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# Perinatal Exposure to Bisphenol A Increases Adult Mammary Gland Progesterone Response and Cell Number

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Bisphenol A [BPA, 2,2,-bis (hydroxyphenyl) propane] is one of the highest-volume chemicals produced worldwide. It is detected in body fluids of more than 90% of the human population. Originally synthesized as an estrogenic compound, it is currently utilized to manufacture food and beverage containers resulting in uptake with food and drinks. There is concern that exposure to low doses of BPA, defined as less than or equal to 5 mg/kg body weight /d, may have developmental effects on various hormone-responsive organs including the mammary gland. Here, we asked whether perinatal exposure to a range of low doses of BPA is sufficient to alter mammary gland hormone response later on in life, with a possible impact on breast cancer risk. To mimic human exposure, we added BPA to the drinking water of C57/Bl6 breeding pairs. Analysis of the mammary glands of their daughters at puberty showed that estrogen-dependent transcriptional events were perturbed and the number of terminal end buds, estrogen-induced proliferative structures, was altered in a dose-dependent fashion. Importantly, adult females showed an increase in mammary epithelial cell numbers comparable to that seen in females exposed to diethylbestrol, a compound exposure to which was previously linked to increased breast cancer risk. Molecularly, the mRNAs encoding Wnt-4 and receptor activator of nuclear factor κB ligand, two key mediators of hormone function implicated in control of mammary stem cell proliferation and carcinogenesis, showed increased induction by progesterone in the mammary tissue of exposed mice. Thus, perinatal exposure to environmentally relevant doses of BPA alters long-term hormone response that may increase the propensity to develop breast cancer. **(*Molecular Endocrinology* 25: 0000–0000, 2011)**

**NURSA Molecule Pages[†]: Nuclear Receptors:** PR; **Ligands:** Bisphenol A.

Bisphenol A [BPA, 2,2,-bis (hydroxyphenyl) propane] originally synthesized as a chemical estrogen (1), is a high-volume chemical with a global production of 4 million tons in 2006 (2). It is used, in particular to manufacture food and beverage containers from which it can leach out. Its pervasive presence in the environment leads to continuous exposure of the human population (3). Uptake is mostly via food and drinks as well as through

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[†] Annotations provided by Nuclear Receptor Signaling Atlas (NURSA) Bioinformatics Resource. Molecule Pages can be accessed on the NURSA website at www.nursa.org.

Abbreviations: BPA, Bisphenol A; bw, body weight; DES, diethylstilbestrol; ER, estrogen receptor; FACS, fluorescence-activated cell sorting; PR, progesterone receptor; RANKL, receptor activator of nuclear factor κB ligand; SLPI, secretory leukoprotease inhibitor; TEB, terminal end bud.

dental fillings and skin contact with thermal paper, widely used for receipts (4).

To date, regulatory bodies in the United States and European Union support safety of low-dose BPA exposure, defined as less than or equal to 5 mg/kg-body weight (bw)/d, the lowest dose used in standard toxicological tests, with a US Enivironmental Protection Agency calculated reference dose of 50 μg/kg-bw/d (5). A number of studies in rodents raise the concern that exposure to low doses of BPA may have developmental effects in various hormone-responsive organs, including the mammary gland, with potential consequences for public health (6–8).

In particular, the hypothesis that perinatal exposures to hormonally active compounds may affect breast cancer risk has been put forward. It is supported by observations made on women exposed to diethylstilbestrol (DES) *in utero*. This estrogenic compound was widely administered to pregnant women in the 1950s and 1960s. The Food and Drug Administration banned DES when uterine exposure to the drug was linked to clear-cell vaginal adenocarcinoma in teenage girls (9). Recently, DES daughters were found to have increased breast cancer risk, with a relative risk of 1.83 after age 40 (10–12). Many of the developmental abnormalities in the reproductive tract observed in human patients were recapitulated in mice and rats by perinatal DES exposure (9, 13) suggesting that rodents can be valuable models for assessing the endocrine disruptive effects of such compounds. Studies in CD-1 mice and rats have linked exposure to low doses of BPA to changes in mammary gland development and an increased propensity to develop mammary carcinomas (6, 7, 14, 15).

Based on these and other concerns, Canada and the European Union banned the use of BPA in baby bottles, yet no general consensus to change current regulations has been reached. The issue is complicated; any assessment of endocrine function is complex and for any biological endpoint the number of confounding factors is large. Hence, large sample sizes are required. Establishing dose-dependent effects, an essential part of standard toxicological assessments, can require enormous numbers of mice and render costs prohibitive.

To assess whether perinatal exposure to low-dose BPA in environmentally relevant conditions affects the mammary gland hormone response, we mimic human exposure, most of which occurs by mouth via food and beverage containers, by adding BPA to the drinking water of breeding C57Bl6 mice. We test a range of doses below those used in standard toxicology testing (5 mg/kg-bw/d) including estimated daily uptake by formula-fed infants (Fig. 1A). To evaluate the long-term impact of this exposure on the mammary gland hormone response, we used novel experimental endpoints such as single-cell analysis by flow cytometry and hormone exposure experiments on freshly isolated organoids, which facilitate statistical analysis. We find that perinatal exposure to environmentally relevant BPA levels has dose-dependent effects on the response to estrogens during puberty and that it alters long-term the response to progesterone with increased mammary epithelial cell numbers in adult females. Molecularly, we identify increased induction of the central mediators of progesterone function, wnt-4 and receptor activator of nuclear factor κB ligand (RANKL). Hence, perinatal exposure to environmentally relevant doses of BPA has long-term effects on the mammary gland with implications for breast cancer risk that need to be carefully evaluated.

## Results

### Experimental setup

To mimic continuous human BPA exposure via the oral route, BPA was added to the drinking water of C57Bl/6 breeding pairs at doses ranging from 2.5 μg/liter to 5 mg/liter. Based on average water intake and average weight (see *Materials and Methods*), this corresponds to 0.6 μg to 1.2 mg/kg-bw/d (Fig. 1A). As a point of reference, the US Department of Health and Human Services estimates daily BPA intake in formula-fed infants to 1–13 μg/kg-bw/d. We included DES (0.12 or 1.2 μg/kg-bw/d) another estrogen receptor (ER)α agonist as a positive control. Women exposed to DES *in utero* were shown to be at increased risk for breast cancer after age 40 (10–12).

The female offspring thus exposed *in utero* and postnatally through milk was transferred to a BPA- and DES-free environment at weaning (d 24 ± 1). For each BPA concentration, four different mothers were used. Because dam treatment results in a single exposure group, we analyzed only one daughter per litter for any given experimental endpoint. To obtain a total number (n) of 18–20 animals per treatment group, the experiment was performed in triplicate.

### Pubertal expression of estrogen-dependent genes

Exposure to low doses of oral BPA had no significant effect on litter size, sex ratio, or body weight at weaning (Supplemental Fig. 1, A–C, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We then analyzed mammary glands during puberty, more specifically at 30 ± 1 d of age, when estrogens drive development (16). Because BPA interacts with the ERα and ERβ and can affect estrogen-induced transcription (17), we assessed mRNA expression levels of two well-characterized estrogen target genes, the progesterone receptor (*PR*) (18) and *amphiregulin* (19);

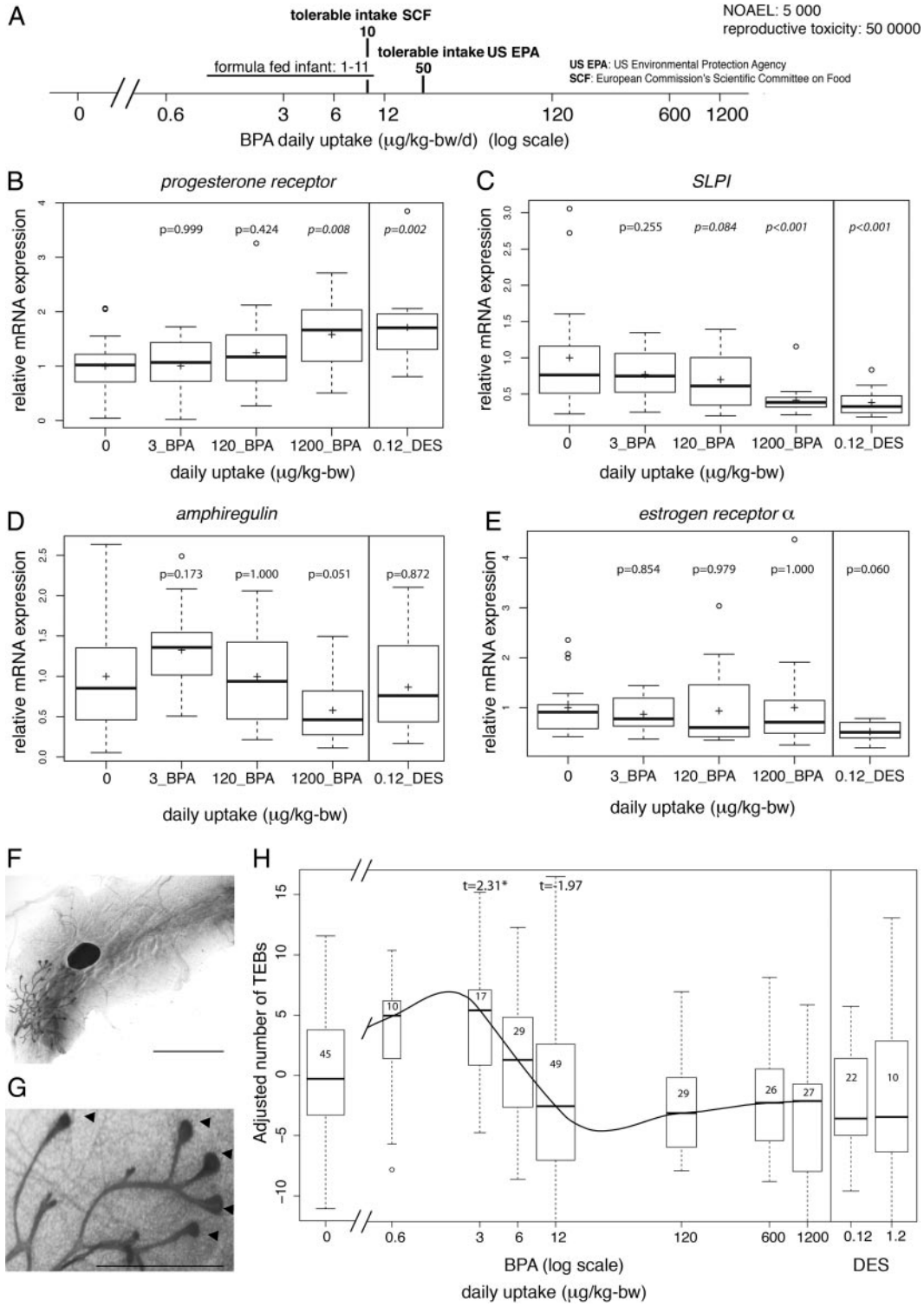


**FIG. 1.** Perinatal BPA exposure and pubertal mammary gland development. A, Range of BPA doses used in the present study expressed as daily uptake (μg/kg-bw). Regulatory benchmarks and estimated human exposure are indicated. B–D, Quantitative RT-PCR analysis of relative mRNA expression of the estrogen-regulated genes *PR*, *SLPI*, and *amphiregulin* as well as ERα in mammary glands of females exposed perinatally to BPA or DES. Values were normalized to cytokeratin 18 (n =18–20 per condition). E and F, Stereomicrograph of a whole-mounted inguinal mammary gland of 30-d-old, unexposed female showing epithelial structures with TEB. E, *Scale bar*, 1 cm. Higher magnification of growing ductal tips; *arrowheads* point to TEB. F, *Scale bar*: 1 mm. G, Effect of BPA and DES on adjusted TEB numbers. The *black curve* represents median smooth. Note, daughters of mothers with a daily intake of 3 μg BPA/kg-bw show an increase in numbers of TEB, which is statistically significant. To evaluate statistical significance, *t* values were determined because the denominator degrees of freedom used to penalize certainty when computing F statistics were unknown given the multilevel data (* indicates statistical significance). NOAEL, No observed adverse effect level.

in addition, we monitored the mRNA specifying secretory leukoprotease inhibitor (*SLP1*), a gene we identified as an estrogen-controlled gene in pubertal mammary glands (Ciarloni L., and C. Brisken, unpublished observations).

Parental daily uptake of 0.12 μg DES/kg-bw resulted in increased *PR* (Fig. 1B) and decreased *SLPI* (secretory leukoprotease inhibitor) (Fig. 1C) mRNA expression but did not significantly affect *amphiregulin* or ERα mRNA expression (Fig. 1, D and E). Similarly, daily uptake of 3, 120, and 1200 μg/kg-bw BPA resulted in dose-dependent effects on *PR* and *SLPI* mRNA expression (Fig. 1, B and C) that were statistically significant and comparable to DES at 1200 μg/kg (n = 18–20). As with DES exposure, ERα mRNA expression was not affected by BPA exposure (Fig. 1E). *Amphiregulin* mRNA expression in BPA-exposed females showed a trend toward a nonmonotonic response, as frequently seen in hormonal responses, which was, however, not statistically significant (n = 18–20) (Fig. 1D). Thus, perinatal low-dose BPA exposure perturbs estrogen signaling in the pubertal mammary gland with positive and negative effects on the transcription of distinct ERα-regulated genes.

## Terminal end buds (TEB)

Estrogen-induced cell proliferation during puberty concentrates at the ductal tips. As a result, they enlarge and form club-like structures that measure between 1.5 to 10 times the diameter of the subtending ducts and are called TEBs (Fig. 1, F and G). To assess whether perinatal BPA exposure affects estrogen function, we determined TEB numbers in a large cohort of mice (n = 10–49 per dose) and analyzed parameters that could potentially confound the analysis. As expected, body weight, one of the factors that determine puberty onset and ovarian estrogen secretion, correlated with number of TEBs, Pearson coefficient 0.31, $P = 1.86e\text{-}06$ (Supplemental Fig. 2A). Inconsistent effects were observed with different litters and between the three experiments (Supplemental Fig. 2, B and C). Perinatal exposure to BPA resulted in a statistically significant increase in adjusted TEB numbers at a dose of 3 μg/kg-bw (t = 2.31) (Fig. 1H). When all BPA doses were considered interdependently as a nonmonotonic function, more specifically, an excess-substrate inhibition function (Supplemental Fig. 2D), and placed as a regressor in a linear mixed effect model, the effect of BPA was significant (t value 3.46) (Supplemental Fig. 2E).

## Mammary cell number in adult females

Next, we examined the long-term outcome in adult mammary glands. In light of the low cell proliferation rates characteristic of the mammary epithelium of adult females that are furthermore susceptible to cyclic changes, difference in proliferation indices due to BPA exposure would be difficult to discern. Hence, we assessed cumulative changes in proliferation *vs.* cell death by quantifying total mammary cell numbers at 3 months of age. To reduce variation, mammary glands from two to three control or BPA-exposed females were pooled and processed in parallel to isolate single cells (20) for each sample point and counted in triplicate by an automated cell counter. A control mouse had on average 1.6 million cells. In glands from BPA-exposed females, cell numbers were on average 50% higher than in the controls; this increase was statistically significant with both low (6/12 μg/kg-bw/d) and relatively high (600/1200 μg/kg-bw/d) parental daily intake (Fig. 2A).

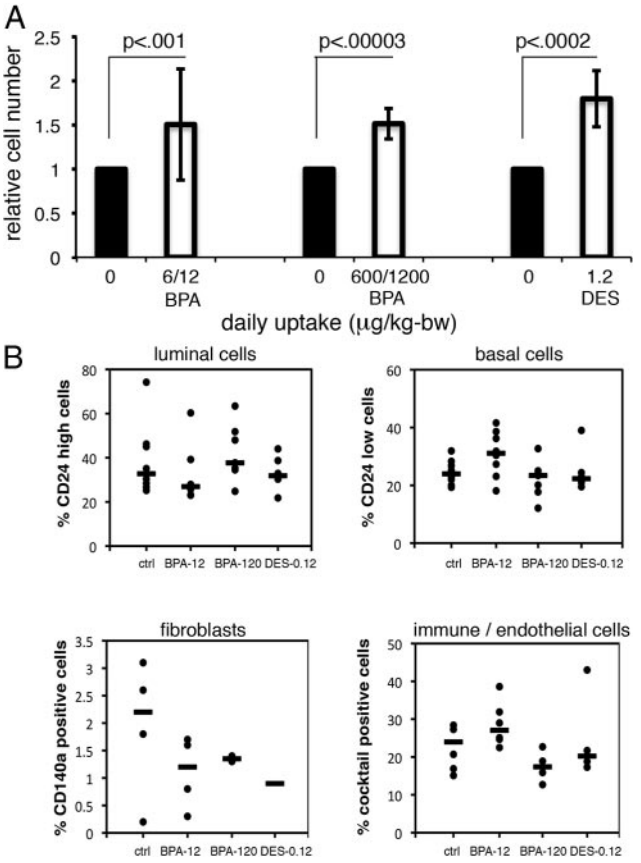


**FIG. 2.** Perinatal BPA exposure and mammary cell populations in adulthood. A, Relative cell numbers in mammary glands from 3-month-old BPA- or DES-exposed or control animals. For each count, two to three animals were pooled per condition. Number of independent experiments is n = 5 f for DES exposure, n = 20 and n = 6, for 6/12 and 600/1200 μg/kg-bw BPA exposure, respectively. B, FACS analysis of single cells derived from mammary glands of exposed and unexposed females. The proportions of distinct cell populations characterized by distinct cell surface antigens are plotted over BPA and DES exposure doses (indicated values are in mg/kg-bw/d). Mammary epithelial cell compartments, luminal (CD24 high) and basal (CD24 low) and the percentage of fibroblasts (CD140a) and combined population of immune and endothelial cells (cocktail: CD45 and CD31) were not significantly altered with $P > 0.06$ for all conditions (from *left to right*: luminal, $P = 0.34/0.67/0.44$; basal, $P = 0.07/0.42/0.94$; fibroblasts, $P = 0.29/0.58/0.34$; immune/endothelial, $P = 0.12/0.17/0.68$). Each *dot* represents one mouse; experiments were performed in triplicate. *Bars* represent median values. ctrl, Control.

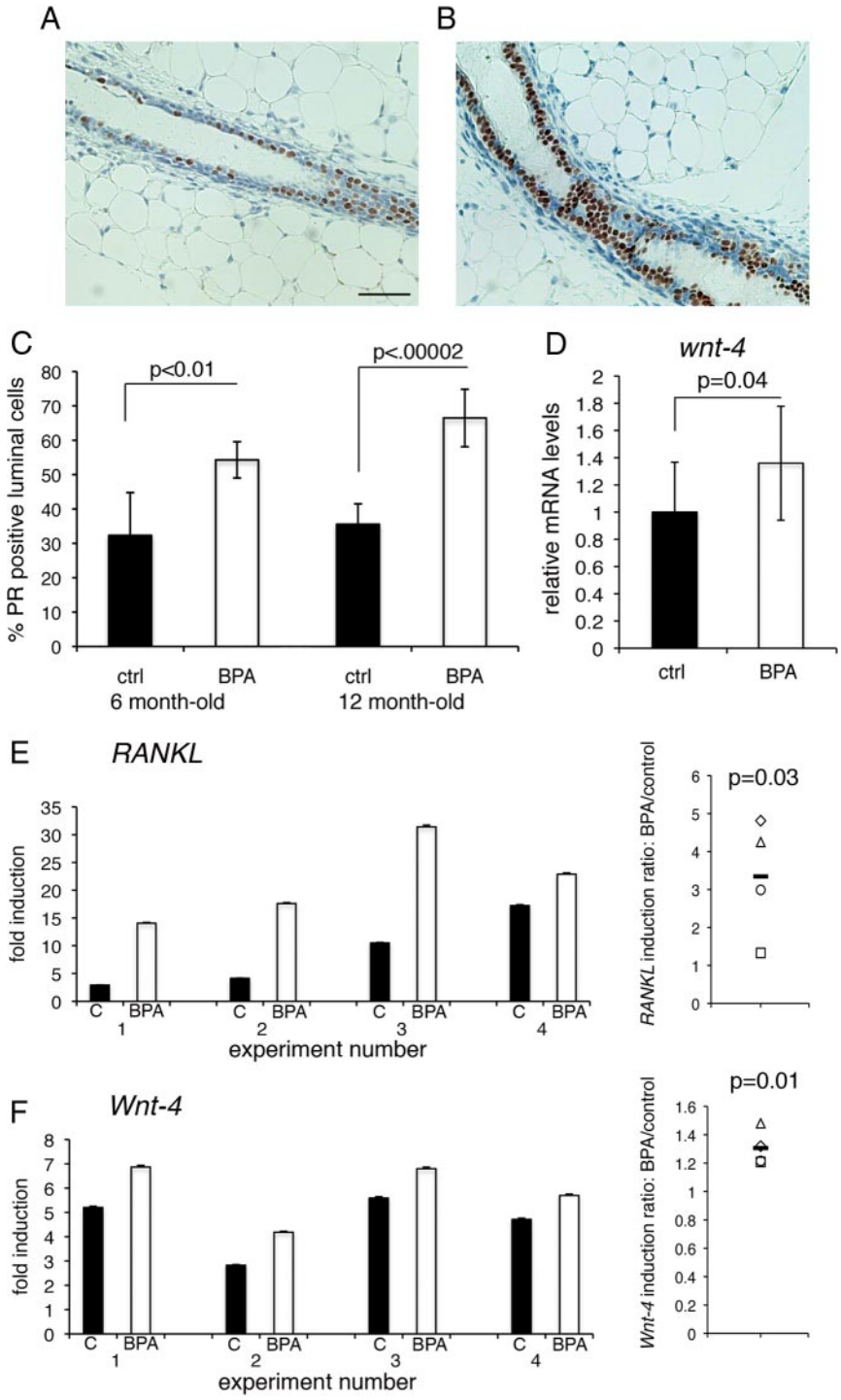


**FIG. 3.** Perinatal BPA exposure alters response to progesterone. A and B, Histological sections of right inguinal mammary glands from control (panel A), or BPA-exposed (6 μg/kg-bw/d) (panel B), 12-month-old females, stained with antibody against *PR*. *Scale bar*, 50 mm. C, *Bar graphs* showing percentage of *PR*-positive luminal epithelial cells in mammary glands of unexposed and BPA-exposed females at 6 and 12 months of age (n = 5–7 per condition). More than 500 cells were counted in three distinct sectors. D, Basal levels of Wnt-4 mRNA are increased in the mammary glands of BPA-exposed (6 μg/kg-bw/d) females *vs.* their age-matched unexposed controls (n = 12). E and F, *Bar graphs* showing induction by R5020 of RANK (panel E) and Wnt-4 mRNA (panel F) in unexposed females (*solid bars*) and in daughters of parents with a daily intake of 6 μg/kg-bw BPA (*open bars*). Four independent experiments are shown. Ratios of RANKL and Wnt-4 mRNA induction in response to R5020 treatment between control and BPA-exposed animals are shown in *right panels*. Note, BPA exposure results in a significant increase in Wnt4 and RANKL induction. *Bars* represent mean values ± SD of triplicate RT-PCR. ctrl, Control.

Mammary glands from DES-exposed females showed a 70% increase in cell number.

Organoids consist of both luminal and basal mammary epithelial cells and contain. in addition, various stromal cell types. Analysis of dissociated mammary glands for multiple cell-surface antigens by fluorescence-activated cell sorting (FACS) revealed no change in the ratio of luminal (CD24 high) *vs.* basal (CD24 low) epithelial, as well as distinct stromal cell populations such as fibroblasts (CD140a+), hematopoetic (CD45+). and endothelial cells (CD31+) in BPA- or DES-treated compared with control mice (Fig. 2B) indicating a proportional increase of various cell types.

### *PR* and downstream signaling mediators

Progesterone is a major proliferative signal in the adult mammary gland (21) and is likely to play an important role in cancer development. The *PR* is expressed on subset of mammary epithelial cells. and the hormone acts via paracrine mechanisms (22). Immunohistochemistry for *PR* revealed a pronounced increase of *PR*-positive cells within the luminal epithelial population (Fig. 3, A and B) of BPA-exposed animals (6 μg/kg-bw/d). The percentage of *PR*-positive cells was 32.3 ± 12.4 and 35.6 ± 5.9% in 6- and 12-month-old unexposed mice, whereas it was increased to 54.3 ± 5.3 ($P < 0.01$) and 66.5 ± 8.4% ($P < 0.00002$) in exposed animals, respectively (Fig. 3C).

Wnt-4 and RANKL are important downstream mediators of progesterone function (21, 23), both of which have recently been implicated in the control of stem cell function (24, 25). Additionally, RANKL has been linked to mammary carcinogenesis (26, 27). Analysis of mRNA expression in mammary glands from 12 age-matched adult females revealed statistically significant increase in Wnt-4 mRNA levels in BPA (6 μg/kg-bw/d)-exposed animals (Fig. 3D). RANKL expression, however,

varied over several orders of magnitude between individual mice so that statistically significant differences between control and BPA-exposed females could not be assessed.

To circumvent the problem of quantifying changes in RANKL expression and to determine whether the increased cell number in BPA-exposed animals reflects increased responsiveness to progesterone, we harvested mammary glands from 3-month-old female offspring of exposed (6 μg/kg-bw/d) and unexposed mothers and briefly homogenized the tissue. Subsequently, the samples were subjected to gentle enzymatic dissociation (see *Materials and Methods*) for 6 h, either in the presence of the progesterone agonist 20 μmol R5020 or vehicle. Real-time RT-PCR of the resulting organoids revealed that both RANKL and Wnt-4 mRNA were consistently increased by the R5020 treatment (Fig. 3, E and F, *solid bars*). The extent of the induction of RANKL mRNA was on average 320% and of Wnt-4 mRNA on average 30% higher in the BPA-exposed than in control tissue (Fig. 3, E and F, *right panels*). Thus, perinatal exposure to BPA enhances the transcriptional response to progesterone in the adult mammary gland.

## Discussion

The present study reveals that perinatal BPA exposure has long-term developmental effects on the mammary gland in C57/Bl6 mice. Our findings are consistent with and extend previous studies in CD1 mice and rats (6, 7, 14). Muñoz-de-Toro *et al.* (14) exposed pregnant dams to 25 and 250 ng BPA/kg-bw/d through sc implantation of an osmotic pump. They observed that the number of TEBs is increased in the offspring at 30 d. Our finding that TEB number is increased in offspring from mice exposed to 600 ng and 3 μg/kg-bw/d orally is in line with this. Importantly, whereas we found that BPA-exposed animals have higher numbers of *PR* expressing mammary epithelial cells at 6 and 12 months of age than unexposed controls, in CD1 mice a similar difference was already detected in 30-d-old females. Moreover, Western blotting of protein lysates from mammary glands of 50-d-old rats that had been exposed postnatally to BPA revealed increased expression of *PR* (7). Taken together, a picture emerges, in which perinatal exposure to BPA results in increased PR expression resulting in increased sensitivity to this hormone. This, likely, accounts for the increased transcription of wnt-4 and RANKL mRNAs we observed and possibly the increased cell number we described. Similarly, the increased side branching observed in 4-month-old BPA-exposed CD1 females (14) and the increased susceptibility to 7,12-dimethylbenz(a)anthracene-induced carcinogenesis (7) may result from an enhanced response to progesterone (27).

Our study illustrates difficulties and complexities involved in discerning perturbations of endocrine function. Expression analysis of three different estrogen-controlled genes *PR*, *SLPI*, and *amphiregulin*, in the pubertal glands of exposed animals, with 18–20 animals sampled per group, revealed statistically significant effects only for the former two genes at the highest dose tested. Interestingly, the graphical representation suggests differential, dose-dependent effects; *PR* expression shows a tendency to increase and *SLPI* expression shows a tendency to decrease with increasing doses of BPA whereas *amphiregulin* expression, as a function of parental BPA intake, can be described as nonmonotonic. We speculate that an increase in sample size might have revealed these tendencies as statistically significant.

The differences observed at the level of individual gene expression highlight the dilemma of finding robust readouts for endocrine disruptors. Indeed, because we used a more complex, biological readout, namely the number of TEB, which reflects estrogen-induced cell proliferation mediated by *amphiregulin* (28), we found statistically significant effects only at one dose (3 μg/kg-bw/d) although we had used more doses and had adjusted for confounding factors. However, as we considered all doses interdependently, based on an excess substrate-inhibition function, the effect of BPA was highly significant ($t = 3.46$). This highlights the need for large datasets and mathematical modeling to discern dose-dependent effects in an inbred mouse strain under controlled experimental conditions.

Progesterone is the major proliferative stimulus in the adult mouse mammary gland, and exposure to this hormone is increasingly recognized as a risk factor in human breast carcinogenesis. Our finding that mammary epithelial cell numbers are increased in adult females exposed to different low doses of BPA early in life suggests increased sensitivity to progesterone in exposed animals. This finding is of concern, in particular, as the effects of BPA on cell numbers are comparable to those of DES, a compound uterine exposure to which has been linked to increased breast cancer risk (10–12). As a likely molecular mechanism underlying the increased proliferative response to progesterone, we find increased expression of Wnt-4 in the mammary tissue of exposed females and increased induction of both RANKL and WNT-4 mRNA in response to progesterone stimulation. Both factors have been implicated in the stimulation of stem cell proliferation, and their downstream signaling pathways are deregulated in mammary carcinogenesis (25–27). Whether the

enhanced mRNA induction in response to progesterone is attributable to the increased number of progesterone receptor-expressing cells and/or reflects an enhanced transcriptional response in individual hormone receptor-positive cells remains to be addressed.

In the present study, BPA exposure occurred *in utero*, through the placenta, and during prepubertal life, through milk. This points to persistent, epigenetic changes as a mechanism underlying the effects observed in adult females; these may occur in the mammary epithelium itself and/or other tissues that, in turn, affect the mammary gland, such as the pituitary gland or the ovaries. Whether there is a particular developmental window during which BPA exposure causes the changes we observed and whether pre- or postnatal exposure is critical remains to be addressed. Our study did not test effects of prolonged exposure throughout adulthood, which may or may not enhance the observed effects.

Exposure of pregnant CD1 dams to 2.4 $\mu$g/kg-bw/d BPA resulted in increased body weight of their daughters at weaning (29), an effect we did not observe. However, we noticed an increase in body weight in the daughters of mothers exposed to low-dose BPA later in life (data not shown). This discrepancy may be related to genetic differences between the two strains, as illustrated by previous observations that effects of perinatal BPA exposure were more pronounced in CD-1 than in C57Bl/6 mice (30).

Taken together, our findings indicate that perinatal exposure to doses of BPA that are currently considered safe for the human population can have long-term, measureable biological effects on the mouse mammary gland. In the course of the experiments in which we followed mice up to more than 1 yr we did not observe palpable tumors, suggesting that BPA exposure is not sufficient to cause mammary carcinomas. We note that the C57Bl/6 mouse strain used for our studies has no predisposition to mammary carcinogenesis (31). Therefore, it is conceivable that similar exposures might result in more striking changes in other mouse strains. In light of the high prevalence of breast cancer, with one in eight women affected, however, minor increases in relative risk, that could result from an increased response to progesterone, although seemingly unimportant at the level of an individual, could have a major impact at the population level.

Clearly, it is impossible to extrapolate our findings in mice directly to other species. Nevertheless, the rodent models, *i.e.* different mouse and rat strains, have provided many insights about mammary gland development and mammary carcinogenesis. In light of our observation that BPA and DES have some comparable effects on the mammary gland of C57Bl/6 females, direct or indirect, the possibility that low-level BPA exposure, may cause an elevated breast cancer risk in the human population as did DES cannot be discounted but should be further explored.

## Materials and Methods

### Mice

All mice were maintained and handled according to Swiss guidelines for animal safety. C57BL6/J mice were bred in a BPA-free environment using polysulfone cages and bottles, autoclaved water, and no paper towels. Breeding pairs consisting of a male and two females were randomly assigned to treatment groups within each cohort. Male pups were killed at 10 d of age. Depending on the litter size, one or two females per litter were killed at 30 $\pm$ 1 d of age (two females used if five or more females exist). The remaining females were kept for analysis at later time points. Female pups were weighed at weaning (24 $\pm$ 1 d) and randomly housed to have multiple treatments in the same cage. Experiments were repeated three times. Five consecutive litters were used for analysis, and all animals born were tracked in relation to mother and littermates, date of birth, litter size, age at termination of males, weight and age at weaning, and at termination of females. Weight was determined at 30 $\pm$ 1 d. Right inguinal mammary gland was whole mounted, and the left one was processed for RNA extraction after lymph node removal.

Stocks of BPA (Sigma, St. Louis, MO) (25 mg/ml) and DES (Sigma) (50 mg/ml) were prediluted in dimethylsulfoxide (Sigma) so that final concentration was always 200 $\mu$l dimethylsulfoxide/liter water.

### Cell counts, progesterone treatment, and FACS

Mammary cell preparations were performed as described elsewhere (20). Cells were counted using Casy TT Cell Counter Analyzer (Roche, Reutlingen, Germany). Two-tailed, paired Student's *t* test was used to calculate statistical significance; *P* values were indicated on the figures. For progesterone treatment, enzymatic digestion with Collagenase 0.25% is performed in the presence of ethanol or R5020 (Sigma) for 6 h at 37 C, and organoids are flash frozen for RNA isolation after a PBS wash. For FACS analysis, biotinylated anti-CD31, anti-CD45, biotinylated or APC conjugated anti-CD140a (eBiosciences, San Diego, CA), phycoerythrin, or fluorescein isothiocyanate conjugated anti-CD24 (BD Biosciences, Palo Alto, CA) were used. Streptavidin-allophycocyanin and Streptavidin-Alexa 750 (eBiosciences) were used to detect biotinylated antibodies. Analyses were done on BD LSR II Flow Cytometer System (BD Biosciences).

### Statistical analysis

Statistical analysis is performed using R environment (32). Observed TEB data are modeled in Mixed-Linear Effect Model with lme4 package [Bates D. and M. Maechtler, 2010, Package "Ime4", http://cran.r-project.org/web/packages/Ime4/Ime4.pdf (accessed November 2010)]. For noninterdependent BPA dose analysis, effects of fixed-factor weight with random-factors generation and experiment number were assessed on control mice and subsequently subtracted from observed TEB to compute adjusted TEB. Adjusted TEB were then modeled with factorized

BPA doses. For interdependence, BPA doses were modeled as a nonmonotonic function in which coefficients A, B, and C were optimized using a nonlinear model. In the resulting Mixed-Effect Linear model of TEB weight figured as fixed factor and generation and round figured as random factors.

## BPA measurements

BPA concentration was measured by ultra-performance liquid chromatography coupled to a tandem mass spectrometer (UPLC-MS/MS). BPA was undetectable in autoclaved water (detection limit, 0.08 $\mu$g/liter), water from commercial Evian plastic bottles (Evian-eau MQ in bottles made with BPA-free plastic) was used as negative control and positive control was our 50 $\mu$g BPA/liter bottle (measures 57.1 $\mu$g/liter).

## BPA administration

BPA was added to the drinking water of C57Bl/6 breeding pairs at doses ranging from 2.5 mg/liter to 5 mg/liter. To determine BPA intake, water consumption in nine cages was measured over four consecutive weeks, and 27 breeding animals were weighed. Average daily water intake and average weight are measured as 7.17 ml and 31.13 g, respectively. Hence, calculated average water intake of 230.32 ml/kg-bw/d is similar to previously published values (34). Based on this calculation, 50 $\mu$g/liter of BPA used in the study is equivalent at 50*0.23 = 11.5 $\mu$g/kg-bw/d consumption. Calculated values were rounded up for simplicity.

## Mammary gland whole mounts as described elsewhere (16)

### *mRNA isolation and quantitative RT-PCR*

Total RNA extraction and cDNA synthesis were performed as described (20). Real-time RT-PCR was performed with SYBR Green PCR Core Reagents (Quanta Biosciences, Gaithersburg, MD) on automated 7900HT Real-time System (Applied Biosystems, Foster City, CA) with liquid handling system (Hamilton) used to prepare 384-well reaction plates. All reactions were done in triplicate. All expression levels were normalized to cytokeratin 18. For each condition RANKL and Wnt4 induction were calculated by comparing ethanol-treated samples with R5020 treated samples. Relative increases in induction were computed as the ratio of inductions in response to R5020 in control and BPA-exposed animals. For all results, two-tailed, paired Student's *t* test was used to calculate statistical significance; *P* values were indicated on the figures. Primers are as follows: *ERα* , 5′-GCACAAGGGTCAGAGAGATG-3′ and 5′-ATAGATCATGGGCGGTTCAG-3′; *PR*, 5′-GGTGGAGGTC GTACAAGCAT-3′ and 5′-CTCATGGGTCACCTGGAGTT-3′; *Amphiregulin*, 5′-GCCATTATGCAGCTACTTTGGAGC-3′ and 5′-TGTTTTTCTTGGGCTTAATCACCT-3′; *Wnt4*, 5′-AGGAGTGCCAATACCAGTTCC-3′ and 5′-CAGTTCTCCA CTGCTGCATG-3′; *CK18*, 5′-CAAGATCATCGAAGACCT GAGGGC-3′ and 5′-TGTTCATACTGGGCACGGATGTCC-3′; *36B4*, 5′-GTGTGTCTGCAGATCGGGTA-3′ and 5′-CAGA TGGATCAGCCACGAAG-3′; *RANKL*, 5′-ACCAGCATCAA AATCCCAAG-3′ and 5′-AAGGGTTGGACACCTGAATG-3′; *SLPI*, 5′-CACAATGCCGTACTGACTGG-3′ and 5′-GACAT-TGGGAGGGTTAAGCA-3′.

## Histological examination and immunohistochemistry

Glands fixed with 4% paraformaldehyde were paraffin embedded. Sections (4 $\mu$m) were stained with anti-*PR* (1:400) (Neomarkers, SP2, Fremont, CA) after antigen retrieval in citrate buffer and revealed with Vectastain Elite kit (Vector Laboratories, Burlingame, CA) on DiscoveryxT (Ventana Medical Systems, Tucson, AZ). Pictures were acquired with a Leica DM2000 microscope (Leica, Inc., Deerfield, IL) and Pixelink PL-A622C camera. Two-tailed, paired Student's *t* test was used to calculate statistical significance; *P* values are indicated on the figures.

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