Original Research

Mammary gland morphology and gene expression signature of weanling male and female rats following exposure to exogenous estradiol

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Abstract

In order to characterize the actions of xenoestrogens, it is essential to possess a solid portrait of the physiological effects of exogenous estradiol. We assessed effects of three doses of exogenous estradiol (E2) (0.1, 1.0 and 10 μg/kg/day) given between postnatal days 21 and 33 on the mammary gland morphology and gene expression profiles of male and female rats compared to vehicle-treated controls. The male mammary gland was more responsive to E2 treatment than in females, with 509 genes regulated >2-fold in a dose-dependent manner in males and only 174 in females. In males, E2 treatment significantly ($P < 0.01$) increased the number of terminal end buds (TEBs) and the expression of proliferating cell nuclear antigen (PCNA) protein ($P < 0.05$), both of which are indicators of proliferation. This change was linked to a significant increase ($P < 0.05$) in the expression of the gene encoding amphiregulin, which is known to induce TEB formation. There was also a dose-dependent increase ($P < 0.001$) in the estrogen-regulated gene encoding the progesterone receptor. In intact females, despite lack of changes in mammary morphology, we observed a dose-dependent increase ($P < 0.05$) in the expression of genes encoding three milk proteins: whey acidic protein, casein beta and casein kappa. There was a significant ($P < 0.05$) downregulation of both estrogen receptors in response to E2 treatment. These results suggest that mammary glands of male rats are very sensitive to exogenous E2 during development post-weaning. The dose-dependent increase observed in amphiregulin and progesterone receptor gene expression was linked to morphological changes and represents a reliable and sensitive tool to evaluate estrogenicity. In contrast, intact weanling female rats were less responsive.

Keywords: 17β-Estradiol, mammary gland, terminal end buds

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Introduction

Xenoestrogens are defined as pharmaceutical or environmental compounds that can mimic the effect of endogenous estrogens. The health impact of exposure to such compounds is a source of concern, particularly with respect to reproductive toxicity and increased cancer risk. For example, exposure to diethylstilbestrol during human pregnancy increases the risk of structural reproductive tract anomalies and of breast and prostate cancer in offspring.[1–3] Several xenobiotics such as environmental contaminants and food components have been investigated for estrogenic properties, including bisphenol A, polychlorinated biphenyls (PCBs), phthalates and the soy-associated isoflavone genistein.[4, 5] However, in order to properly assess the estrogenicity of a xenobiotic, it is essential to possess an accurate portrait of the effect of exogenously administered 'endogenous' estrogens.

Estrogens are steroid hormones with a wide range of physiological effects. 17β− estradiol (E2) is the principal estrogen in non-pregnant females of reproductive age, while estrone and estriol have roles in menopause and

pregnancy, respectively. E2 is produced in the ovary, or synthesized via steroid precursors in the male testes and in adipocytes in both sexes.[6–9] Examples of the endocrine roles of E2 are the development of secondary sexual characteristics and the modulation of lipid metabolism and bone turnover.[6] The first estrogen receptor to have been discovered, estrogen receptor alpha (ERα; *Esr1*), is essential for mammary and reproductive tract maturation. ERα knockout mice of both sexes are sub- or infertile (reviewed by Couse and Korach [10]). The second estrogen receptor, estrogen receptor beta (ERβ; *Esr2*), is ubiquitously expressed in the body, but is not essential for sexual maturation.[10, 11] ERα activation leads to the production of growth factors such as epidermal growth factor and to cell proliferation. In contrast, signaling through some isoforms of ERβ has a dominant inhibitory function on ERα transcriptional activity when both receptors are expressed in the same cell.[12] The traditional screening test for xenoestrogens is the uterotrophic assay. As the uterus expresses almost exclusively ERα, this test ignores activation of ERβ. Caveats are also associated with *in vitro* assays, which most commonly use ERα-positive malignant cell lines. Although uterotrophic and *in vitro* assays are useful as initial screening for xenoestrogens, they are insufficient to assess the *in vivo* behaviour and safety of potentially estrogenic compounds. Thus, additional tools to evaluate specific estrogen-induced endpoints *in vivo* are needed.

The mammary gland is particularly sensitive to estrogens. The rat mammary gland expresses both ERα and ERβ, with a predominance of ERβ except during lactation.[13] Nonetheless, *Esr2* −/− mice develop normal mammary tissues.[14] ERα, together with insulin-like growth factor 1, regulates ductal morphogenesis, as evidenced by the rudimentary ductal system found in *Esr1* −/− and *Igf1* −/− mice.[15, 16] Despite being associated with proliferation, ERα does not co-localize with markers of proliferation, such as proliferating cell nuclear antigen (PCNA) in mammary epithelial cells because the receptor is downregulated following stimulation with E2.[17] In female rodents, perinatal exposure to estrogens increases the density of terminal end buds (TEBs) in the mammary gland.[18–20] The same was observed in male rats exposed to ethinylestradiol perinatally.[19] Such increases are associated with an increased risk of tumour formation later in life.[4, 21] Other reports have described mammary gland hyperplasia, increase in ductal branching, lobular enlargement, or epithelial proliferation in the male mammary gland associated with the phytoestrogen genistein.[22–24] In humans, exposure to estrogens in adult women, as well as lifelong exposure to high E2 levels, increases the rate of postmenopausal breast cancer and breast cancer recurrence.[25–27] In men, an increase in the risk of developing male breast cancer has been associated with exposure to elevated E2 levels in conditions such as prostate cancer, obesity, Klinefelter's syndrome (XXY) and in transsexual individuals taking estrogens.[28–30] However, at least two studies have examined short term estrogen exposure in immature animals and both have identified a reduction in mammary tumour risk.[31, 32] A meta-analysis also revealed no association between levels of estrogen in young women and premenopausal breast cancer.[26] Similarly, exposure to genistein perinatally in animal models increased mammary gland tumour rates, but reduced it when exposure occurred prior to puberty.[31, 33–35] The timing of exposure to estrogens thus seems to have a significant influence on the response of the mammary gland.

The mammary gland is an excellent estrogen-sensitive target that can be equally analysed in both sexes. There are several reports of changes in gene expression associated with the xenoestrogens bisphenol A, butyl benzyl phthalate and isoflavones in the female mammary gland, as assessed by microarray.[36–40] Gene expression changes have also been assessed in the male rat mammary glands exposed to the xenoestrogens genistein and methoxychlor *in utero* until puberty.[23, 41] Microarrays have proven to be an excellent tool for such toxicological assessment. However, caution must be exercised when extrapolating results, as not all significant changes are necessarily biologically relevant.[42] For this reason, gene expression variations that are linked to physiological or morphological alterations are preferable, especially if these occur at sufficiently low doses to reflect environmental exposures. Morphological changes associated with estrogens are indeed observed in the mammary gland (change in TEB number), which makes it a good potential target organ. Furthermore, the current literature points to the period between weaning and puberty as a window of susceptibility to endocrine changes in this tissue.

There are few reports on the effects of endogenous estrogens on the mammary gland when exogenously administered and this situation hinders research on xenoestrogens.[40, 43] In order to understand how hormonal disrupting compounds affect the mammary gland before puberty and to identify new potentially useful indicators of estrogenic responses, we studied structural and molecular changes following E2 treatment during this phase of life. In this experiment, we describe changes induced by the administration of three doses of exogenous E2 on the morphology and gene expression in the mammary gland of male and female Sprague–Dawley rats exposed between weaning (PND21) and the expected onset of puberty (PND33). This study provides reliable indicators of estrogenic action in both sexes in the maturing mammary gland.

