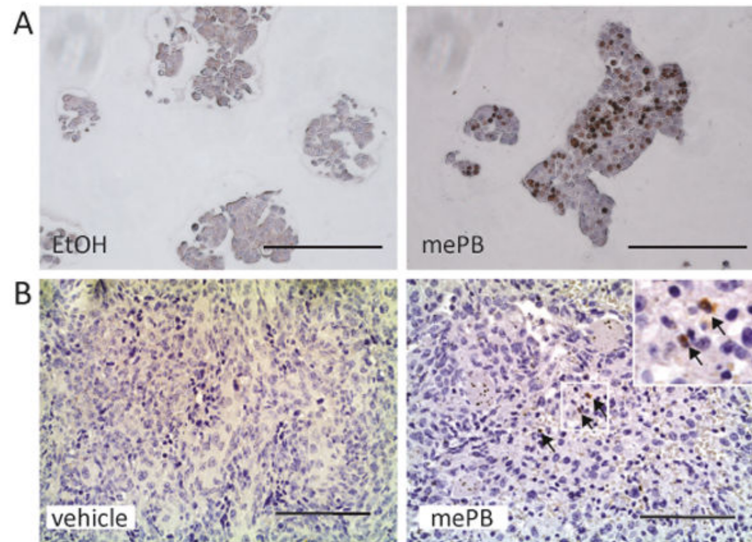

Figure 5.

mePB increases NANOG expression in mammospheres and tumor xenografts derived from MCF-7 cells. A, Immunohistochemistry of NANOG protein in mammospheres treated with EtOH (control) or 10 nM mePB for 10 days and B, MCF-7 tumor xenografts developed in mice with placebo pellet (control) or mePB pellet. Magnification bar, 200μm.

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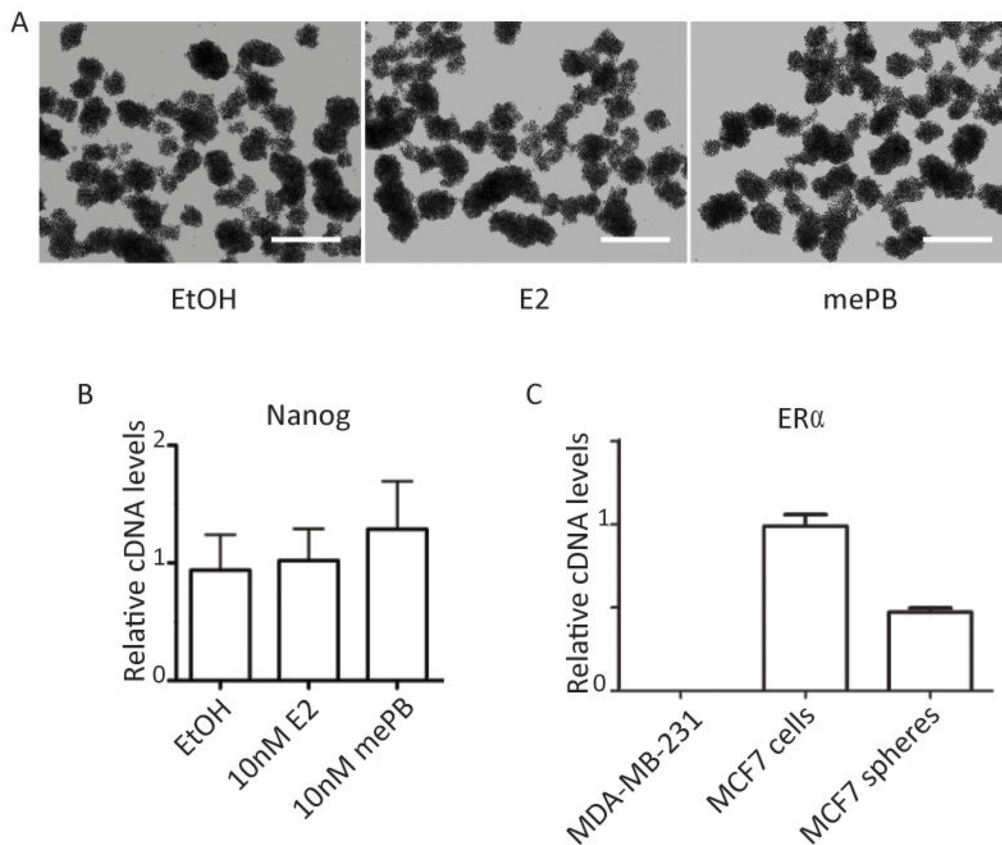

Figure 6.

mePB does not increase MDA-MB-231 mammosphere size. A, MDA-MB-231 mammospheres treated with 10 nM E2 or mePB have similar size as vehicle control treated mammospheres. Magnification bar, 400 μm. B, Nanog levels were analyzed by qPCR using cDNAs isolated from MDA-MB-231 mammospheres treated with E2 or mePB. C, ERα levels were compared by quantitative RT-PCR using cDNAs isolated from MDA-MB-231 cells, adherent MCF-7 cells and MCF-7 mammospheres harvested at day 10 in culture. *, $P<0.002$.

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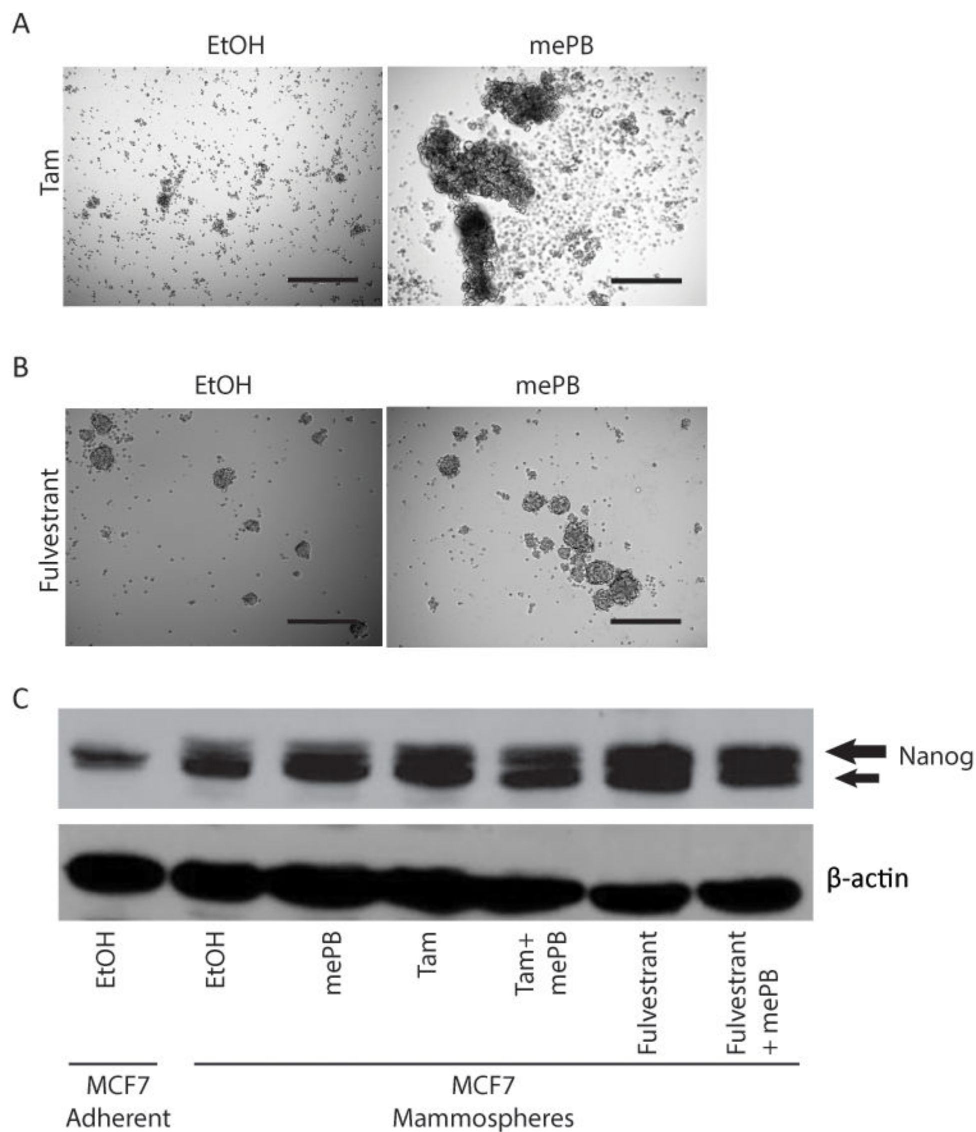

Figure 7.

ER antagonists increase MCF-7 mammosphere size. **A**, Mammospheres treated with 1 μM Tam and 10 nM mePB are bigger than mammospheres treated with 1 μM Tam. B, Mammospheres treated with 100 nM fulvestrant and 10 nM mePB are bigger than mammospheres treated with 100 nM fulvestrant. **C,** Immunoblot analysis of NANOG protein expression in lysates harvested from adherent MCF-7 cells and MCF-7 mammospheres treated with 10 nM mePB, 1 μM Tam and/or 100 nM fulvestrant as indicated. Large arrow indicates NANOG protein.