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Pubertal Bisphenol A Exposure Alters Murine Mammary Stem Cell (MaSC) Function Leading to Early Neoplasia in Regenerated Glands

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Abstract

Perinatal exposure to bisphenol A (BPA) has been shown to cause aberrant mammary gland morphogenesis and mammary neoplastic transformation. Yet, the underlying mechanism is poorly understood. We tested the hypothesis that mammary glands exposed to BPA during a susceptible window may lead to its susceptibility to tumorigenesis through a stem-cell mediated mechanism. We exposed 21-day-old Balb/c mice to BPA by gavage (25 µg/kg/day) during puberty for 3 weeks, and a subset of animals were further challenged with one oral dose (30 mg/kg) of 7,12-dimethylbenz[a]anthracene (DMBA) at 2 months of age. Primary mammary cells were isolated at 6 weeks, and 2 and 4 months of age for MaSC quantification and function analysis. Pubertal exposure to the low-dose BPA increased lateral branches and hyperplasia in adult mammary glands and caused an acute increase of MaSC in 6-week-old glands and a delayed increase of luminal progenitors in 4-month-old adult gland. Most importantly, pubertal BPA exposure altered the function of MaSC from different age groups, causing early neoplastic lesions in their regenerated glands similar to those induced by DMBA exposure, which indicates that MaSCs are susceptible to BPA-induced transformation. Deep sequencing analysis on MaSC-enriched mammospheres identified a set of aberrantly expressed genes associated with early neoplastic lesions in human breast cancer patients. Thus, our study for the first time shows that pubertal BPA exposure altered MaSC gene expression and function such that they induced early neoplastic transformation.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Keywords

Bisphenol A; Breast Cancer; Mammary stem cell; Early neoplastic lesion; Risk assessment; RNAseq; Mammosphere

INTRODUCTION

Bisphenol A (BPA) is a xenoestrogen that is ubiquitous in the environment and human body (1). The structural similarity between BPA and the more potent synthetic estrogen diethylstilbestrol has prompted researchers to suspect that BPA may promote carcinogenesis in humans (2, 3). Indeed, BPA has been implicated to be one of the significant environmental risk factors for increasing breast cancer incidence in the past decade. Perinatal exposure to low, environmentally relevant doses of BPA in mice or rats resulted in increased terminal ducts and terminal end buds (TEBs) (4); accelerated puberty onset and prolonged estrous cycle (5); increased numbers of lateral branches and sensitivity of the developing mammary gland to endogenous estrogen (6); increased adult mammary gland progesterone response and epithelial cell number (7); induction of the preneoplastic ductal hyperplasia and carcinoma in situ (8, 9), as well as increased susceptibility to tumorigenesis (10, 11). Recently, BPA-induced adverse effects on mammary glands have also been recapitulated in a more human relevant animal model -the rhesus monkey (12). In addition, low dose BPA exposure on the early differentiation stage of mammary epithelial cells has been associated with possible cancer risk in the in vitro model of human embryonic stem cell (13). Together, these findings indicate that exposure to BPA during fetal life may contribute to the increased incidence of breast cancer in adulthood (14).

Despite the prevailing evidence supporting BPA as a risk factor for mammary tumors, the cellular mechanisms underlying these BPA-induced morphogenetic changes and susceptibility to tumorigenesis is poorly understood. The recently identified murine mammary stem cells (MaSCs) are found to be present in fetal and adult mammary glands, and have the potential to drive gland development during puberty, growth and remodeling during pregnancy (15-17). Modulation of oncogenes and tumor suppressors has been shown to increase MaSC self-renewal (18-20) suggesting that alteration of MaSC frequency and function may lead to transformation and tumorigenesis. In fact, gene expression profiles of different types of breast cancer have been shown to correspond to gene expression profiles of MaSCs or luminal progenitors (LPs) (19, 21, 22). Therefore, MaSCs and LPs could be the targets for BPA-induced tumorigenesis.

Currently, little is known about whether and how xenoestrogens may alter the number and function of MaSCs during mammary gland development and maturation. Although BPA has been reported to act in other developmental windows such as prepubertal (10), early adulthood (23), or adult stage of mice (24), most studies have focused on the perinatal exposure period. It is known that the development of mammary epithelial ducts primarily occurs during puberty and is likely to be accompanied with extensive MaSC proliferation, yet it remains unknown whether BPA exposure at puberty would also cause similar adverse effects. In the present study, we used Balb/c mice to evaluate the effect of low dose BPA exposure at puberty on mammary gland development and transformation potential, and whether any adverse effects observed are related to changes of the number and/or function of MaSCs. Our findings revealed that pubertal BPA exposure essentially recapitulated the phenotypic changes resulted from perinatal BPA exposure and these morphogenetic changes have a stem cell origin.

Materials and Methods

Mice and BPA exposure

Animal care and use were conducted according to established guidelines approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. Female Balb/c mice (Charles River, Raleigh, NC) were housed in polypropylene cages with glass water bottles (both polycarbonate/BPA free), and fed with low phytoestrogen (< 20 mg/kg) Harlan Teklad 2016 diet (Harlan Teklad Global Diets, Wilmington, DE). Beginning at the age of 21 days (week 3) and continuing through week 6, these pups were intragastrically gavaged daily with sesame oil (vehicle control) or BPA at 25 µg/kg/day. Beyond puberty, all mice were continued on low-phytoestrogen diet until the end of the experiment. At 2 months of age, a subgroup of vehicle control and pubertal BPA-treated mice were given a single oral gavage of 7,12-dimethylbenz[a]anthracene (DMBA) at 30 mg/kg. Mammary glands were harvested at 6 weeks (immediately after BPA treatment), 2 months, and 4 months of age for analysis (Fig. 1A).

BPA dose

We used BPA exposure at a low dose of 25 µg/kg/day in this study as BPA exhibits nonmonotonic dose response, and only low doses of BPA have been shown to be associated with carcinogenesis (23, 25). Based on previous pharmacokinetic data in CD-1 mice and difference in BPA metabolism between infant and adult rodents (26), our dose of 25 µg/kg/day by oral gavage is predicted to lead to an average serum concentration of unconjugated (bioactive) BPA of about 0.175 ng/mL in young mice at pubertal age. This value is comparable to levels of unconjugated BPA measured in human serum (in the ng/ml range) (27).

Mammary cell preparation

The detailed protocol was described in our previous publication (28). In brief, dissected inguinal and thoracic mammary glands were digested in dissociation medium and treated sequentially with dispase and DNase I. Cell suspension was filtered through a 40-micron mesh before being labeled with antibodies (see below).

Antibodies

Antibodies included anti-CD24-PE, anti-CD49f-FITC, biotinylated anti-CD31/CD45/Ter119 cocktail (StemCell Technologies), anti-CD16/CD32 (BD Pharmingen). APC-conjugated streptavidin (Invitrogen) was used to visualize the biotinylated antibody cocktail.

Cell labeling and flow cytometry sorting

MaSCs were enriched and isolated from endothelial (CD31) and hematopoietic (CD45 and TER119) lineage-depleted (Lin⁻) mammary epithelial cells using cell surface markers of CD24 and CD49f (16). In detail, cells were first incubated with anti-CD16/CD32 (Fcγ III/II receptor) for 10 min on ice to reduce Fc receptor-mediated binding, followed by a 15-min incubation on ice with the biotinylated CD31/CD45/Ter119 antibody cocktail. After washing, cells were incubated with anti-CD24-PE, anti-CD49f-FITC, and streptavidin-APC on ice for 10 min. After one more wash, cells were sorted (FACSaria IIIu) according to the gates illustrated in Fig. 1A. The efficient isolation between luminal and basal cells using CD24/CD49f were confirmed with high percentage of hollow or solid structures formed respectively (Fig. 1B) in the SFD assay (see below).

Sphere formation and differentiation (SFD) assay

We recently developed this SFD assay as an in vitro alternative to in vivo repopulation assay for MaSC identification (29). In brief, sorted cells were cultured in ultralow attachment 96-well plates (Corning) with mouse EpiCult-B medium (150 μ L per well) supplemented with 2% B27 (Invitrogen), 20 ng/mL bFGF, 20 ng/mL EGF, 10 μ g/mL heparin, 10 μ g/mL insulin, 1 μ g/mL hydrocortisone, and 50 μ g/mL gentamicin (referred to as mammosphere or MMS medium). After 7-days of suspension culture, mammospheres were counted and harvested by centrifugation at 400 $\times$ g. A total of 30 to 50 individual spheres were resuspended in 60 μ L Matrigel (BD Biosciences) for sphere differentiation. The sphere-Matrigel drop was allowed to solidify inside a 37°C incubator for 15 min, covered with MMS medium supplemented with 5% FBS, and incubated at 37°C for 9 days. The number of solid and hollow 3D structures was counted in at least 3 wells (approximately 120 spheres).

Stem/progenitor cell quantification

The MaSC frequency was calculated based on our SFD assay (29). In brief, MaSC-enriched basal cell derived mammospheres that can be further differentiated into solid structures upon 3D culture in Matrigel were found to originate from single MaSC. Thus, MaSC frequency within the epithelial cell population (expressed as %MaSC_e) can be derived by the following formula. In our previous study, we also found that mammospheres derived from LP-enriched luminal cells can form hollow structures upon 3D culture, and these types of structures were considered representative of LPs by others (15, 16, 19). Therefore, we enumerate LP frequency (%LP_e) in a similar way to that of %MaSC_e.

1. Sphere formation efficiency (SFE) = No. of spheres per 1,000 cells
2. %MaSC_e = (SFE/1000 $\times$ %3D solid) $\times$ (%Basal cell/%Total epithelial cell) $\times$ 100
3. %LP_e = (SFE/1000 $\times$ %3D hollow) $\times$ (%Luminal cell/%Total epithelial cell) $\times$ 100

The %(3D solid) or %(3D hollow) was defined as the percentage of spheres that formed a solid or hollow structure in Matrigel culture of the total number of spheres plated, respectively. The %basal or %luminal cell was the percentage of cells gated as Lin⁻CD24⁺CD49^{hi} (basal) or Lin⁻CD24^{hi}CD49^{low} (luminal), and the %total epithelial cells was the sum of percentage cells gated as basal and luminal.

Mammary colony forming cell (Ma-CFC) assay

This Ma-CFC assay was used to estimate the colony forming cell (= progenitor cell) within the mammary cells (30). Specifically, one thousand sorted cells were plated into each well of the 6-well plates containing MMS medium supplemented with 5% FBS in the presence of 10,000 cells/cm² irradiated NIH-3T3 cells. After 24 h the media was replaced with serum-free MMS medium, and 8 days later the colonies were fixed with 100% cold methanol for 1 min, stained with 10% Giemsa for 30 min, and counted.

Cleared fat pad transplant and analysis

Stem cells in single 3D solid structures were injected into the inguinal glands of 21-day-old virgin female mice cleared of endogenous epithelium as we previously reported (29). An outgrowth, defined as an epithelial structure with both ductal and lobular structures, was evaluated after 8-10 weeks by whole mount staining. For the 6-week and 2-month time points, the Balb/c mice were used as recipients. For the 4-month time point, we used nude mice as recipients to create a more permissive environment for potential formation of solid tumors by MaSCs from BPA- and/or DMBA-treated mice. To ensure that the cleared fat pads were completely free of endogenous MaSCs, we first did whole mount staining on the

fragments of the removed fat pads to see whether a rudimentary epithelial structure (Fig. S1) was present, which would indicate the removal of the endogenous gland. We also checked the unique ductal growth pattern of the transplants after they were harvested. The regenerated glands from endogenous uncleared mammary ducts usually display unidirectional growth similar to primary glands. In contrast, regenerated glands from donor stem cells usually give rise to bi-directional ductal growth (31). These quality control procedures were instituted for each experiment after we had confirmed constant success in our technique of clearing endogenous MaSCs using various fluorescence protein-labeled MaSCs in the *in vivo* repopulation assay as previously reported (29).

Estrous cycle

To control the possible effect of diestrus phase on MaSC frequency, all mice were subjected to vaginal smear for estrous cycle analysis immediately prior to euthanasia. Vaginal smears were collected by flushing the vagina gently with 30 μ L PBS, air dried, fixed with methanol, and stained with Giemsa (1:20 V/V). Estrus cycles were then staged with a Nikon microscope (200 x) based on the method described previously (Nelson et al., 1982).

Whole mount and H&E staining

For primary glands, the pair of thoracic glands from each mouse was used for whole mount Carmine Alum staining and Hematoxylin and eosin (H&E) staining. The remaining mammary glands were used for mammary cell isolation. For regenerated glands, whole mount staining was used to identify and score positive outgrowths. All positive outgrowths were subject to H&E staining for histological analysis. Ductal branching morphogenesis of primary glands was assessed by branching point, calculated based on whole mount images as described previously (6). In brief, for any given gland, 10 branches were randomly selected, and for each branch, the number of branching points was counted, and the branch length (from the first branching point to the last branching point, mm) was measured, and then the number of branching point per mm of branch length was calculated for each branch. The level of branching for each gland was expressed by the mean value of 10 branches. Pre-neoplastic transformation was indicated by different degrees of ductal hyperplasia, which was assessed based on H&E images by a breast cancer pathologist. In brief, normal ductal structure was characterized by an outside myoepithelial cell layer and an inside luminal epithelial cell layer (Fig. 1C). Hyperplastic lesions with only a few extra layers of epithelium present were considered mild (Fig. 1D), and those with dilated ducts completely filled with uniform cells were considered severe and termed as atypical ductal hyperplasia (ADH; Fig. 1E), or as ductal carcinoma in situ (DCIS; Fig. 1F) if cytologic atypia (numerous mitotic figures, nuclear enlargement, and coarse chromatin pattern) was present along with necrosis. The frequency of hyperplasia was calculated by dividing the number of hyperplastic ducts by the total number of ducts $\times$ 100. A minimal of 20 ductal structures was counted for each gland.

mRNA-sequencing sample preparation and analysis

Mammospheres formed from MaSC enriched basal cells of animals sacrificed at 4-months of age were subjected to RNAseq analysis. Total RNA was isolated using miRNeasy Mini kit (Qiagen, cat #217004). Sequencing libraries were constructed using the Illumina TruSeq RNA sample preparation protocol (Illumina, Catalog no. RS-122-2002). The resulting libraries were sequenced on an Illumina HiSeqTM 2000 using a standard single-end 50 bp sequencing protocol. The reads were aligned to the reference mouse genome (UCSC build MM9) with TopHat (32). No more than 2 mismatches were allowed in the alignment. HTseq (33) was used to count gene expression (reads), and DEseq (34) was used to find differentially expressed genes after performing median normalization. Differential expressed

genes were identified with adjusted P -value < 0.05 for multiple tests by Benjamini-Hochberg method for controlling false discovery rate (35) and sequence read abundance per gene above 40% within each sample. Heatmap of gene expression levels were generated with R/heatmap2 (36). Hierarchical clustering was performed on differentially expressed genes and 8 clusters were identified by R/cutree function. To carry out functional analysis, clusters with fewer genes or similar expression levels were combined first, and individual clusters were then analyzed further for functional enrichment of gene ontology (GO) with Database for Annotation, Visualization and Integrated Discovery (DAVID) (37) to determine common and unique functions and processes. DAVID's enrichment clusters with higher enrichment scores (> 2) were examined and representative functions were selected to note on the bi-hierarchical clustering heatmap. The differentially expressed genes were further uploaded into Ingenuity Pathways Analysis software (IPA, Ingenuity® Systems) for identification of potentially important biological mechanisms and pathways perturbed by BPA exposure.

Data analysis

Initial data analysis with two-way ANOVA indicated that time and DMBA had significant effects on some parameters we measured. To control these two factors, student's t -test was used to compare differences between control and BPA treated groups. Results were presented as means $\pm$ SEM and probability values of $P < 0.05$ were considered to be significant unless specified otherwise.

Results

BPA increases lateral branching and hyperplasia in primary adult mammary glands

Pubertal BPA exposure recapitulated the phenotypic changes of increased lateral branching and hyperplastic lesions in 4-month old adult glands (Supplementary Fig. S2) as those from the in utero BPA exposure studies (6, 8). We did not observe significant changes in glands harvested at 6 weeks or 2 months (data not shown). When we challenged the BPA-treated mice with one single oral dose of 30 mg/kg DMBA at 2-months of age, the number of lateral branches was increased non-significantly in comparison with mice only treated with BPA (Supplementary Fig. S2A,B), but %hyperplasia was increased by 2.4-fold in BPA and DMBA combined group in comparison with those that only received BPA or DMBA (Supplementary Fig. S2C). Treatment with DMBA alone had no effect on branching point.

BPA alters mammary stem/progenitor cells and leads to an increase of luminal progenitors

Pubertal BPA exposure increased basal MaSC fraction for mammary glands harvested at 6 weeks as indicated by the expansion of basal cell pool and increased sphere forming efficiency (SFE), which led to an ultimate increase of %MaSC_e (see formula [2] in Methods) in BPA-treated glands (Fig. 2). On average, %MaSC_e increased from 1 MaSC in 582 total epithelial cells in the 6-week old control glands to 1 MaSC in 299 total epithelial cells in the BPA exposed glands. However, this effect on MaSCs was acute and short-lived, and was not observed in the glands harvested at later time points (Fig. 2). On the other hand, %LP_e (see formula [3] in Methods) was significantly higher in the glands harvested from 4-month-old BPA-treated mice though luminal cell pool was initially decreased at 6-week-old BPA-treated glands (Fig. 2). Challenge with DMBA had no significant effect on the number of MaSCs and LPs.

It is known that progesterone can induce MaSC expansion and mice at the luteal diestrus phase usually had an increased MaSC pool when compared with other estrous phases such as proestrus, estrus and metestrus (38). In this study, we found a total of 3 animals at diestrus phase, with 2 from the 4-month old control group and 1 from the 4-month old

DMBA-treated group. We did not observe an expansion of MaSC pool from the one animal at the DMBA-treated group, but we found an approximately 2-3 fold increase of MaSC pool from the two animals in the 4-month control group comparing to animals at other estrus phases. However, excluding these two animals from the control group did not result in a significant difference of %MaSC between control and BPA-treated group.

Previously, the mammary colony forming cell (Ma-CFC) assay has been routinely used to provide an in vitro readout for luminal progenitors from the luminal cells. However, unlike the luminal cells from other strains of mice such as C57BL/6 or FVB, luminal cells from Balb/c gave rise to very few colonies: on average of 10 colonies per 1000 cells, which is far less than the number of colonies found in C57BL/6 (100-300 per 1000 cells, our unpublished data). In addition, these colonies are also much smaller than those formed from C57BL/6 (Fig. S3). We thus concluded that this Ma-CFC assay is not adequate to evaluate luminal progenitors for Balb/c mice.

BPA alters stem cell function and leads to early neoplastic lesion formation in regenerated glands

We further examined the stem cell function changes upon pubertal BPA exposure by in vivo repopulation assay. We have previously demonstrated that individual 3D solid structures derived from basal cell formed mammospheres contain MaSCs that can repopulate cleared mammary fat pad (CFP) with high efficiency (29). Thus, we used 3D solid structures for in vivo transplantation. Upon transplant of a single 3D solid structure per CFP, we observed no significant difference in positive outgrowths (Fig. 3A) and %fill of outgrowth (Fig. 3B, C) from MaSCs harvested at different time points between control and BPA-treated animals.

Subsequent histological examination showed significant increase of hyperplastic lesions in the regenerated glands from BPA treated MaSCs vs. control MaSCs derived from 6-week (65.3% vs. 29.8%), 2-month (41.6% vs. 13.4%) and 4-month (72.1% vs. 30.8%) glands (Fig. 4A). Treatment with DMBA plus BPA resulted in higher frequency of hyperplastic lesions (83.6%) than those with DMBA alone (59.0%), which was also significantly higher than that of 4-month control without BPA or DMBA (30.8%). Quantification of ADH/DCIS, the more severe forms of hyperplastic lesions, showed zero incidence in regenerated glands from control MaSCs derived from 2-month and 4-month glands, and approximately 1% ADH/DCIS from the 6-week glands (Fig. 4B). However, %ADH/DCIS was elevated in regenerated glands from BPA or DMBA exposed MaSCs harvested at all time points with the highest average value (6.3%) found in BPA-treated MaSCs harvested at 4 months of age (Fig. 4B). Stem cells harvested from DMBA-treated glands also yielded significantly higher %ADH/DCIS in the regenerated glands when compared to the age-matched controls. However, combined treatment of BPA and DMBA did not exhibit an additive effect in %ADH/DCIS (Fig. 4B).

BPA induces pre-cancerous gene signature in mammary stem cell-enriched mammospheres

To elucidate transcriptional changes underlying the observed ADH/DCIS in the regenerated glands derived from BPA exposed MaSCs, we performed RNAseq analysis of mRNA isolated from basal cell derived mammospheres (highly enriched with MaSCs). Analysis of the differentially expressed genes resulted in a total of 40 genes that were significantly misregulated (adjusted $P < 0.05$) by BPA exposure when compared to the control (Supplementary Fig. S4A, Table 1). Treatment with DMBA alone or BPA in combination with DMBA resulted in 172 and 79 misregulated genes, respectively, when compared to the control (Fig. S4A). Only 9 genes (highlighted in bold in Table 1) were shared by BPA and

DMBA, among which *Abca1*, *Arhgef6*, *Fcgbp*, *Mmp13*, *Scd1*, *Smoc1* are common to all treatment groups (Fig. S4A).

The clustering of different samples revealed that the BPA-treated group was more similar to controls than to DMBA, and the BPA+DMBA exposed group was more similar to DMBA (Fig. S4B). The heat map clusters also showed that there were fewer differentially expressed genes in BPA compared to DMBA or BPA+DMBA (Fig. S4B). Pathway analysis using DAVID was applied to each cluster to elucidate the statistical enrichment for functional perturbations upon BPA and/or DMBA exposure. The upregulated categories by BPA exposure include cell adhesion and lipid transport. The downregulated categories by BPA exposure include cytoskeleton organization, regulation of apoptosis, cell growth, cell adhesion, enzyme inhibition activity, and acute inflammation response (Fig. S4C). Except for cluster 6, DMBA exposure exhibited almost opposite functional perturbations compared to BPA (Fig. S4C).

Differentially expressed genes were also uploaded into IPA software to identify the biological processes disrupted by BPA and/or DMBA (Supplementary Table S1). The most significantly impacted diseases and disorders, molecular and cellular functions, canonical pathways, and upstream regulators all exhibited extreme similarity between DMBA and BPA+DMBA treated groups. In contrast, BPA-treated group revealed different processes being perturbed except for the most significantly impacted molecular and cellular functions where similar processes were found among all three groups (Supplementary Table S1). The common canonical pathway shared by all treatment groups was the LXR/RAR activation (Supplementary Table S1).

To reveal how these transcriptional changes relate to the ADH/DCIS phenotype, we further examined genes related to mammary tumor or breast cancer (Table 2). Our findings revealed that a similar set of genes was misregulated by DMBA or BPA+DMBA; however, genes affected by BPA alone were distinct from those of DMBA except for *Fcgbp* and *Mmp13*. A total of 15 genes, except for *Mmp12*, were significantly downregulated by BPA exposure when compared to the control. Most significantly, 9 out of these 15 genes have been reported to be downregulated in cells from DCIS when compared with normal ductal epithelial cells during early tumorigenesis in human breast (39). These 9 genes are *Atf3*, *Dusp1*, *Egr1*, *Fos*, *Fosb*, *Klf4*, *Nr4a1*, *Rgs2*, *Zfp36*.

Discussion

Our study showed that pubertal low dose BPA exposure recapitulated the morphogenic changes associated with perinatal low dose BPA exposure in mice or rats; resulted in increased luminal progenitors in primary adult glands; and stably altered MaSC function leading to early neoplasia in the regenerated glands. These findings suggest that puberty consists of an additional window of susceptibility for BPA induced adverse effects on mammary gland, and MaSCs could be one of the possible culprits responsible for BPA induced tumorigenesis.

Recent study with gene expression profiling indicated that an aberrant LP population could be a target for transformation in BRCA1-associated basal tumors (19). Consistent with this observation, breast tissue from BRCA1 mutation carriers generally showed an increase in LPs relative to the total epithelial cells (40). In our study, pubertal BPA exposure led to an expanded LP pool as well as increased incidence of hyperplastic lesions. These findings indicate a possible correlation between increased LPs in mammary epithelium and mammary gland transformation potential.

Whether the increased LP associated with pubertal BPA exposure will indeed contribute to the development of basal-like tumors remains to be tested. The expansion of LPs in the 4-month glands does not appear to be derived from an initial LP expansion because our data showed an initial decrease of luminal cells from 6-week old glands and no changes of luminal cells or LP from the 2-month old glands. Future studies are necessary to elucidate how this late expansion of LPs occurs.

The significant expansion of MaSC pool right after BPA treatment is consistent with published studies in that BPA induced changes such as increased TEBs, prolonged diestrus stage, and increased progesterone receptor (PR) expression have all been linked to the induction of MaSC expansion (38, 41). We also confirmed upregulation of PR in stromal cells collected right after BPA exposure (data not shown). The reason that this expanded MaSC pool was not sustained into adult mammary glands could be due to the withdrawal of BPA treatment after puberty. This may also help explain why the uterine horn exhibited significant hypertrophy when assessed at 6 weeks but returned to normal when assessed at later time points (Supplementary Fig. S5). It is possible that continuous BPA exposure is required to sustain an expanded MaSC pool like the transgenic MMTV-Wnt 1 mice, which harbors an expanded MaSC pool due to constitutive activation of the Wnt pathway (15). Future studies are necessary to further explore this phenomenon as well as the underlying mechanism responsible for MaSC expansion.

On the other hand, our study showed that it was the change of MaSC function, not the number, which resulted in preneoplastic lesions in the regenerated glands. In particular, the more severe forms of hyperplastic lesions such as ADH/DCIS were manifested in these regenerated glands derived from BPA exposed MaSCs. These intraductal hyperplasias are considered the precursors of carcinomas, both in rodents and human beings, and have been shown to develop into palpable tumors when transplanted into hosts with intact ovaries (42). Thus, our findings indicate that pubertal BPA exposure induced MaSC to undergo early neoplastic transformation. The complete absence of ADH/DCIS in the regenerated glands from age matched control MaSCs at 2 and 4 months of age also indicated that MaSCs without any perturbation are resistant to alteration under the current in vivo transplantation scheme. The small incidence of ADH/DCIS that occurred in the regenerated glands from the 6-week-old control MaSC could be due to the rather proliferative state of the MaSCs at 6 weeks of age, given the need for extensive ductal elongation in this particular developmental window. Of note, the regenerated glands derived from the 4-month-old MaSCs displayed consistently higher incidence of hyperplastic lesions than the 4-month-old primary glands in the control (30.8% vs. 12.1%), BPA (72.1% vs. 21.9%), DMBA (59.0% vs. 23.8%) or DMBA+BPA (83.6% vs. 53%). This may be in part due to the permissive environment of the recipient nude mice we used for the 4-month time point as 30.8% hyperplasia in the regenerated glands from the control MaSCs is higher than what we have normally observed in regenerated glands derived from 2- to 4-month-old MaSCs, which is normally less than 20% in isogenic transplantation. This, however, could also indicate the importance of stromal environment in hyperplastic lesion induction for preneoplastic MaSCs. Although immune compromised nude mouse mammary glands are more permissive to tumorigenesis than those of the immune component Balb/c mice, the early neoplastic lesions we observed in nude mice were similar in stage as those in the syngeneic Balb/c mice except that higher percent of hyperplastic lesions were observed in nude mice than in Balb/c mice. We also did not observe noticeable growth pattern difference of the transplanted 3D solid structures in regenerating the glands in nude mice and Balb/c mice.

To reveal the underlying transcriptional changes that may account for altered MaSC function upon short-term BPA exposure during puberty, we did whole transcriptome sequencing on MaSC-enriched mammospheres from the 4-month-old age group, where the

highest %ADH/DCIS occurred in the MaSC-regenerated glands. Interestingly, only 40 genes were significantly altered between control and BPA-treated groups, and most of them were underexpressed in the BPA-treated samples. We took several approaches (gene ontology, DAVID, IPA) to identify potentially functional significance of the differentially expressed genes. The most overrepresented gene ontology molecular functions and biological process categories are related to DNA binding and cell adhesion functions (Table 1). This is consistent with the relatively large number of transcription factors that are differentially expressed, including AP-1 components (*Fosb*, *Fos*), Kruppel-like factors (*Klf4*), nuclear hormone receptors (*Nr4a1*), and zinc-finger proteins (*Zfp36*) as well as many extracellular matrix structural constituents (e.g., *Col12a1*, *Col3a1*, *Col15a1*, *Col6a3*, *Col5a2*). Other significant categories included lipid transport, protein binding, cell differentiation, and response to cytokine stimulus (Table 1).

Although transcription regulators are frequently implicated in breast carcinogenesis (43), cancer was not listed among the top 5 affected diseases and disorders in any of our 3 treatment groups upon IPA analysis (Table S1). When we focused on a subset (15 out of 40 genes) of differentially expressed genes related to mammary tumor or breast cancer, we found striking similarities between our finding and that of an early study where the authors looked for gene expression differences between histologically normal epithelium as well as DCIS of breast cancer patients and breast-cancer free controls (39). First, most genes in their study were also downregulated in the earlier lesions vs. normal controls. Second, the nine downregulated genes in our study (*Fos*, *Fosb*, *Atf3*, *Rgs2*, *Dusp1*, *Nr4a1*, *Egr1*, *Klf4*, *Zfp36*) were found to be similarly downregulated in the earlier lesions in their study. Third, the top two downregulated genes in our study (*Fosb*, *Fos*) were also notably ranked as the top two downregulated genes (21-fold decrease for *FOSB* and 10-fold decrease for *FOS*) in their study. The convergence of similar sets of genes between these two studies suggests that these genes may play important roles in preventing early stages of breast carcinogenesis. Immunohistochemistry confirmed lower expression of FosB and c-Fos in the outgrowths regenerated from BPA-treated MaSCs than from control MaSCs (Supplementary Fig. S6). Future functional validation of these signature genes could enhance our understanding of important functional alterations present early in carcinogenesis, reveal targets for cancer prevention, and improve cancer risk assessment.

Although we have focused our study on gene expression changes related to MaSCs, this does not imply that MaSCs are direct targets of BPA. BPA exposure in utero promoted fat pad maturation and altered the localization of collagen in mice (1). Additional research concluded that estrogen receptors are present predominantly in the stromal compartment during prenatal development (44). Thus, BPA may affect MaSC function indirectly by altering the stem cell niche (stroma). Future studies may take advantage of the cleared fat pad regeneration model system to evaluate whether BPA exposed stroma will initiate similar changes on donor MaSCs that are naïve to BPA exposure.

Previously, DMBA has been used to induce tumor formation in perinatal BPA exposed glands, but we did not observe flank tumors in either primary or regenerated glands in this study. One possible reason could be the time point when we harvested the glands, which might be too young for tumors to occur as tumors often develop at much later time points such as 12 months in studies using DMBA for tumor induction (10, 11). Another possible reason could be the difference between pubertal and perinatal (or fetal stage) MaSCs because recent lineage tracing studies indicate that multipotent MaSCs are only present in fetus (45). Thus, the effect of BPA on MaSCs during puberty could be much less devastating than its effect on fetal MaSCs. Differences in MaSC susceptibility at different developmental stages may also help explain why DMBA, a very potent carcinogen, when administered at 2 months of age (with MaSCs presumably at a relatively quiescent stage) did

not elicit as much ADH/DCIS incidence as pubertal BPA exposure, and neither did it elicit any additive effect on neoplastic lesions in MaSC-regenerated glands when combined with pubertal BPA exposure. Therefore, we predict that the window of susceptibility for tumorigenic transformation involving mammary stem cells will most likely to be associated with periods of mammary tissue development or remodeling involving extensive MaSC proliferation/differentiation such as those during perinatal, puberty, and pregnancy.

To our knowledge, this is the first study to link BPA induced morphogenic changes and tumor susceptibility in mammary glands to mammary stem cells. Our findings indicate that MaSCs are vulnerable targets for estrogen-mimic BPA during mammary gland's early developmental stages, which have significant implications in breast cancer etiology and disease prevention. For example, knowing there is a susceptible window during development when MaSCs could be instigated to generate neoplastic lesions late in life will allow public policy to be directed to a particular age group for cancer prevention. In addition, the striking similarity between the aberrantly expressed genes in BPA-exposed MaSC and those found in tumor-adjacent, normal epithelial tissues from breast cancer patients suggests that these genes may play important roles in early stages of breast carcinogenesis. Functional validation of these signature genes will open up future venues to explore clinically relevant diagnostic tools (e.g, RT-PCR or immunohistochemistry staining technique for a particular gene or gene set in biopsy) for early identification of susceptible individuals. Our study also provides a robust model system for screening environmental risk factors of human breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schemes of experimental flow (A), %solid and %hollow structures formed in 3D culture from basal and luminal derived spheres (B), and H&E histology analysis showing normal duct (C), hyperplastic lesion (D), atypical ductal hyperplasia (ADH) (E), and ductal carcinoma in situ (DCIS) (F). Scale bars, 50 μ m in C-F.

Figure 2.

Cell frequency and sphere formation efficiency (spheres per 1,000 cells) of basal ($CD24^+CD49^{hi}$) or luminal ($CD24^{hi}CD49^{low}$) cells as well as stem cell (%MaSC_e) and luminal progenitor (%LP_e) frequency in total epithelial cell (TE, equal to the sum of basal and luminal cells) (mean $\pm$ SEM, $n = 5$). Asterisks indicate significant difference between control and BPA at $P < 0.05$.

Figure 3. Results of in vivo cleared mammary fat pad (CFP) transplantation of single 3D solid structure. (A) Positive take of outgrowth from control or BPA/DMBA exposed MaSCs in the in vivo transplant (IVT) assay. (B) Representative whole mount staining pictures showing different percentage of fill in cleared fat pad with regenerated glands from control or BPA/DMBA exposed MaSCs. (C) Bar graphs showing the distribution of percentage of fill CFP for all regenerated glands derived from each treatment group.

Figure 4. Percent hyperplasia (A) and ADH/DCIS (B) of regenerated glands (mean $\pm$ SEM, $n = 4-10$). Asterisks indicate significant difference between control and BPA at $P < 0.05$.

Table 1List of differentially expressed genes in BPA-treated group ($P < 0.05$)

Symbol	Gene ontology	Log fold change	P-value
<i>Fosb</i>	DNA binding	-3.659	1.10E-104
<i>Fos</i>	DNA binding	-2.379	6.08E-56
<i>Atf3</i>	DNA binding	-1.476	9.43E-09
<i>Nr4a1</i>	DNA binding	-0.924	2.47E-03
<i>Egr1</i>	DNA binding	-0.856	8.77E-06
<i>Klf4</i>	DNA binding	-0.814	3.90E-03
<i>Zfp36</i>	DNA binding	-0.786	3.78E-03
<i>Spon1</i>	Cell adhesion	-0.903	2.67E-06
<i>Smoc1</i>	Cell adhesion	-0.724	1.18E-02
<i>Col12a1</i>	Cell adhesion	-0.600	1.55E-02
<i>Col3a1</i>	Cell adhesion	-0.597	5.83E-03
<i>Fbln2</i>	Cell adhesion	-0.545	2.47E-02
<i>Col15a1</i>	Cell adhesion	-0.542	3.61E-02
<i>Spp1</i>	Cell adhesion	-0.528	3.34E-02
<i>Col6a3</i>	Cell adhesion	-0.525	3.77E-02
<i>Col5a2</i>	Cell adhesion	-0.520	4.90E-02
<i>Thbs4</i>	collagen, heparin, integrin, laminin-1 binding	1.281	5.09E-09
<i>Ccdc80</i>	fibronectin binding	-0.710	4.47E-03
<i>Thy1</i>	integrin binding	-0.679	1.55E-02
<i>Mmp12</i>	metallopeptidase activity	0.981	2.34E-02
<i>Mmp13</i>	metallopeptidase activity	-1.505	9.50E-13
<i>Rgs2</i>	GTPase activator activity; protein binding	-1.247	3.31E-03
<i>Socs3</i>	protein binding	-0.791	1.23E-02
<i>Mid1</i>	protein binding	-0.680	8.21E-04
<i>Fcgbp</i>	protein binding	-0.547	2.34E-02
<i>Serping1</i>	serine-type endopeptidase inhibitor activity	-0.832	3.77E-02
<i>Serpma3n</i>	serine-type endopeptidase inhibitor activity	-0.733	3.64E-02
<i>Serpinf1</i>	serine-type endopeptidase inhibitor activity	-0.596	1.55E-02
<i>Dusp1</i>	phosphatase activity	-1.034	3.94E-08
<i>Frem1</i>	carbohydrate or metal ion binding	-1.016	1.30E-04
<i>Hp</i>	catalytic activity; hemoglobin binding	-0.906	4.04E-05
<i>Hc</i>	endopeptidase inhibitor activity	-0.844	4.95E-02
<i>Arhgef6</i>	rho guanyl-nucleotide exchange factor activity	-0.818	4.34E-03
<i>Abca1</i>	ATP binding; phospholipid transporter activity	-0.805	8.77E-06
<i>Car12</i>	carbonate dehydratase activity	-0.740	1.07E-03
<i>Pla1a</i>	catalytic activity	-0.691	1.39E-02
<i>Lpl</i>	lipoprotein lipase activity	-0.687	4.24E-02
<i>Gpx3</i>	glutathione peroxidase activity	-0.675	3.40E-02
<i>Ctsk</i>	hydrolase activity	-0.645	3.78E-03

Symbol	Gene ontology	Log fold change	P-value
<i>Scd1</i>	stearoyl-CoA 9-desaturase activity	-0.600	4.85E-03

Genes in bold are shared with DMBA treated group.

Table 2

List and log₂ fold change of genes related to mammary tumor or breast cancer for different treatment groups (P < 0.05)

Symbol	BPA	Symbol	DMBA	BPA+DMBA
<i>Fosb</i>	-3.659	<i>Igfbp5</i>	-2.706	-1.638
<i>Fos</i>	-2.379	<i>Fcgbp</i>	-2.589	-2.190
<i>Mmp13</i>	-1.505	<i>Mmp13</i>	-2.152	-1.398
<i>Atf3</i>	-1.476	<i>Lamc3</i>	-2.123	--
<i>Rgs2</i>	-1.247	<i>Muc1</i>	-2.078	-1.686
<i>Dusp1</i>	-1.034	<i>Dixdc1</i>	-1.914	-1.875
<i>Nr4a1</i>	-0.924	<i>Pdk4</i>	-1.888	-1.757
<i>Hp</i>	-0.906	<i>Slc6a2</i>	-1.844	-1.709
<i>Egr1</i>	-0.856	<i>Hp</i>	-1.714	--
<i>Klf4</i>	-0.814	<i>Arrdc3</i>	-1.528	-1.245
<i>Zfp36</i>	-0.786	<i>Foxi1</i>	-1.441	--
<i>Serpinf1</i>	-0.596	<i>Aldh1a3</i>	1.276	1.355
<i>Fcgbp</i>	-0.547	<i>Trp63</i>	1.298	--
<i>Spp1</i>	-0.528	<i>Adams1</i>	1.358	--
<i>Mmp12</i>	0.981	<i>Bmp7</i>	1.426	--
		<i>Igfbp3</i>	1.457	1.266
		<i>Krt14</i>	1.570	1.449
		<i>Slpi</i>	1.576	--
		<i>Pgr</i>	1.675	--
		<i>Cdh3</i>	1.688	1.386
		<i>S100a14</i>	1.766	1.396
		<i>Myb</i>	1.878	--
		<i>Klk6</i>	1.909	--
		<i>Apoe</i>	2.195	1.499
		<i>Krt5</i>	2.256	1.993
		<i>Krt17</i>	2.328	1.824
		<i>Spon2</i>	2.481	--
		<i>Cst6</i>	2.766	1.819
		<i>Krt6a</i>	2.949	2.707
		<i>Krt15</i>	3.072	2.186

Genes in bold are shared by all treatment groups.