Materials and methods

Animal care and experiment design

Time-impregnated Sprague–Dawley rats arrived at our facility at gestational day 4 (Harlan Industries, Indianapolis, IN) and fed an AIN-93 G formulated diet,[44] except that corn oil replaced soybean oil.[45] Upon birth, animals suckled until weaned onto AIN-93 G on PND21. The animals were separated by sex and were randomly assigned to one of four treatment groups ($n = 10$ for males and $n = 7$ for females): they were subcutaneously infused with polyethylene glycol vehicle (Sigma-Aldrich, Saint-Louis, MO) or with E2 using Alzet 2002 mini-osmotic pumps (Alza Corp., Mountain Vw, ieCA) that were calibrated to produce an E2 dose of 0, 0.1, 1.0 or 10 μg/kg/day. On PND33, the male and female rats were anesthetized with Nembutal

(Pentobarbital; 100 mg/kg, i.p.) and euthanized by decapitation. This corresponds to the average time of vaginal opening in control Sprague–Dawley females in our hands. Serum and organs were collected and frozen at −80°C until analysis. One abdominal mammary gland pair (no. 4) was removed from each animal. One was used for the preparation of whole mounts and the other was formalin-fixed for light microscopy. Mammary gland epithelial area and fat pad areas were measured using an Olympus XZS10 microscope with DP21 imaging system (Olympus, Inc., Center Valley, PA). TEBs were counted from the distal portions of the mammary gland whole mounts examined under an Olympus BX40 microscope, and numbers were determined in five contiguous 100 × fields (diameter 2 mm).

Serum hormones

Serum hormones were measured using 200 μL of frozen serum following manufacturer's instructions (E2: DLS-4800, testosterone:DSL4100, progesterone:DSL-3400, luteinizing hormone:DSL-10-4600). All kits were purchased from Beckman Coulter, Indianapolis, IN.

Mammary microarray set

RNA preparation was performed as described in Singhal *et al.*[46] Briefly, RNA was extracted from frozen mammary tissue using TRI reagent (Molecular Research Center Inc, Cincinnati, OH) and treated with the RNeasy Mini Kit (Qiagen, Valencia, CA). First- and second-strand cDNA syntheses, biotin-labelled cRNA synthesis, fragmentation of cRNA and hybridization reactions were performed using the one-cycle cDNA synthesis kit (Affymetrix Inc, Santa Clara, CA). In addition, 8 μg of purified mRNA was used to synthesize cDNA. Labelled cRNA was synthesized from cDNA using a GeneChip IVT labelling kit (Affymetrix) according to the manufacturer's instructions, and 20 μg of cRNA was fragmented in 5× fragmentation buffer with RNase for 35 min. For each treatment group, cRNA from six individual rats/treatment group were combined into three different pools of two animals each providing $n = 3$/treatment for statistical comparison. cRNA from each of the pools were hybridized to an Affymetrix GeneChip Rat Genome 230 2.0 for 16 h at 45°C in a hybridization oven set at 60 rpm. The probe array was washed and stained using the Affymetrix kit in a GeneChip fluidics station 450 and scanned using GeneChip Scanner 3000. For each of the 31,099 genes on the Affymetrix Rat genome 230 2.0 array, the data on probe-level intensities were extracted using GeneChip Operating Software obtained from Affymetrix and CEL data files generated. The microarray data are available as GEO Accession no. GSE40713 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (NCBI) (http:/www.ncbi.nlm.nih.gov/geo/) The files containing the probe-level intensities were processed using the robust multiarray analysis algorithm (GeneSpring 11.5.1, Agilent Technologies Inc., Wilmington, MA) for background correction, normalization and log transformation.[47] Subsequently, the data were subjected to per-chip and per-gene normalization using GeneSpring normalization

Table 1 Serum hormones and organ weights from male and female rats, PND33

	Serum E2 (pg/mL)	Serum P (ng/mL)	Serum T (ng/mL)	Serum LH (mIU/mL)	Body weight (g)	Liver	Thymus	Spleen	Abdominal fat	Gonadal fat	Testes	Prostate	Uterus
Males													
0 μg/kg/day	5.52 ± 0.63	1.44	0.321 ± 0.125	0.308 ± 0.083	152.0 ± 5.3	5.2 ± 0.1	0.38 ± 0.02	0.36 ± 0.02	0.39 ± 0.04	0.51 ± 0.04	0.83 ± 0.04	0.16 ± 0.01	–
		0.36											
0.1 μg/kg/day	4.64 ± 0.52	1.45	0.342 ± 0.085	1.441** ± 0.431	159.9 ± 3.7	5.1 ± 0.3	0.34 ± 0.02	0.37 ± 0.02	0.43 ± 0.03	0.54 ± 0.03	0.83 ± 0.02	0.08 ± 0.01	–
		0.50											
1.0 μg/kg/day	6.74 ± 0.22	1.37	0.301 ± 0.076	0.343 ± 0.145	158.8 ± 4.2	5.1 ± 0.1	0.33 ± 0.01	0.36 ± 0.01	0.40 ± 0.03	0.53 ± 0.04	0.86 ± 0.02	0.10 ± 0.01	–
		0.38											
10 μg/kg/day	12.75*** ± 2.19	1.91	0.026** ± 0.008	0.321 ± 0.110	139.6 ± 4.6	5.2 ± 0.1	0.28** ± 0.02	0.30* ± 0.01	0.27 ± 0.04	0.48 ± 0.06	0.51*** ± 0.06	0.05 ± 0.01	–
		0.38											
Females													
0 μg/kg/day	6.89 ± 0.90	48.33	–	–	136.9 ± 7.1	4.6 ± 0.2	0.35 ± 0.02	0.32 ± 0.02	0.20 ± 0.03	0.22 ± 0.04	–	–	0.26 ± 0.03
		3.58											
0.1 μg/kg/day	8.62 ± 0.91	19.71	–	–	134.9 ± 5.0	4.9 ± 0.1	0.46* ± 0.04	0.35 ± 0.01	0.24 ± 0.03	0.32 ± 0.05	–	–	0.37 ± 0.09
		7.47											
1.0 μg/kg/day	7.71 ± 1.19	3.22*	–	–	129.1 ± 3.0	5.2* ± 0.1	0.36 ± 0.03	0.42 ± 0.05	0.28 ± 0.03	0.44 ± 0.09	–	–	0.35 ± 0.04
		0.86											
10 μg/kg/day	19.18*** ± 1.56	2.05*	–	–	117.4* ± 4.0	5.3*** ± 0.1	0.27 ± 0.02	0.27 ± 0.01	0.27 ± 0.03	0.33 ± 0.07	–	–	0.26 ± 0.01
		0.36											

Note: Mean ± standard error. Organ weights are expressed as percentage of body weight. $n = 10$ for males and $n = 7$ for females (except serum E2; $n = 7, 7, 6, 6, 6, 7, 7, 6$). Dunnett tests comparing each group to control (0 μg/kg/day): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
P: progesterone; T: testosterone; LH: luteinizing hormone.

algorithms. A list of differentially expressed genes was generated by performing an unpaired *t*-test. These comparative lists were used to assess differences between various treatments: 0.1 E2 versus vehicle; 1.0 E2 versus vehicle and 10 E2 versus vehicle, criteria: $>+2$ or <-2 (i.e. ± 2), and $P<0.05$. Male and female glands were analysed separately. From these lists, we proceed to estimate fold changes for each of the groups and map those into a grid for classification. A simple supervised classifier was developed in Python to a supervised algorithm to classify the response to different concentrations of E2. Each probe-set (or gene) was placed in a dose-response class if its fold change was either increasing or decreasing (classes d+ or d−). Alternatively, the probe-set was placed on a non-linear response class if the middle dose behaved differently than the other two (classes n+ or n−). Finally, those genes that expressed almost the same fold change irrespective of the E2 dose were placed on the constant class (see Supplementary Bioinformatics Methods). After this classification procedure was performed, each class was analysed in DAVID for functional annotation and gene ontology (GO) analyses. Heat maps for classification were generated with Circos.[48]

Microarray validation by real-time quantitative PCR

Total RNA (1 μg) was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. cDNA samples were amplified using conditions described earlier.[49] Quantitative real-time PCR was performed on genes that were of particular interest to this study. Expression levels of genes were normalized to the gene levels of control genes Rps13 and Srp14, averaged with geNorm.[50] Primers used were: *Rps13* F: 5′-CCCCGAGGATCTCTACCATTT-3′ R: 5′-TCAGAATCAGGCGGAATTTAGC-3′, *Srp14* F: 5′-GAACA AGTTTCAGATGGCCTATTCA-3′ R:5′-GTGCTGGTTTGCT CTTCTTACTCTT-3′, *Areg* F: 5′-AGCCTAGCTGATGGCAA TTCAGGA-3′R:5′-ACTTCTGGAGCCTTCGCTGAAAGA-3′, *Pgr* F: 5′-TGGTTCCGCCACTCATCA-3′, R: 5′-TGGTCA GCAAAGAGCTGGAAG-3′, *Csn2* F:5′-TATGCCCAGACC ATCTCTTGCAGT-3′, R: 5′-AGGAAGGGCATCTGTTTG TGCTTG-3′, *Fabp1* F:5′-ACCTCATCCAGAAAGGGAAG GA-3′, R:5′-GTGGATCACCTTGGACCCATA-3′, *Esr1* F: 5′-GACAATCGACGCCAGAA-3′, R: 5′-ACAGCACAGTAGC GAGTCTCCTT-3′, *Esr2* F: 5′-AGAGTCCCTGGTGTGA AGCAAG-3′, R: 5′-GACAGCGCAGAAGTGAGCATC-3′.

Western blotting

Tissue lysates were prepared from 100 mg mammary tissues homogenized in RIPA buffer. Samples from animals belonging to the same group were separated into two individual pools and 20 μg of the pooled samples were applied to 10% SDS-PAGE gels. Proteins were transferred onto a PVDF membrane. Primary and secondary antibodies were obtained from Santa Cruz Biotechnologies and detected by chemoluminescence (SuperSignal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA). For PCNA protein, five pools of two male animals per group were utilized in Western immunoblots for statistical analysis.

Statistical analysis

Experimental outcomes are summarized as mean ± standard errors (SE). Data analysis was stratified by sex. For each outcome, ANOVA was used to compare means across experimental groups. *Post hoc* analyses of significant ANOVA values ($P \leq 0.05$) were performed using Dunnett's many-to-one test for organ weight and mammary morphology data. Dunnett's test compares the mean outcome from the control group (rats not infused with E2) to those from each of the experimental treatment groups while maintaining the overall experiment-wise error rate at 0.05.[51,52] Statistical analyses were performed using the Stata statistical package version 12.0 (Stata Corporation, College Station, TX). For real-time quantitative PCR and Western immunoblot of PCNA, analysis was performed using one way ANOVA with Tukey's *post hoc* analysis (Sigma Plot).

Table 2 Mammary morphology in male and female rats on PND33 following exposure to increasing doses of E2

	n	Terminal end buds	*n*	Epithelial area (mm^2)	*n*	Fat pad area (mm^2)	*n*	Epithelial/fat pad area
Males								
0 μg/kg/day	10	24.4 ± 8.5	9	12.8 ± 1.6	9	21.6 ± 1.8	9	0.60 ± 0.06
0.1 μg/kg/day	7	20.7 ± 6.1	10	16.7 ± 1.0	10	25.1 ± 1.0	10	0.67 ± 0.03
1.0 μg/kg/day	8	56.9** ± 4.6	9	17.1 ± 2.1	9	23.5 ± 2.1	9	0.71 ± 0.03
10 μg/kg/day	8	51.9** ± 3.0	9	19.8** ± 1.1	9	25.3 ± 1.1	9	0.78** ± 0.03
Females								
0 μg/kg/day	6	54.3 ± 9.9	6	17.5 ± 1.6	6	24.9 ± 2.3	6	0.70 ± 0.04
0.1 μg/kg/day	5	59.2 ± 9.0	7	16.9 ± 1.0	7	23.9 ± 1.7	7	0.71 ± 0.02
1.0 μg/kg/day	6	45.8 ± 4.8	7	19.0 ± 2.0	7	24.9 ± 1.9	7	0.75 ± 0.04
10 μg/kg/day	6	39.7 ± 3.9	6	18.7 ± 1.5	6	23.5 ± 1.8	6	0.79 ± 0.02

Note: Mean ± standard error. Dunnett tests comparing each group to control (0 μg/kg/day).
**$P < 0.01$.

Results

Effects of E2-treatment on reproductive development and endocrinology and mammary gland morphology

All hormonal and physiological data are presented in Table 1. Serum hormone concentrations were measured in test animals at the end of the experiment on PND33. For E2, only the 10 μg E2/kg/day dose increased serum E2 levels compared to controls in both sexes ($P < 0.001$). Serum progesterone was reduced in a dose-dependent fashion by E2 only in female rats, and the difference was significant in the two highest E2 doses ($P < 0.001$). In males, serum testosterone was reduced at the highest dose of E2, while serum luteinizing hormone (LH) was greater than controls only with the lowest dose of E2 ($P < 0.005$). The majority of significant changes in relative organ weight were observed only at the highest tested dose. In males, there was no significant change in body weight. Relative testes weight was reduced in the 10 E2 group versus controls ($P < 0.001$) and the relative thymus and spleen weights were reduced ($P = 0.01$ and $P = 0.039$, respectively). In contrast, there was a decrease in body weight with the highest E2 dose ($P = 0.030$) in females but the weight of the uterus did not differ from controls. There was an increase in relative liver weight at PND33 with increasing E2 dose ($P = 0.012$ for 1.0 E2 and $P < 0.001$ for 10 E2, not significant for 0.1 E2), and the relative thymus weight was greater only in the 0.1 E2 group compared with controls ($P = 0.024$). In females, vaginal

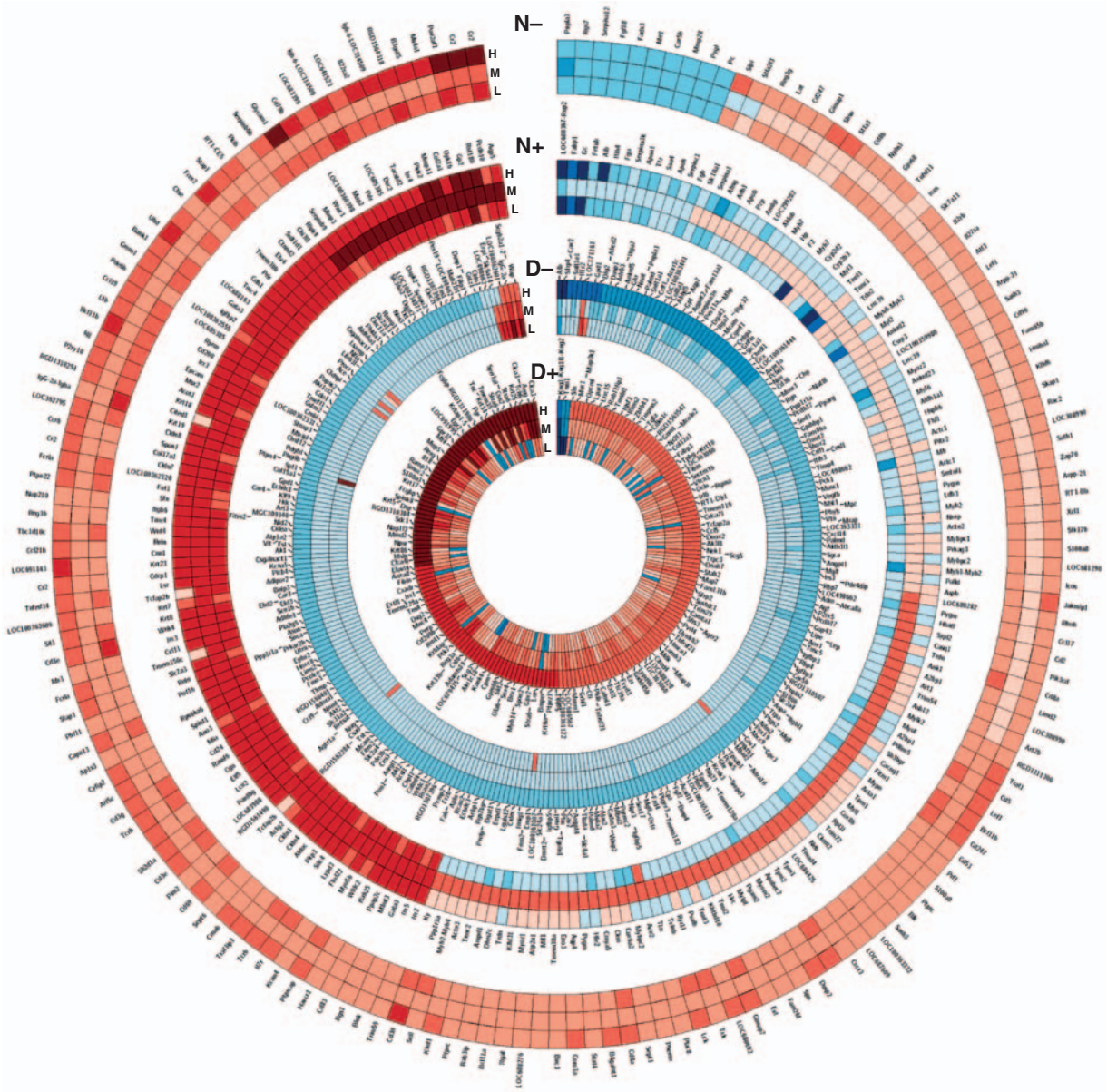

Figure 1 Microarray heat maps of genes changed by >2 fold by E2 in the weanling mammary gland of male rats. Genes were separated in circular bands according to the classification scheme: N+/N− represent the non-linear response classes and D+/D− represent the dose-dependent response classes (see text for details). Letters L, M and H on each circle refer to estradiol concentrations: L: low (0.1 μg/kg/day of E2); M: medium (1.0 μg/kg/day of E2); H: high (10 μg/kg/day of E2). (A color version of this figure is available in the online journal)

opening was significantly accelerated by E2 treatment and was observed in 100% of the animals by PND27 in the two highest doses of E2 (1.0 and 10), compared with PND 32-33 in control and low dose (0.1) E2-treated rats.

We assessed mammary gland physiology in treated and untreated animals. Results are presented in Table 2. In males, we observed an increase in the number of TEBs, the most proliferative part of the mammary gland,[53] in the 1.0 and 10 E2 groups compared with the control group ($P = 0.007$). There was also a significant increase in the epithelial area and in the ratio between the epithelial and the fat pad area in the 10 E2 group compared to controls ($P = 0.007$). In females, no statistical difference was observed in the number of TEBs, the epithelial area, the fat pad area or the ratio between the epithelial and fat pad area.

Classification of microarray genes

In order to identify optimal genes for the detection of an estrogenic response in mammary glands, we performed two rounds of selection. First, we identified genes that were modulated two-fold or more by at least one of our E2 doses. Second, we classified genes for which expression was modulated in a non-linear (Figures 1 and 2, layers n+ and n−) or in a dose-dependent manner (Figures 1 and 2, layers d+ and d−). The genes regulated in a dose-dependent manner represent robust E2-responsive genes

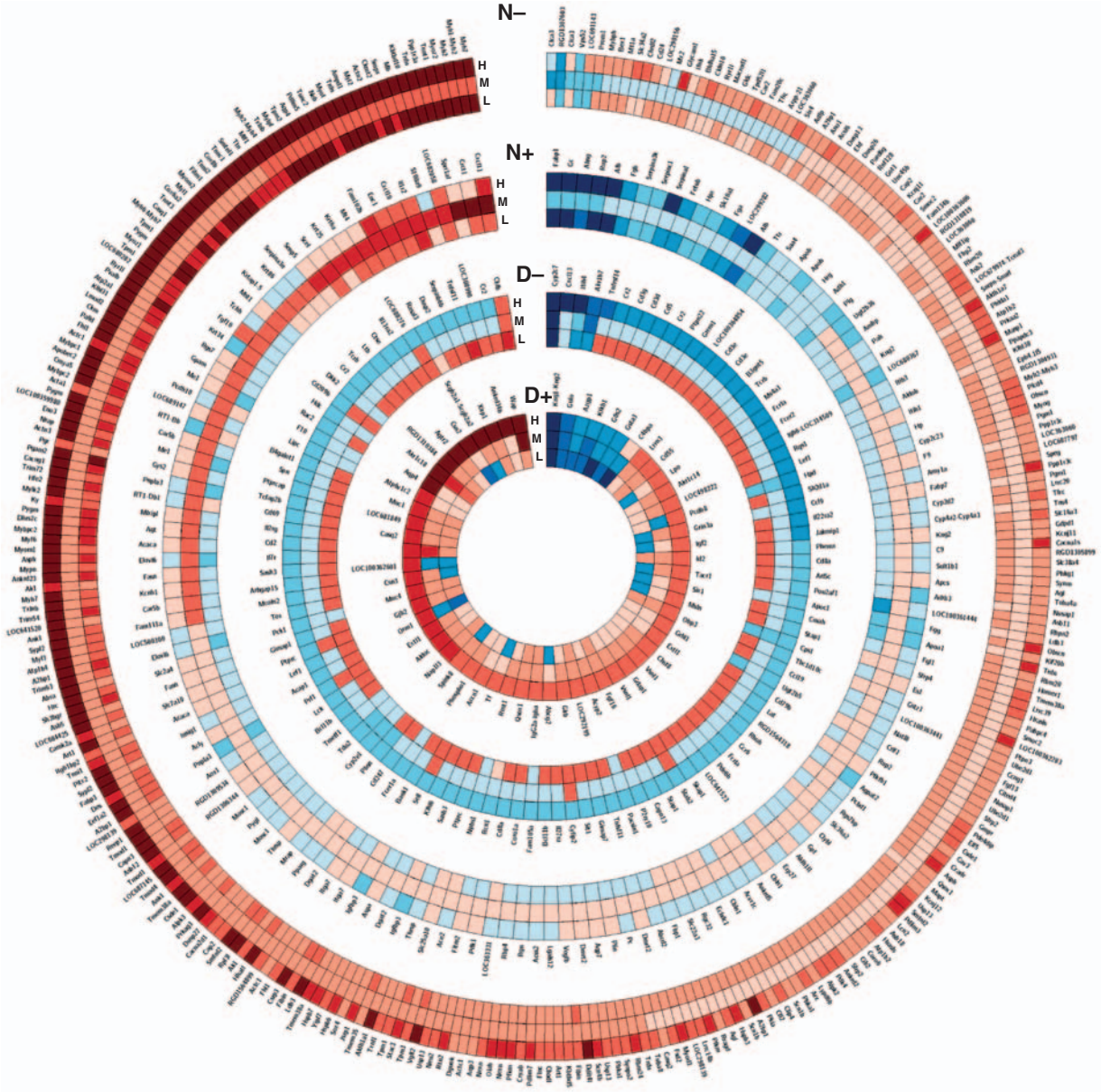

Figure 2 Microarray heat maps for genes changed by >2 fold by E2 in the weanling mammary gland of female rats. Genes were separated in circular bands according to the classification scheme: N+/N− represent the non-linear response classes and D+/D− represent the dose-dependent response classes (see text for details). Letters L, M and H on each circle refer to estradiol concentrations: L: low (0.1 μg/kg/day of E2); M: medium (1.0 μg/kg/day of E2); H: high (10 μg/kg/day of E2). (A color version of this figure is available in the online journal)

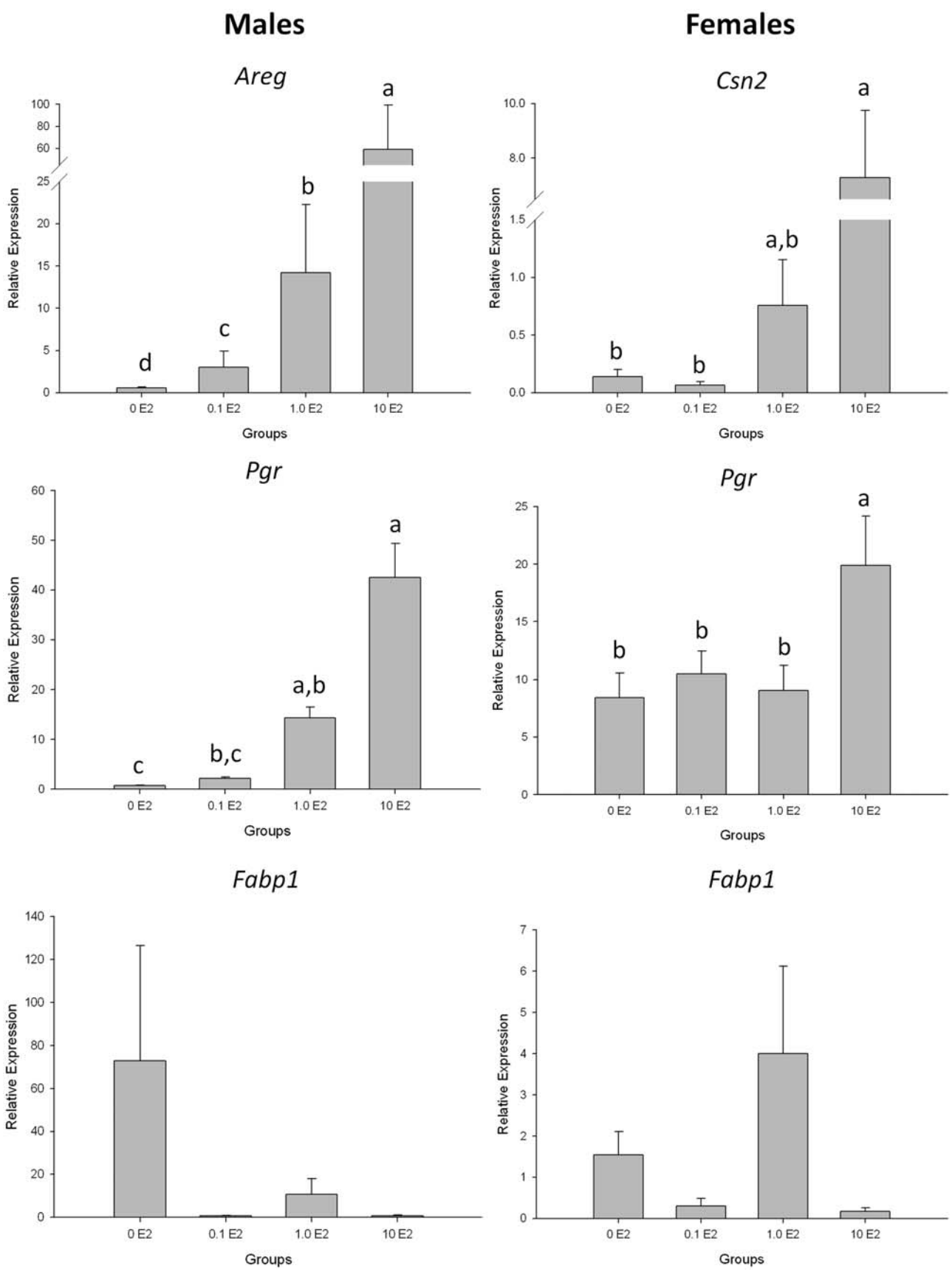

Figure 3 mRNA levels of E2-responsive genes in the weanling rat mammary gland. Real-time quantitative PCR. Different letters indicate a difference significant at $P \leq 0.05$. $n=5$ for males (5 pools of 2 animals) and $N=7$ for females by one way ANOVA followed by Tukey's *post hoc* analysis. Expression normalized to the housekeeping genes Srp14 and Rps13

with a predictable expression pattern and reproducible results. In male mammary glands, we identified 509 genes for which the expression differed modified in a dose-dependent fashion by E2 (194 upregulated and 315 downregulated) and 174 in females (61 upregulated and 115 downregulated). These results and the amplitude of the fold changes observed for the three E2 doses are provided as Supplementary Tables 1 and 2. In males, this analysis highlighted a dose-dependent increase in the expression of amphiregulin (*Areg*), an ERα-responsive gene known to regulate TEB development.[54] Confirmation of the microarray results by qPCR and Western blot are presented in Figures 3 and 4, respectively. There was also a dose-dependent increase in the expression of the progesterone receptor (*Pgr*), a well-described E2-responsive gene in the mammary gland.[55] This was accompanied by an increase for the marker of proliferation PCNA at the protein level in males for the 1.0 and 10 E2 groups versus control and 0.1 E2 ($n = 5$, $P < 0.05$ except 10 E2 versus control; $P = 0.076$), but there was no difference in females (Figure 4). In females, *Areg* expression was unchanged (data not shown) while *Pgr* was increased only at the highest dose tested ($P < 0.05$). We identified large dose-dependent increases in three major milk proteins: whey acidic protein (*Wap*), casein beta (*Csn2*) and casein kappa (*Csn3*) ($P < 0.05$) only in the female mammary gland (Figures 3 and 4).

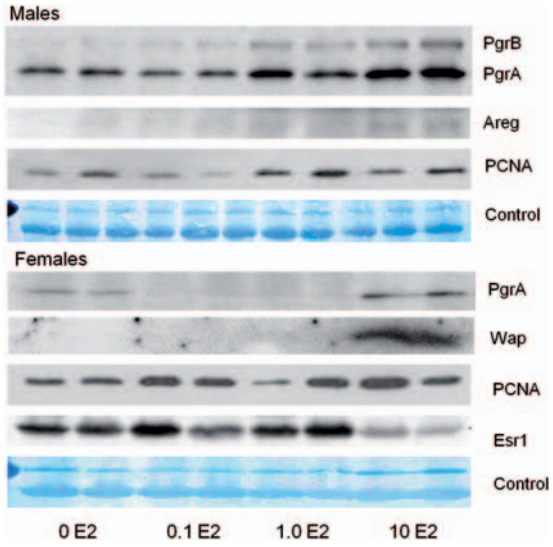

Figure 4 Protein levels of E2-responsive genes in the weanling rat mammary gland. Whole cell lysate was used for Western blotting, 20 μg/lane on a 10% SDS-PAGE gel detected with chemoluminescence. Each lane represents a different pool of three animals. Loading controls obtained by amido-black staining of total protein are included
(A color version of this figure is available in the online journal)

Gene ontology analysis

Results of GO analysis on the genes regulated by the E2 treatment are summarized in Table 3 (full results are presented in Supplementary Table 3). In males, the GO analysis revealed that the E2 treatment had a dose-dependent effect on mammary gland genes associated with blood pressure (reflective of an impact of E2 on mammary vasculature), hormonal response and lipid metabolism. In females, despite the dose-dependent increase in three major milk proteins, there were no GO terms significantly associated with differentiation. The GO analysis retrieved almost exclusively immune system-related activities.

A total of 392 genes in males and 468 in females were regulated by E2 in a non-linear fashion (Figures 1 and 2 and Supplementary Tables 1 and 2). In both males and females, the GO analysis revealed that there was an E2-induced, non-linear activation of the muscle-associated genes (Table 4). In males, we saw a non-linear impact on the immune system similar to the one we observed in a dose-dependent fashion in females. We noticed that for a number

Table 3 Selected GO terms associated with the dose-dependent genes

Term	Term ID	*P* value	FDR	Genes
Males				
Regulation of blood pressure	0008217	3.53 E-09	6.23 E-06	*Ace, Adipor2, Adora1, Adra1d, Agt, Aoc3, Apln, Atp1a2, Ephx2, Lep, Pcsk5, Serpina3n, Sult1a1, Sult1e1*
Response to hormone stimulus	0009725	4.69 E-09	8.27 E-06	*Akr1c18, Acvr1c, Adipor2, Adm, Adra1d, Agtr1a, Ak1, Ak2, Akap12, Akr1cl2, Aldh1l1, Ankrd5, Areg, Asns, Bmp4, Car4, Cav1, Cd36, Cdo1, Cebpa, Cited4, Cxcl14, Dusp1, Egfr, F3, Fabp3, Fam131b, Fam13a1, Ghr, Klf9, Lep, Mdk, Pck1, Pde3b, Pfkfb1, Pgr, Pla2g5, Rbp4, Rerg, S100b, Sdc1, Serpina3n, Slit2, Sphk1, Steap3, Sult1a1, Sult1e1, Tf, Tnc, Wfdc1*
Regulation of lipid metabolic process	0019216	1.33 E-07	2.35 E-04	*Akr1c18, Abcd2, Abhd5, Adipor2, Adora1, Agt, Akr1cl2, Angpt1, Angpt4, Ankrd5, Atp1a2, C1qtnf2, Cav1, Cidea, Fam131b, Fam13a1, Fitm2, Lep, Pde3b, Pla2g5, Pnpla2, Pnpla3, Snca*
Females				
Positive regulation of immune system process	0002684	1.48 E-15	2.39 E-12	*Ccl19, Cd5, Cd69, Coro1a, Cr2, Cxcl13, Fcer1a, Gimap1, Gimap7, Klhl6, Lck, LOC100364854, Ptpn22, Sash3, Sash3, Serpinb6b, Skap1, Spn*

FDR: False discovery rate.

of the genes behaving in a non-linear manner, in both sexes, the middle dose used (1.0 μg E2/kg/day) had a lesser effect than either the low or high dose (Figure 1 and 2, outer layers). This was the case for the gene encoding the fatty acid binding protein 1 (*Fabp1*) in both males and females, for which we obtained the same pattern of expression with qPCR (Figure 3).

Estrogen receptor expression

As reported earlier, E2 treatment decreased *Esr1* expression at the mRNA level in the mammary gland (Figure 5).[31, 56] Qualitative analysis of protein levels of ERα in whole cell lysates in females supported the mRNA results (Figure 4). *Esr2* mRNA was also significantly decreased in two of our three treatment groups in males (Figure 5). When we pooled the data from males and females, we observed a decrease in the ratio of mRNA expression from ERα to ERβ with increasing E2 doses (Figure 5). In addition, we used our classified microarray results to evaluate how many of previously described ERα and ERβ target genes in human mammary MCF7 cells were differentially expressed in our data-set.[57] Of the 489 genes modulated by either ERα or ERβ in Grober *et al.*, only 26 were differentially expressed in the mammary gland of males and 13 in the mammary gland of females, corresponding to only 8% of the ER targets identified in transformed mammary epithelial cells *in vitro* (Table 5).

Discussion

In order to establish potentially more sensitive molecular target endpoints for exposure to xenoestrogens, we studied the effects of exposure to increasing E2 doses from weaning until puberty in the mammary gland of male and female rats. Intact rather than gonadexctomized animals were utilized to mimic real life environmental exposures where xenoestrogens would compete with or be additive to endogenous estrogens. We chose to administer three concentrations, 0.1, 1.0 and 10 μg E2/kg/day, to represent both low to high levels of chronic exposures and assessed both morphological changes and specific gene expression patterns in the mammary gland. We also examined overall effects of exogenous E2 on reproductive physiology and endocrinology.

We observed extensive sexual dimorphism in the overall response to E2 in weanling rats. There was no overlap between males and females in the changes that were observed in either body or organ weight, or in mammary gland morphology. However, we did observe an acceleration of female vaginal opening and a dose-dependent decrease in serum progesterone in females and a decrease in serum testosterone concentrations in males confirming the efficacy of E2 treatment on reproductive endocrinology and physiology.

In females, the E2-induced, dose-dependent decrease in serum progesterone is consistent with previously reported direct effects of estrogens to suppress progesterone production in granulosa cells in the ovary.[58] The accelerated vaginal opening that we observed is also a well-established response to estrogen treatment in weanling animals[45] and is consistent with an LH surge induced by a positive feedback of E2 on the hypothalamic–pituitary axis.[39–49] In males, the suppression of serum testosterone by E2 is consistent with previous reports of the direct testicular effects of estrogens.[59, 60] Interestingly, we also observed that E2 induced a non-linear response in serum LH, with an increase at the lowest dose followed by suppression to control levels at higher doses. Previously published studies of E2 treatment in weanling male rats report either no effects or a reduction in serum LH.[61–63] However, these studies only used a single high E2 dose. The non-linear behaviour of LH may be caused by a negative feedback of androgens on LH secretion at high doses in males, but not in females.[61–63]

Looking at the changes in mammary gland gene expression induced by E2 in weanling animals of both sexes,

Table 4 Selected GO terms associated with the non-linear genes

Term	Term ID	*P* value	FDR	Genes
Males				
Muscle system process	0003012	8.75 E-20	1.50 E-16	*Mybpc2, Myl2, Tnnc2, Myl3, Mybpc1, Tnnc1, Myl1, Pgam2, Trim72, Ttn, Tpm1, Myom2, Mb, Actc1, Acta1, Myh1, Myh2, Mylk2, Mylpf, Myh7, Actn2, Actn3, Myh6, Tnni2, Tnnt3, Tnnt1*
Positive regulation of immune system process	0002684	6.42 E-11	1.10 E-07	*Itgal, Il27Ra, Fcer2, Cd247, Ptpn22, Il7R, Skap1, Sh2D1A, Klhl6, Cd69, Icos, Zap70, Cd24, Cd5, Klrd1, Spn, Ptprc, Cd3E, Trat1, Gimap1, Cd83, Coro1A, Lck, Cd79B, Sash3*
Females				
Muscle system process	0003012	4.71 E-24	8.13 E-21	*Myl2, Tnnc2, Mybpc2, Mybpc1, Tnnc1, Myl3, Myl1, Pgam2, Trim72, Ttn, Tpm1, Myom2, Agt, Myom1, Mb, Actc1, Acta1, Myh1, Myh2, Mylpf, Mylk2, Myh7, Actn2, Actn3, Myh6, Trim63, Homer1, Tnni2, Tnnt3, Tnnt1*
Glucose metabolic process	0006006	1.87 E-09	3.22 E-06	*Prkag3, Pdk1, Rbp4, Cryab, Phkg1, Phka1, Pdk4, Aldob, Fbp1, Pgam2, Fbp2, Pfkm, Kcnj11, Ppp1R3C, Pygm, Pygl, Pgm1, Gys2, Eno3, Agl, Pc*

FDR: False discovery rate.

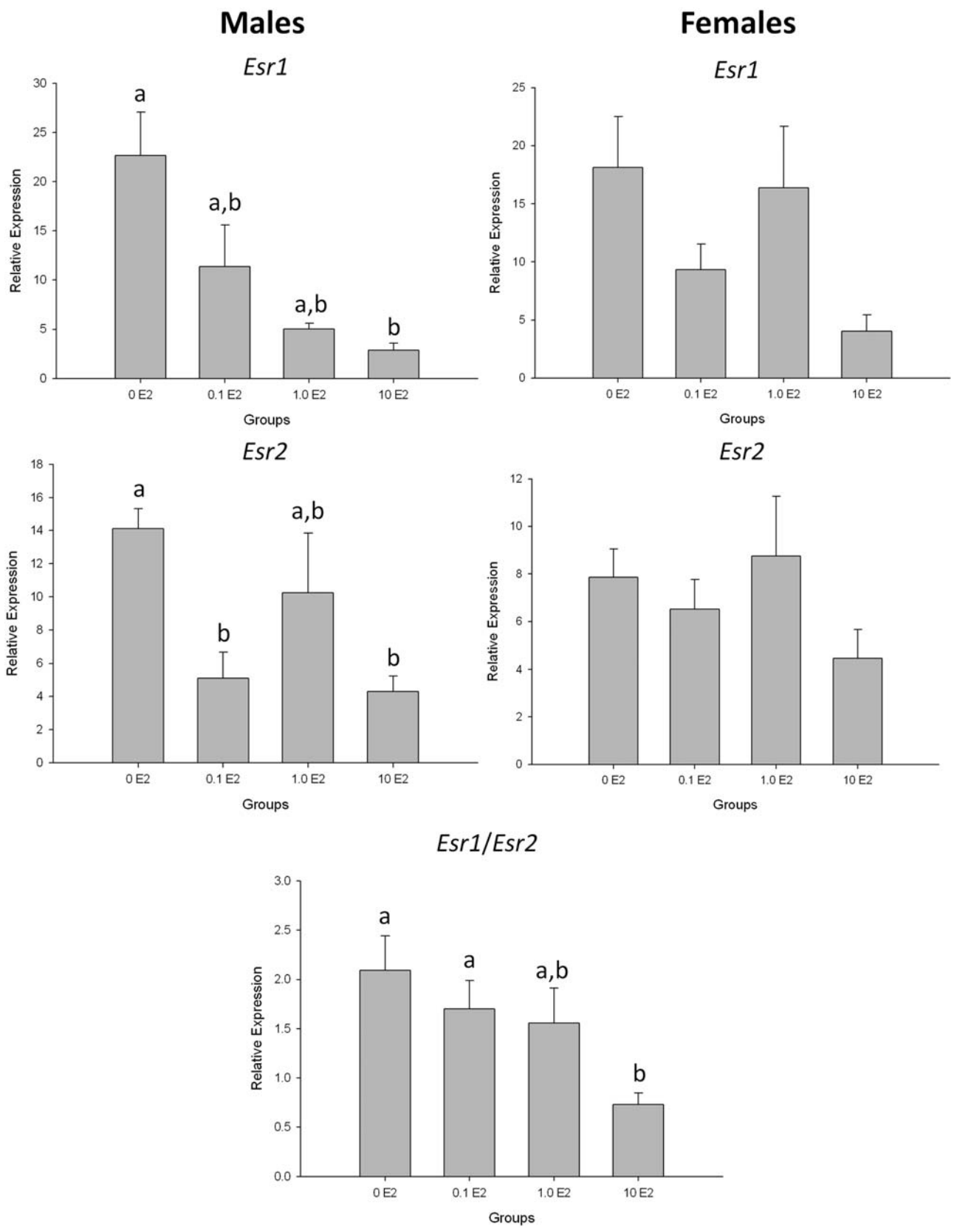

Figure 5 mRNA levels of estrogen receptors *Esr1* (ERα) and *Esr2* (ERβ) in the weanling rat mammary gland following estrogen treatment. Real-time quantitative PCR. Different letters indicate a difference significant at $P \leq 0.05$. $n = 5$ for males (5 pools of 2 animals) and $n = 7$ for females by one way ANOVA followed by Tukey's *post hoc* analysis. Expression normalized to the housekeeping genes Srp14 and Rps13

Table 5 Estrogen receptor target genes

ERα				ERβ			
Males							
n+	n−	d+	d−	n+	n−	d+	d−
Cgn	*Atf3*	*Plod2*	*Adora1*	*Cdh1*	*Mx1*	*Fxyd3*	*Adora1*
Rprm	*Fgf18*	*Rerg*	*Ghr*	*Cgn*	*Pkib*	*Plod2*	*Akr1cl2*
	Pkib	*Slit2*	*Wisp2*	*Ctnnd2*		*Vtcn1*	*Cebpa*
		Vtcn1		*Mb*			*Chpt1*
				Mypn			*Klf9*
				Ripk4			*Mbnl2*
							Ptger3
							Serpina3n
							Wisp2
Females							
n+	n−	d+	d−	n+	n−	d+	d−
Elovl6	*Cap2*			*Enc1*	*Cap2*	*Cd55*	*Pacsin1*
Enc1	*Mapt*			*Serpina3n*	*Mb*	*Qsox1*	
	Tpd52l1				*Mypn*		
					Qsox1		
					Rhpn2		
					Tpd52l1		

n: non-linear; d: dose-dependent; + : increasing −: decreasing.

the male mammary gland was more responsive to the doses tested both in terms of the number of gene expression changes and morphological changes. Both sexes showed E2-specific markers, such as milk proteins in females and *Areg* and *Pgr* in males, but there was very little overlap between males and females. Overall, the quality of the dose–response observed in the male animals suggests that the male mammary gland may be a better target to screen for estrogenic responses during this developmental window.

While no E2-induced changes in mammary gland morphology were observed in females, there was a doubling of TEBs in male mammary gland at the two highest E2 doses. An increase in TEB number is generally associated with an increased susceptibility to breast cancer, although studies have mainly been performed in female animals. [20, 38, 64,65] Others have recently identified a similar increase in the number of TEBs in male rats treated perinatally with ethinylestradiol, while no changes were observed in females.[19] However, these changes were seen only at doses equivalent to five times our largest dose of E2, indicating that the period between weaning and puberty might be a more sensitive window of exposure. Increased E2 serum levels are not linked with an increased risk of breast cancer in premenopausal women,[26] so the absence of TEB increase and the poor responsiveness observed in females is perhaps not unexpected.

The increase in TEBs seen in males was supported at the gene expression level by a dose-dependent upregulation of *Areg*, involved in the differentiation of TEBs. There was also a dose-dependent upregulation of *Pgr* expression in males. The response of *Pgr* to E2 has been well described.[66, 67] In females, effect of E2 on *Pgr* expression was observed only at the highest dose tested, consistent with the observation that the female mammary gland is less responsive to E2 than the male mammary gland. The expression of *Pgr* was reported to be elevated in TEBs[68] and to increase proliferation in the mammary epithelium.[23, 69] This correlates with the increase in mammary gland epithelial area observed in males in this study, and with results by others.[23] Increases in *Areg* and *Pgr* in the weanling male mammary gland were directly linked to a morphological alteration: an augmentation in TEB number. These observations are all indications of an increased proliferative activity induced by the E2 treatment, as supported by an increase in the expression of PCNA protein. Thus, the combined measurement of *Areg* and *Pgr* gene expression in the weanling male mammary appears to provide an easy, reliable and sensitive indicator of an estrogenic response *in vivo* during this developmental window and should be considered for use in screening potential xenoestrogens.

In the intact weanling females, there was a dose-dependent upregulation of three components of milk protein, *Wap*, *Csn2* and *Csn3* in female animals. Concordant results in female rats of the same age have been reported by others following a single subcutaneous injection of 10 μg diethylstilbestrol at PND2.[20] We also observed a strong, non-linear upregulation in gene expression associated with the muscular system. The same GO term was observed in males. However, gene expression was decreased in males compared to controls. For example, *Myh7* encodes the beta subunit the myosin heavy chain[70] and was upregulated 10-fold in females and decreased 10-fold in males with the 0.1 E2 dose. Reports by others have linked elevated estrogens to increased muscle mass, and specifically to an increased expression of myosin heavy chain in skeletal muscles.[71,72]

Alternatively, the upregulation of muscle-related genes in female mammary glands might be reflective of an expansion of myoepithelial cells, whose contraction stimulates the movement of milk from the ducts to the nipple during lactation. This hypothesis is supported by the increase in milk proteins. We thus suggest that these changes reflect an increase in differentiation in the mammary gland of weanling females treated with E2, as opposed to the changes in proliferation seen in males. However, the increase in milk proteins in females was not accompanied by changes in morphology. It thus represents a tool for the screening of xenoestrogens in intact females, but inferior in quality to what we observed in males. Changes seen in mammary gene expression profiles after administration of exogenous E2 are likely caused by direct E2 actions on mammary cells. However, potential indirect effects may also result from the observed non-linear effects of E2 on serum LH in the male or as a result of the reduction in serum progesterone concentrations in the female.

Surprisingly, we observed that chronic E2 treatment elicited remarkably little response in many well-described estrogen receptor targets previously identified in *in vitro* studies using the transformed human mammary epithelial MCF-7 cell line.[57] This may reflect a dilution of estrogen effects when comparing the actions on multiple cell types in the mammary gland *in vivo* compared to specific effects on epithelial cells *in vitro*. The differences in gene expression might be associated with species differences in estrogen responses between rats and humans or differences in estrogen signalling in transformed cells. Alternatively, the lack of concordance with acute *in vitro* studies may be an indication of tissue adaptation to chronic *in vivo* estrogen overexposure via downregulation of ER expression. The repressive effect of exogenous E2 on the mRNA and protein expression of ERα and ERβ has been well documented both *in vitro* and *in vivo*.[31,56,73]

In summary, exogenous E2 administration to weanling rats produced distinct mammary gland gene expression signatures for males and females. Greater modulation of morphology and gene expression by exogenous E2 was observed in males than in intact females in the weanling rat. There was an increase in the number of TEBs in males correlated with a robust upregulation of the genes *Areg* and *Pgr*. In females, the milk proteins *Wap*, *Csn2* and *Csn3* were upregulated in a dose-dependent fashion by treatment, but there were no corresponding morphological changes. The information presented here may provide basis to generate potentially useful screening tools for specific effects of xenoestrogens in the mammary gland during this developmental window, especially in males. The long-term functional significance of these sex-specific actions of E2 in the developing mammary remains to be further elucidated.

Author contributions: MJJR and TMB conceived and designed the study; IRM, NS and JV performed laboratory experiments; HG-A wrote and performed the classification algorithm, IRM, MJJR, LH, KS, MAC, HG-A and TMB analysed data and interpreted the results; IRM drafted the manuscript; IRM and HG-A created the figures; MJJR, HG-A, KS, MAC, HG-A and TMB edited and revised manuscript; IRM, MJJR, LH, KS, MAC, HG-A and TMB approved the final version of the manuscript.

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REFERENCES

1. Doherty LF, Bromer JG, Zhou Y, Aldad TS, Taylor HS. In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: An epigenetic mechanism linking endocrine disruptors to breast cancer. *Horm Cancer* 2010;**1**:146–55
2. Schrager S, Potter BE. Diethylstilbestrol exposure. *Am Fam Physician* 2004;**69**:2395–400
3. Titus-Ernstoff L, Hatch EE, Hoover RN, Palmer J, Greenberg ER, Ricker W, Kaufman R, Noller K, Herbst AL, Colton T, Hartge P. Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy. *Br J Cancer* 2001;**84**:126–33
4. Fenton SE. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. *Endocrinology* 2006;**147**:S18–24
5. Roy JR, Chakraborty S, Chakraborty TR. Estrogen-like endocrine disrupting chemicals affecting puberty in humans – A review. *Med Sci Monit* 2009;**15**:RA137–45
6. de Ronde W, Pols HA, van Leeuwen JP, de Jong FH. The importance of oestrogens in males. *Clin Endocrinol (Oxf)* 2003;**58**:529–42
7. Forney JP, Milewich L, Chen GT, Garlock JL, Schwarz BE, Edman CD, MacDonald PC. Aromatization of androstenedione to estrone by human adipose tissue in vitro. *Correlation with adipose tissue mass, age, and endometrial neoplasia.* J Clin Endocrinol Metab 1981;**53**:192–9
8. Schindler AE, Ebert A, Friedrich E. Conversion of androstenedione to estrone by human tissue. *J Clin Endocrinol Metab* 1972;**35**:627–30
9. Siiteri PK. Adipose tissue as a source of hormones. *Am J Clin Nutr* 1987;**45**:277–82
10. Couse JF, Korach KS. Estrogen receptor null mice: What have we learned and where will they lead us? *Endocr Rev* 1999;**20**:358–417
11. Taylor AH, Al Azzawi F. Immunolocalisation of oestrogen receptor beta in human tissues. *J Mol Endocrinol* 2000;**24**:145–55
12. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999;**140**:5566–78
13. Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson JA. Estrogen receptors alpha and beta in the rodent mammary gland. *Proc Natl Acad Sci USA* 2000;**97**:337–42
14. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci USA* 1998;**95**:15677–82
15. Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW, Smith EP. Estrogen receptor gene disruption: Molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res* 1996;**51**:159–86
16. Ruan W, Kleinberg DL. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. *Endocrinology* 1999;**140**:5075–81
17. Cheng G, Weihua Z, Warner M, Gustafsson JA. Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. *Proc Natl Acad Sci USA* 2004;**101**:3739–46
18. Hilakivi-Clarke L, Cho E, Clarke R. Maternal genistein exposure mimics the effects of estrogen on mammary gland development in female mouse offspring. *Oncol Rep* 1998;**5**:609–16

19. Mandrup KR, Hass U, Christiansen S and Boberg J. Perinatal ethinyl oestradiol alters mammary gland development in male and female Wistar rats. *Int J Androl* 2012;**35**:385–96
20. Umekita Y, Souda M, Hatanaka K, Hamada T, Yoshioka T, Kawaguchi H, