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Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells

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Abstract

Substantial evidence indicates that exposure to bisphenol A (BPA) during early development may increase breast cancer risk later in life. The changes may persist into puberty and adulthood, suggesting an epigenetic process being imposed in differentiated breast epithelial cells. The molecular mechanisms by which early memory of BPA exposure is imprinted in breast progenitor cells and then passed onto their epithelial progeny are not well understood. The aim of this study was to examine epigenetic changes in breast epithelial cells treated with low-dose BPA. We also investigated the effect of BPA on the ERα signaling pathway and global gene expression profiles. Compared to control cells, nuclear internalization of ERα was observed in epithelial cells preexposed to BPA. We identified 170 genes with similar expression changes in response to BPA. Functional analysis confirms that gene suppression was mediated in part through an ERα-dependent pathway. As a result of exposure to BPA or other estrogen-like chemicals, the expression of *lysosomal-associated membrane protein 3* (*LAMP3*) became epigenetically silenced in breast epithelial cells. Furthermore, increased DNA methylation in the *LAMP3* CpG island was this repressive mark preferentially occurred in ERα-positive breast tumors. These results suggest that the *in vitro* system developed in our laboratory is a valuable tool for exposure studies of BPA and other xenoestrogens in human cells. Individual and geographical differences may contribute to altered patterns of gene expression and DNA methylation in susceptible loci. Combination of our exposure model with epigenetic analysis and other biochemical assays can give insight into the heritable effect of low-dose BPA in human cells.

Keywords

Bisphenol A; Estrogen; DNA methylation; Epigenetics; Breast cancer

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Conflict of interest statement

All authors declare that they have no competing financial interests.

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Introduction

Bisphenol A (BPA), first synthesized by A. P. Dianin in 1891, has been widely used as a cross-linking reagent in the manufacture of epoxy resins since 1950s (Vogel, 2009). It is extensively used in a board range of products, including toys, water pipes, drinking bottles, baby bottles, food containers, tubing, and dental sealants (Welshons *et al.*, 2006). Presently, the worldwide production of BPA exceeds 3 billion kilograms per year (Vandenberg *et al.*, 2009). Studies have shown that BPA can be released from incomplete polymerization upon heating or leached out through normal use (Mountfort *et al.*, 1997; Kang *et al.*, 2003; Goodson *et al.*, 2004). Because of its ubiquity in environment, low levels of BPA can be detected in 92.6% of urine samples (≥ 6 years of age ranging from 0.4 – 149 μg/L) in the National Health and Nutrition Examination Survey (NHANES) 2003–2004 (Calafat *et al.*, 2008; CDC, 2009). Animal studies have shown that these low levels of BPA exposure may alter developmental programs of sensitive end organs, like mammary and prostate gland, during critical stages of early development (Markey *et al.*, 2001; Nikaido *et al.*, 2004; Timms *et al.*, 2005). The changes may persist into puberty and adulthood, suggesting an imprinting process being imposed in differentiated breast epithelial cells (Markey *et al.*, 2001; Munoz-de-Toro *et al.*, 2005).

The molecular mechanisms by which early memory of BPA exposures can be imprinted in breast progenitor cells and then passed onto their epithelial progeny are not well understood. One distinct possibility is through epigenetic remodeling of DNA structure without altering the nucleotide sequence itself. The changes, including DNA methylation, frequently occur in GC-rich promoter CpG islands of transcriptionally repressed genes (Ohm and Baylin, 2007; Widschwendter *et al.*, 2007). Studies have suggested that DNA methylation of a promoter CpG island, or promoter hypermethylation, can be initiated in progenitor genomes and heritably passed onto the differentiated progeny (Jones and Baylin, 2007; Marotta and Polyak, 2009). This epigenetic process is known to cause phenotypic variations among individuals and contributes to the development of pathological conditions, like cancer (Feinberg *et al.*, 2006; Esteller, 2007).

Previous studies of epigenetic effects of BPA preexposure mainly rely on animal models and epidemiological surveys (Ho *et al.*, 2006; Dolinoy *et al.*, 2007; Prins *et al.*, 2008; Yaoi *et al.*, 2008). Whereas these observations strongly implicate that the exposure to low-dose BPA is potentially harmful to human health, the challenge encountered is validation studies of the findings in primary human cells. In this regard, we have recently established a human preexposure model for epigenetic studies (Cheng *et al.*, 2008; Hsu *et al.*, 2009). In the model, breast progenitor cells were first exposed to different environmental chemicals, and then these cells were differentiated into epithelial cells in the absence of these environmental stimulants. We hypothesize that slow-dividing progenitor cells have a longer life span and thus are more susceptible to environmental injuries and can transmit this injured memory to their differentiated progeny through epigenetic mechanisms. In our previous studies, the preexposure to 17β-estradiol (E2) and diethylstilbestrol (DES) may trigger epigenetic repression of protein-coding genes and non-coding microRNAs, some of which exhibit promoter hypermethylation in breast cancer cells (Cheng *et al.*, 2008; Hsu *et al.*, 2009).

Here, we extended this preexposure study to evaluate epigenetic effects of low-dose BPA in human breast epithelial cells. As a result of chronic exposure to BPA, activation of estrogen receptor α (ERα)-mediated signaling and subsequent alterations of responsive gene expression were observed in the differentiated epithelial progeny. This heritable influence on gene expression was similarly observed in ERα-positive breast tumors.

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Materials and Methods

Tissue samples and cell culture

Breast tissues, obtained from individuals undergoing mastectomy or reduction mammoplasty, were collected in accordance with the protocols approved by the Institutional Review Boards of the Ohio State University and the National Taiwan University Hospital. For isolation of breast progenitor cells, non-cancerous tissues (age: 17–42 years old) were enzymatically dissociated via collagenase digestion as described previously (Hsu *et al.*, 2009). Single cells were grown into floating spherical colonies (2–10,000 cells per colony), called mammospheres, in ultra-low attachment dishes (Corning, Lowell, MA) in serum-free medium. These mammospheres, enriched in breast progenitor cells (Dontu *et al.*, 2004), were exposed with BPA (4 nM) (Sigma, St. Louis, MO), diethylstilbestrol (DES, 70 nM), daidzein (10 μM), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 0.1 nM), 4-nonylphenol (NP, 1 μM), N-butyl-benzyl phthalate (BBP, 10 μM), di(2-ethylhexyl)-phthalate (DEHP, 10 μM), 4,4′-dichloro-biphnyl (PCB, 0.1 nM) or DMSO in phenol red-free medium for 3 weeks (medium changed twice a week). The concentration of each chemical was selected based on the literature review. Cell viability assay indicated that there is no toxicity effect of each compound under the concentration we selected (HarrEus *et al.*, 2002; Rohrdanz *et al.*, 2002; Buteau-Lozano *et al.*, 2008) After the exposure, mammospheres were washed with PBS to remove BPA and then placed on a collagen-coated dish in phenol red-free DMEM/F12 medium containing 5% charcoal-dextran-treated FBS (Hyclone, Waltham, MA) for 2–3 weeks. Under this condition, progenitor cells were differentiated into breast epithelial cells, or called mammosphere-derived epithelial cells (MDECs). A panel of 48 breast cancer cell lines, procured through the Integrative Cancer Biology Program of the National Cancer Institute, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and routinely propagated in culture dishes for epigenetic analyses.

Immunofluorescence staining

Approximately 5,000 MDECs were seeded on a collagen I-coated coverslip (BD Biosciences, San Jose, CA) for overnight and then fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 3% bovine serum albumin (Fisher Scientific, Pittsburgh, PA) for 1 h, the coverslip was incubated with anti-ERα antibody (D-12, 1:50) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. The corresponding secondary FITC-conjugated antibody (Invitrogen, Carlsbad, CA) was applied followed by 4′,6-diamidino-2-phenylindole staining (DAPI) (Invitrogen) to localize cell nuclei. The images were captured by confocal laser microscope (Zeiss LSM510) (Zeiss, Thornwood, NY), and percentages of ERα subcellular localization were calculated in 10 different optical fields (~100 cells) by two independent researchers.

Western blot analysis

MDECs preexposed to BPA or DMSO were collected and protein lysates were made. 30 μg of lysate were immunoblotted with antibody against phospho-p42/44 MAPK (1:1000), phospho-Akt (1:2000) (Cell Signaling Technology). GAPDH (Santa Cruz Biotechnology) was used as loading control. Cy5-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibody (GE Healthcare, Pittsburgh, PA) were used for multiplex detection. The membranes were scanned by Typhoon 9400 scanner (GE Healthcare).

Gene expression microarray

Total RNAs of ten independent MDECs, including BPA-preexposed and control, were isolated with Trizol (Invitrogen) according to the manufacturer's instructions. RNA (5 μg/

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sample) was used for microarray hybridization to the Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) by the Microarray Core Facility at the Ohio State University Comprehensive Cancer Center (Columbus, OH). Gene expression estimates of the 54,675 probe sets on arrays were obtained using robust multi-array analysis (RMA) method with quantile normalization and background correction (Irizarry *et al.*, 2003). Gene expression microarray data of 48 breast cancer cell lines (BCC48, Neve et al., 2006) and breast tumors (GSE2109, International Genomics Consortium, http://www.intgen.org/expo) were available for downloading. Qualtile normalization and background correction were also applied to these individual datasets.

Comparison between BPA-preexposed and control samples was performed using BRB Array Tools software developed by the Biometric Research Branch of the National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools). A paired *t*-test with random variance model was applied in order to identify differentially expressed genes between BPA-preexposed and control samples. Initial filtering was performed by selecting genes with $P < 0.05$ and by removing genes with lower expression for all samples (genes with expression values less than or equal to 100 for all samples were removed). An un-paired *t*-test was applied for the BCC48 and GSE2109 datasets in order to identify differentially expressed genes between ERα-positive and ERα-negative samples. The final 170 loci were obtained by restricting the initial list of candidates to commonly expressed genes in breast cancer based on two microarray datasets, BCC48 and GSE2109. Functional and network analyses of these genes were performed using Ingenuity Systems' IPA software (Ingenuity Systems Inc., www.ingenuity.com).

Epigenetic treatments and reverse transcription-quantitative PCR (RT-qPCR)

MCF-7 breast cancer cells were treated with 1 μM 5-aza-2′-deoxycytidine (DAC) in phenol red-free MEM containing 10% FBS and 6 ng/ml insulin. During the final 24 h, some cells were additionally treated with 0.5 μM trichostatin A (TSA). RNA (1 μg) was isolated and reversely transcribed into cDNA using the SuperScript III Reverse Transcriptase (Invitrogen). RT-qPCR was performed by using 2x SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on a 7500 Real-Time PCR System apparatus (Applied Biosystems). Levels of the *36B4* mRNA transcript were also measured as internal controls (Akamine *et al.*, 2007). The reactions were performed in triplicate, and the standard deviation was calculated using the Comparative Method (ABI Prism 7700 Sequence Detection System User Bulletin #2). Primer sequences and conditions for amplification are available in Supplemental Table S1.

DNA methylation analysis by Pyrosequencing

To determine methylation levels of candidate genes in samples, the Pyrosequencing system (Qiagen, Valencia, CA) was used to detect methylated CpG sites in sequencing reactions (Tost and Gut, 2007). Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA). Bisulfite-treated DNA was amplified with specific primers for each gene of interest. The Pyro Mark Assay Design program and the Pyro Q-CpG software were used for primer designs and data analysis, respectively. Average methylation levels of individual CpG sites for each DNA sample were calculated.

Statistical analysis

All data derived from subcellular localization, RT-qPCR, and Pyrosequencing were presented as mean ± SD of n independent measurements. Statistical comparisons between two groups (DMSO *vs.* BPA) were made by Student's *t* test using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). To avoid any violation of normal distribution

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assumption for DNA methylation analysis, we applied non-parametric Mann-Whitney rank-sum test (GraphPad Prism 6). A significance was assigned if $P < 0.05$.

Results

Effect of low-dose BPA on the nuclear localization of ERα in MDECs

Environmental chemicals, such as BPA, are known to act as estrogenic ligands that activate or deactivate gene transcription in breast epithelial cells (Soto *et al.*, 2006; Dairkee *et al.*, 2008). To determine whether BPA causes an estrogen-like effect, we performed immunofluorescence analyses in MDECs (un-exposed) transiently treated with different doses (ranges: 1 – 1,000 nM) of BPA at 0, 5, 30, 60, and 120 min (Figure 1A). BPA, as a weak estrogenic ligand, caused maximized ERα internalization at 30 min in a higher dose (1,000 nM) of exposure.

Because prolonged exposure of breast progenitor cells to xenoestrogens also causes ERα internalization in their differentiated progeny (Hsu *et al.*, 2009), we determined whether BPA has this effect. Progenitor-containing mammospheres from an individual (#124) were continuously exposed to 4 nM BPA for 3 weeks. After the exposure, BPA was removed, and progenitor cells underwent epithelial differentiation in the collagen-coated dishes for 2–3 weeks. Immunofluorescence analysis showed an increase of ERα-positive population during the mammosphere and MDEC stages (Figure 1C). After 7-d preexposure, the majority (>90%) of mammospheres were ERα-negative (yellow bar in Figure 1C). However, cell lineages were greatly shifted from ERα-negative to ERα-positive (green-plus-red bar, > 80%) after 42-d incubation. Among ERα-positive cells at 42-d, nuclear expression of ERα (green bar/green-plus-red bar, 78%) in BPA-preexoposed MDECs was increased compared to that of control (DMSO) cells (11.6%), suggesting that BPA preexposure contributes to ERα internalization in MDECs (Figure 1B). As a control, we also observed similar effect in E2 (70 nM)-preexposed MDECs.

When the analysis was extended to different primary MDECs (n = 9), we noticed individual variations in response to this low-dose BPA preexposure. As shown in Figure 1D, five (#96, 124, 98, 99, and 117) of these MDEC sets exhibited greater effects (up to 80%) of ERα internalization compared to the other four sets (#111, 120, 113, and 119) showing lesser effects (18 – 40%). This initial result suggests that as a weak estrogenic ligand, high-dose BPA (at least 1000 nM) is needed to acutely activate ERα-mediated signaling while chronic exposure of a lower dose (4 nM) can similarly bring about this signal transduction in breast epithelial cells. Furthermore, our observations indicate that the genetic background of individuals may influence differential responses to the exposure of low-dose BPA.

It is known that other exogenous stimulants, such as growth factors, may act through mitogen-activated protein kinase (MAPK) or Akt pathways to promote the nuclear internalization of ERα for transcriptional regulation in proliferating cells (Lannigan, 2003; Murphy *et al.*, 2009). In this regard, we observed an increased level of phospho-p42/44 MAPK, likely attributed to this internalization in BPA-preexposed MDECs without further ligand stimulation (Figure 1E).

Effect of low-dose BPA on differential gene expression in MDECs

To investigate whether this effect altered gene expression, we conducted microarray analysis in ten sets of preexposed (BPA, 4 nM) and control MDECs using the Affymetrix Human Genome U133 Plus 2.0 Array. One set of the MDECs (#119) was removed from gene expression analysis because of the low nuclear localization of ERα. Differential expression of genes at $P < 0.05$ within 9 set samples was scored, yielding a total of 2,234 candidate loci (1,162 down-regulated and 1,072 up-regulated) likely influenced by this BPA preexposure.

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Scatter plots and the number of differentially expressed genes for individual MDECs are presented in Figure 2. Consistent with the observation of ERα internalization, we observed individual variations of gene expression in these primary MDECs preexposed to low-dose BPA. In this regard, greater numbers of differentially expressed genes were seen in #124, 99, 100, and 128 (i.e., the high-responder group) while the rest of six primary MDECs had fewer changes of expression (i.e., the low- responder group). We additionally compared these expression profiles with the status of ERα internalization available for seven MDEC sets (#124, 99, 117, 120, 111, 113 and 119). Though not statistically significant, we observed a general trend that greater degrees of ERα internalization seemed to be associated with increased numbers of differentially expressed genes in MDECs.

Effect of BPA-influenced gene signatures in ERα-positive breast cancer

In silico analysis was conducted to determine whether specific expression profiles of BPA-influenced genes are associated with the development of breast cancer. When the 2,234 candidate loci were compared with those of two microarray datasets, BCC48 (Neve *et al.*, 2006) and GSE2109 (International Genomics Consortium, http://www.intgen.org/expo), we found a total of 170 BPA-influenced genes (57 up-regulated and 113 down-regulated), the aberrant expression of which may contribute to breast tumorigenesis (Figure 3A and B; see also Supplemental Table S2). Hierarchical clustering of 48 breast cancer cell lines (i.e., BCC48) and 244 breast tumors (i.e., GSE2109) revealed that specific up- and down-regulated patterns of these 170 genes are distinctly related to ERα-positive cell lines (Figure 3C and D). This observation further indicates that 1) BPA may aberrantly regulate gene expression through an ERα-dependent pathway and 2) this regulatory mechanism may be epigenetically imprinted in ERα-positive breast cancer.

To validate this potential imprinting effect, we choose 15 down-regulated genes for expression analysis (Figure 4). The reason to focus on these loci was that the BPA-influenced repression might be associated with hypermethylation of their CpG islands, which are located in the transcription start sites of these selected genes. First, RT-qPCR was used to confirm the expression status of these loci in the aforementioned six MDECs preexposed to BPA (4 nM). The expression of these loci was consistently down-regulated in three high-responders, #124, 99, and 128 ($P < 0.05$). Down-regulation of these loci, however, could not be confirmed in one high-responder (#100), likely attributed to a small sampling of down-regulated loci. Though this down-regulation was also seen in the low-responder group by the sensitive RT-qPCR assay, the repressive effect was usually less apparent (e.g., #120 and 113). In the rest of low-responders (#111, 129, 117, and 119), significant changes of expression between pre-exposed and control MDECs were not noted.

Epigenetic repression of a BPA-influenced locus, LAMP3, in ERα-positive breast cancer cells

To further investigate a potential role of epigenetic repression, we focused the expression analysis on a candidate gene, *lysosomal-associated membrane protein 3* (*LAMP3*), in the well-characterized ERα-positive MCF-7 cell line. (see functional analysis of this gene in MCF-7 cells in supplemental Figure S1). A low level of *LAMP3* expression was detected in MCF-7 cells. To determine whether this reduced expression is mediated by epigenetic mechanisms, we treated these cells with the demethylating agent DAC (1 μM) and/or the histone deacetylase inhibitor TSA (0.5 μM), known to reactivate epigenetically repressed genes (Dworkin *et al.*, 2009; Huang *et al.*, 2009). As shown in Figure 5A (lanes 1–4), the expression of *LAMP3* was significantly reactivated by single treatments (i.e., DAC or TSA, $P < 0.01$). Furthermore, synergistic re-expression of this gene was observed in cells with the combined treatment (DAC plus TSA, $P < 0.001$). Additional results of 7 other repressed genes are presented in supplemental Figure S2.

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To investigate whether this epigenetic repression could be attributed to an estrogen-mediated pathway, MCF-7 cells were additionally treated with E2 and/or an ERα antagonist, ICI182780. The subsequent E2 treatment led to re-silencing of *LAMP3*, suggesting a role of estrogen signaling in mediating this epigenetic repression (Figure 5A, lanes 5–8). Treatment of ICI182780 abolished the down-regulation, additionally indicating that this regulation is partly mediated through an ERα-dependent pathway (Figure 5A, lane 9). The repression was partially attenuated in the presence of additional epigenetic treatments (i.e., DAC and TSA, lanes 10–12).

Based on the results of these pharmacological experiments, our observations suggest that 1) estrogen signaling initiates the repression of the BPA-influenced loci in breast epithelial cells; 2) this repression is partly mediated trough an ERα-dependent pathway; and 3) persistent repression of the BPA-influenced loci in cancer cells may be further maintained by DNA methylation and histone modifications.

LAMP3 repression in MDECs preexposed to other estrogen-like chemicals

To determine whether long-term exposure of other estrogen-like chemicals can additionally initiate this epigenetic repression, mammospheres were exposed to diethylstilbestrol (DES, 70 nM), daidzein (10 μM), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 0.1 nM), 4-nonylphenol (NP, 1 μM), N-butyl-benzyl phthalate (BBP, 10 μM), di(2-ethylhexyl)-phthalate (DEHP, 10 μM), and 4,4′-dichloro-biphnyl (PCB, 0.1 nM) for 3 weeks. After the exposure, MDECs were subjected to RT-qPCR analysis for *LAMP3* expression. As shown in Figure 5B, downregulation of *LAMP3* was confirmed in MDECs preexposed to these estrogen-like chemicals (2.3 to 12.5-fold decrease). Suppressive effects varied for the different environmental exposures, indicating differential sensitivity of progenitors to these chemicals.

Promoter hypermethylation of LAMP3 in ERα-positive breast cancer

To confirm the *in vitro* epigenetic findings, we conducted DNA methylation analysis in the promoter CpG island regions of *LAMP3* loci, in 48 breast cancer cell lines, 484 primary breast tumors (Taiwan cohort, $n = 336$; US cohort, $n = 148$), and 10 noncancerous breast tissues as normal controls. Pyrosequencing analysis of *LAMP3* (9 CpG sites) revealed that DNA methylation levels were significantly increased in breast cancer cell lines relative to those of normal controls (Supplemental Figure S3B). Moreover, promoter hypermethylation of *LAMP3* ($P = 0.008$) was significantly associated with the ERα-positive status. In close agreement with these results, hypermethylation of *LAMP3* was observed in ERα-positive tumors in the US cohort ($P < 0.0001$) (Figure 6A, and Supplemental Figure S3A) and in the Taiwan cohort (Figures 6B, and Supplemental S3A). The cut-off points of age groups used in the further analysis were based on menopausal status - premenopausal (age <50 years) and postmenopausal (age >50 years) groups. The young age group defined by age <35 years appears to have distinct biological characteristics and display poor prognosis compared to those ≥35 years. Interestingly, while the hypermethylation event occurred in both age groups (35–50 and >50 years old) in the US cohort, this trend was only seen in the old age group (>50 years old) of the Taiwan cohort. Association of this hypermethylation with other clinicopathological features of patients was not apparent.

Discussion

When acutely exposed to estrogenic ligands, signal transduction is mediated in part through nuclear hormone receptors, such as ERα (Bjornstrom and Sjoberg, 2005). We have previously shown that the hallmark of this signal transduction is the translocation of cytoplasmic ERα into the nucleus of a normal breast epithelial cell (Hsu *et al.*, 2009). Unlike

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E2 and DES, BPA is considered to be a weak estrogenic ligand based on the present immunofluorescence analysis and previous receptor binding assays (Okada *et al.*, 2008). In our case, up to 1000 nM BPA is needed to initiate this ligand-dependent function, which mobilizes ERα into the nucleus for transcriptional activation and deactivation (Bjornstrom and Sjoberg, 2005; Okada *et al.*, 2008).

We also observed that long-term exposure of breast progenitor cells to low-dose BPA (4 nM) is capable of triggering ERα internalization later observed in the differentiated progeny. In this case, exogenous stimulants (e.g., growth factors) may elicit ligand-independent activation by promoting the nuclear internalization of phosphorylated ERα for transcriptional regulation in proliferating cells (Lannigan, 2003; Murphy *et al.*, 2009). This ligand-independent genomic function likely co-regulates a subset of target genes (e.g., *LAMP3*) governed through the ligand-dependent pathway. We speculate that persistent exposure of progenitor cells to low-dose PBA likely renders a permanent alteration of their differentiated transcriptomes that are maintained by epigenetic mechanisms. Deacetylated modifications of histone and promoter hypermethylation are heritably established in an inactive gene while acetylated histone and promoter hypomethylation may be present to mark an active locus (Jones and Baylin, 2007; Vaissiere *et al.*, 2008). However, individuals may have different susceptibility to these epigenetic modifications. Based on our expression profile analysis of primary MDECs, the high-responder group is more sensitive than the low-responder group to the BPA preexposure. Whereas genetic variations in response to xenoestrogens are well documented in different strains of mice or rats (Richter *et al.*, 2007; Tyl, 2009), this study provides the first evidence that differential susceptibility to low-dose BPA exposure may also be present in human populations.

It has been observed that exposure to low-dose BPA during early stages of mammary gland development may increase the risk of developing breast neoplasm in adult animals (Durando *et al.*, 2007; Murray *et al.*, 2007). Supporting this finding, we have identified 170 human candidate genes that may play a critical role in tumorigenesis. Ingenuity pathway analysis has uncovered their putative functions primarily related to aminoacyl-tRNA biosynthesis, circadian rhythm signaling, GM-CSF signaling, and HER-2 signaling, the deregulation of which can promote the development of breast cancer (see Supplemental Figure S4). The expression of some of these BPA-influenced genes may be epigenetically imprinted in breast cancer cells. We were able to validate one candidate gene, *LAMP3*, the promoter hypermethylation of which is preferentially linked to transcriptional silencing in ERα breast cancer cells. This gene is known to encode proteins associated with cell mobility and adhesion, and its overexpression is usually linked to invasiveness in cancer (Kanao *et al.*, 2005). Since *LAMP3* may not be epigenetically silenced in ERα-negative tumors, its aberrant expression could contribute to more aggressive phenotypes in this type of breast cancer. The hypermethylation finding of *LAMP3* independently observed in MCF-7 cells and primary breast tumors suggests that this epigenetic event can be initiated in normal breast epithelial cells and then heritably passed on to cancer cells during the course of malignant progression. We further speculate that DNA methylation of *LAMP3* is potentially acquired as a result of long-term exposure of progenitor cells to BPA and other xenoestrogens.

Interestingly, promoter hypermethylation of this locus was found to be associated with older ERα-positive breast patients in the US cohort and Taiwan cohort. However, the levels of DNA methylation distribution showed significantly differences between age groups. This epigenetic disparity could be attributed in part to the geographical differences of breast cancer incidence in these cohorts. Compared to the US patient population, there has been an increased trend of ERα-positive young breast cancers (<50 years old) in Taiwan (Lin *et al.*, 2009). Future population study is needed to additionally determine whether different

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exposure history of BPA and other related chemicals contribute to this epigenetic disparity in the two patient populations.

Conclusions

In the present study, we have shown that the mammospheres exposure system is a valuable tool for validation studies of BPA findings based on animal models. We observed heritable effects of low-dose BPA on the nuclear localization of ERα and differential gene expression in primary MDECs. Long-term exposure of breast progenitor cells to BPA may promote ligand-independent ERα actions in differentiated progeny. Furthermore, genetic variations of individuals may contribute to differential susceptibility of breast epithelial cells to the environmental exposure. We have also identified 170 BPA-influenced genes that likely play a role in the development of ERα-positive breast cancer. These loci are potential biomarkers for assessing the risk of developing breast cancer from exposure to other environmental chemicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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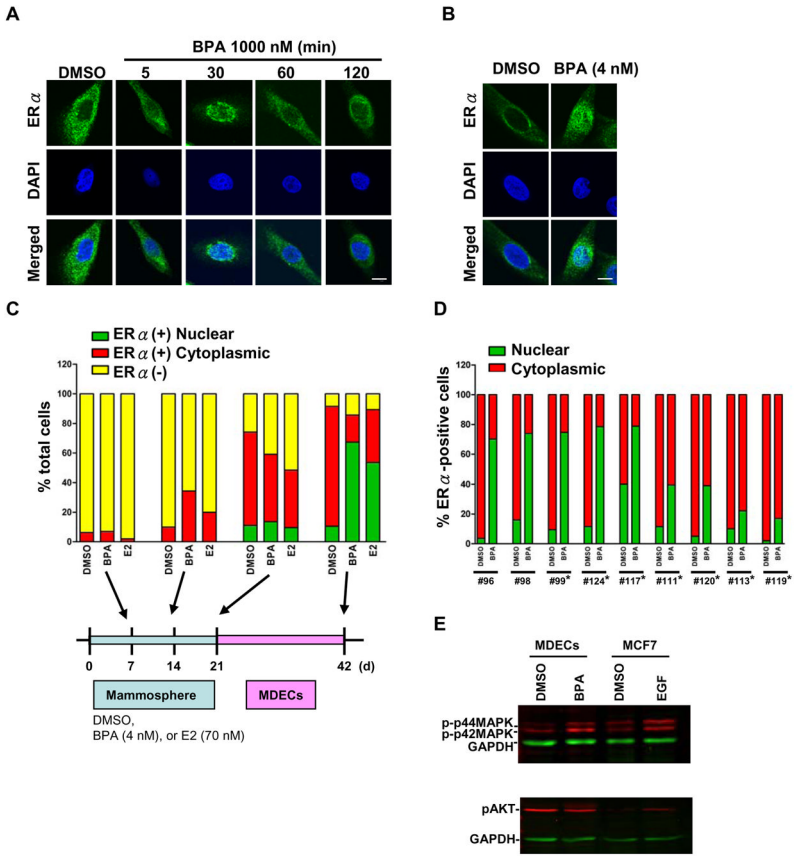

Fig. 1.

Preexposure of MDECs to bisphenol A (BPA) and immunofluorescence analysis of nuclear ERα. (A) Subcellular localization of ERα in MDECs on acute BPA treatment. MDECs were exposed to BPA (1000 nM) for the indicated time periods. The observed translocation of ERα protein (green) from the cytoplasm to nucleus is indicative of functional estrogen signaling. Nuclei were stained with DAPI (blue). Bar = 10 μm. (B) Mammospheres were treated with BPA 4 nM or DMSO for 3 weeks. After the exposure, mammospheres were washed with PBS to remove BPA and then placed on a collagen coated dishes for differentiation. Immunofluorescence staining showed that translocation of ERα protein (green) from the cytoplasm to nucleus was observed. Nuclei were stained with DAPI (blue). Bar = 10 μm. (C) Increased internalization of ERα. in BPA-preexposed MDECs (#124). Mammospheres were treated with DMSO, BPA (4 nM) or E2 (70 nM) for 3 weeks. The distribution of ERα in mammospheres was monitored each week as indicated in the bottom graphic (7, 14, and 21 d). After the exposure, BPA was removed, and progenitor cells underwent epithelial differentiation in the collagen-coated dishes for 3 weeks. ERα localization was also monitored as indicated in figure (42 d). Yellow bars indicate the percentage of ERα-negative cells within the total population. Green bars (nuclear ERα) and red bars (cytoplasmic ERα) represent ERα-positive cells within total population. (D) Increased nuclear localization of ERα in BPA-preexposed MDECs. After the preexposure to BPA (4 nM) or DMSO, MDECs were subjected to immunofluorescence analysis. (C–D) The percentage of subcellular localization of ERα-positive cells, independently scored by two researchers, is shown. These results were collected from 9 independent sets of MDECs samples. *, indicates samples were also subjected to gene expression analysis. (E) BPA induced p42/44 phosphorylation. Phosphorylated levels of p42/44 MAPK and Akt were

analyzed in MDECs preexposed to BPA (4 nM) or DMSO by western blotting. GAPDH was used as loading control.

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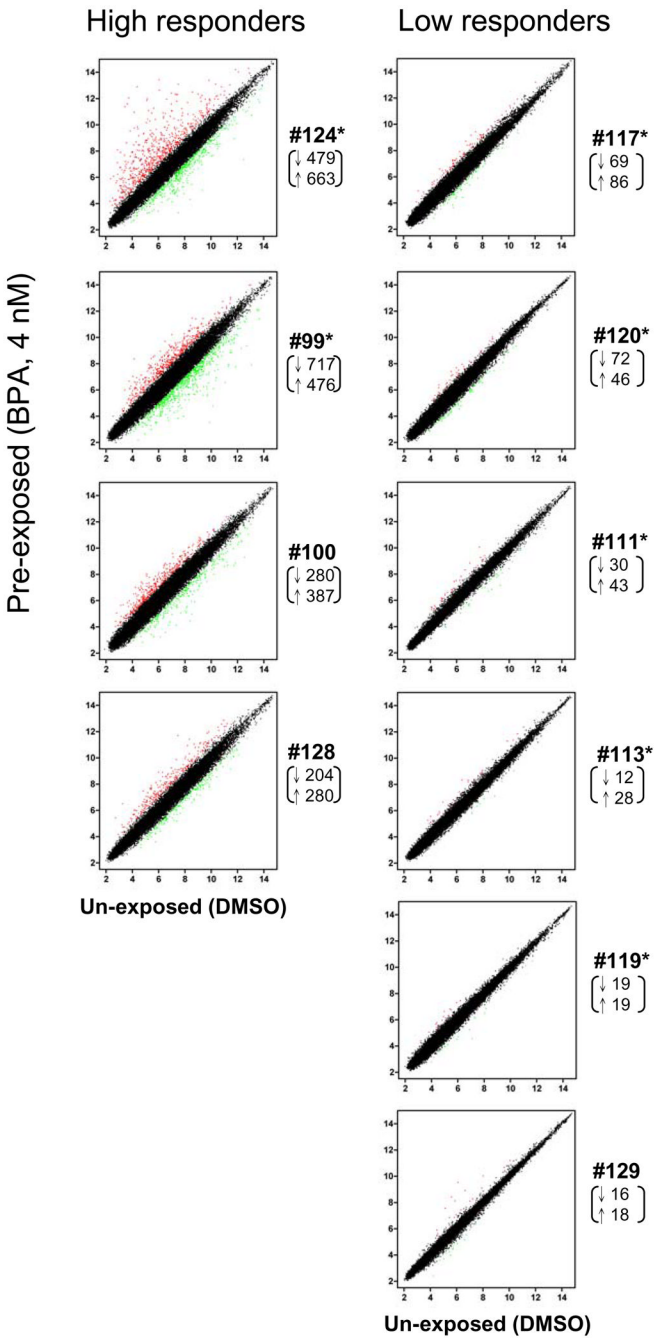

Fig. 2.

Gene expression profiling of ten sets of the MDECs. A total of genes in control (DMSO) and BPA-preexposed cells are shown in the scatter plot. The number of significant down-regulated (↓, green dots) and up-regulated (↑, red dots) genes (2 fold differences between DMSO *vs.* BPA) are shown below the ID#. *, samples were also analyzed for ERα nuclear localization.

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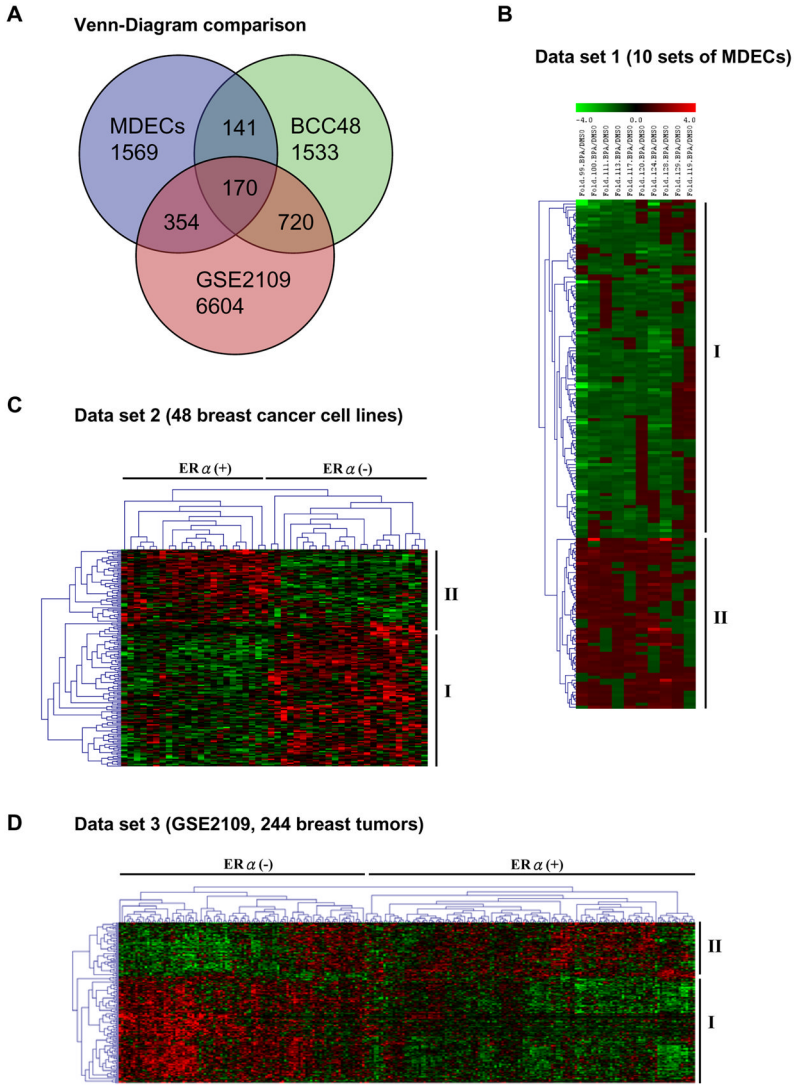

Fig. 3.

Comparison of gene expression profiles in BPA-preexposed MDECs, 48 breast cancer cell lines (Neve et al. 2006) and breast tumor samples (GSE2109). (A) Venn diagram showing 170 common genes identified in separate analyses of the three different data sets, i.e., MDECs (2234 genes altered by BPA), breast cancer cell lines (2564 genes preferential difference between ERα-positive and ERα-negative), and primary tumor samples (7848 genes preferential difference between ERα-positive and ERα-negative). Gene tree cluster analysis was performed on the 170 genes altered by BPA in MDECs (B), ERα-stratified cell line (C) and patient tumor data sets (D). This analysis identified 113 repressed (panel B, group I) and 57 activated (panel B, group II) genes in BPA-preexposed MDECs. Group I genes were also identified in ERα-positive breast cancer cell lines (panel C) and primary tumor samples (panel D); group II genes were identified in ERα-negative breast cancer cell lines (panel C) and primary tumor samples (panel D). Color bar, magnitude of gene expression; green, repression; red, stimulation.

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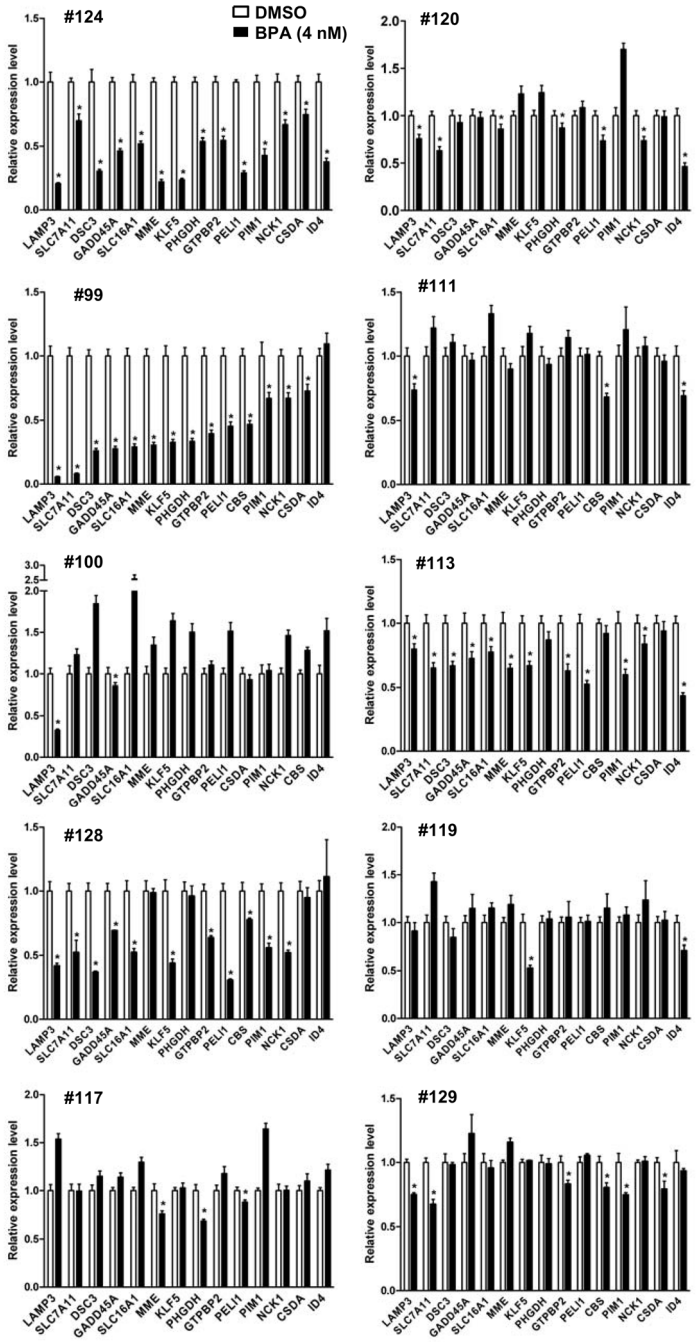

Fig. 4.

Validation of differentially expressed loci by RT-qPCR. Gene-specific RT-qPCR on 10 independent sets of DMSO and BPA-preexposed MDECs was conducted to validate 15 down-regulated loci. Data were analyzed by ΔΔCt method using *36B4* as the internal control and are presented relative to DMSO treatment for each MDEC sample. Mean ± SD (n = 3). *, $P < 0.05$ (Student's *t* test) for down-regulated genes compared with DMSO treated control.

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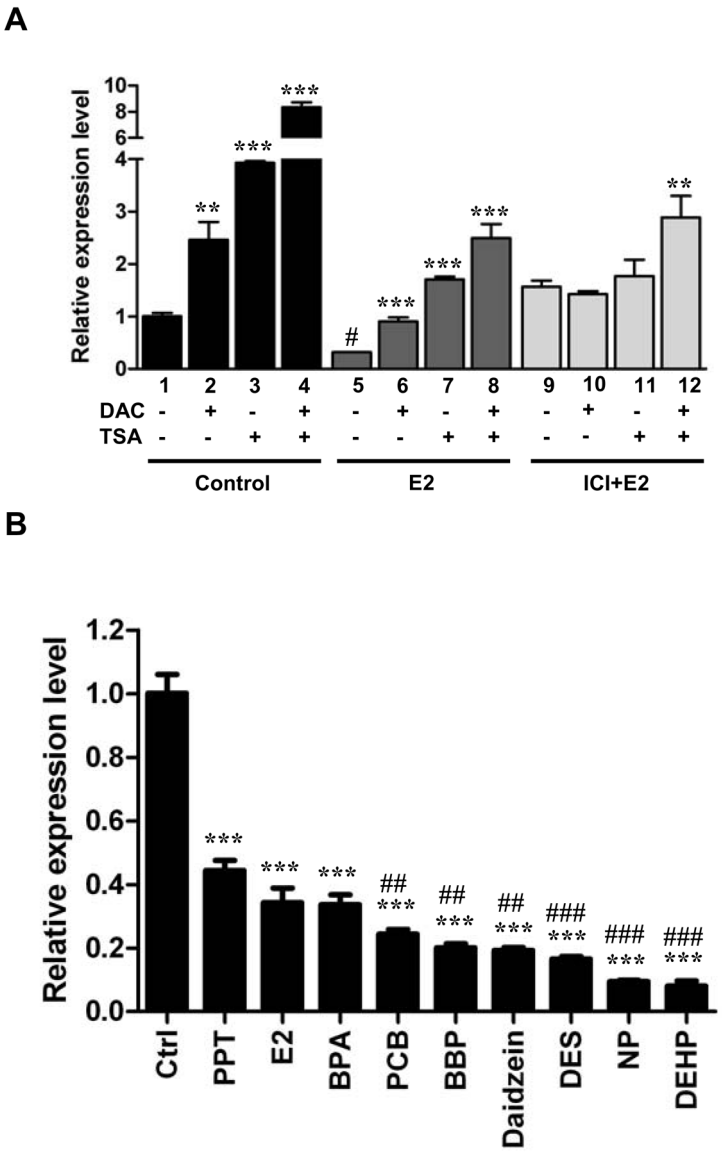

Fig. 5.

Epigenetic reactivation of ERα-mediated *LAMP3* repression in MCF-7. (A) MCF-7 were treated with DAC (1 μM), TSA (1 μM) and/or ERα antagonist, ICI182780 (ICI, 1 μM) 6 hr before E2 stimulation. Total RNA was subjected to RT-qPCR analysis. *36B4* was used as internal control. Mean ± SD; **, $P < 0.01$, ***, $P < 0.001$ compared with DMSO treated control (lane 1–4), E2 treated alone (lane 5–8), and ICI+E2 alone (lane 9–12). #, $P < 0.001$ compared lane 5 (E2 treatment) with lane 1 (DMSO). (B) Mammospheres were exposed to E2 (70 nM), diethylstilbestrol (DES, 70 nM), daidzein (10 μM), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 0.1 nM), 4-nonylphenol (NP, 1 μM), N-butyl-benzyl phthalate (BBP, 10 μM), di(2-ethylhexyl)-phthalate (DEHP, 10 μM), and 4,4′-dichloro-biphnyl (PCB, 0.1 nM) for 3 weeks. After the exposure, the MDECs were subjected to RT-qPCR analysis for *LAMP3* expression. Mean ± SD; ***, $P < 0.001$ compared with DMSO treated control. ##, $P < 0.01$, ###, $P < 0.001$ compared with BPA treated sample.

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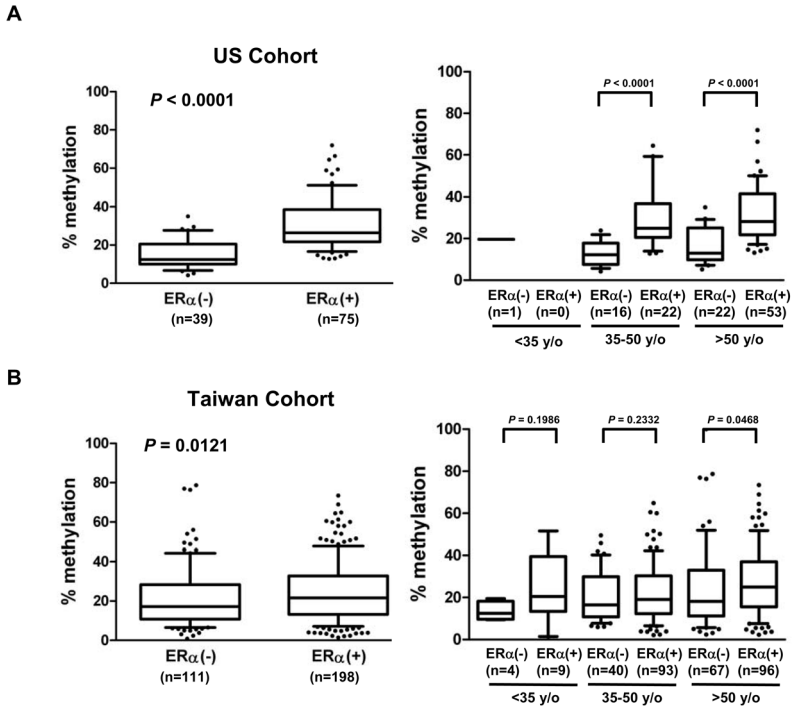

Fig. 6.

DNA methylation analysis of *LAMP3*. Quantitative methylation profiles of tumor samples from the US and Taiwan cohorts are shown in supplemental figure S3A. (A) Box plots indicate that the level of *LAMP3* promoter methylation is positively correlated with ERα status in primary tumors from the US cohort (left panel). A positive correlation between *LAMP3* methylation and ERα status is also observed in patient age 35–50 years, and >50 years (right panel). (B) Box plots indicate that the level of *LAMP3* promoter methylation is positively correlated with ERα status in primary tumors from the Taiwan cohort (left panel). Further analysis shows a positive correlation between *LAMP3* methylation and ERα status is observed in the age >50 years (right panel).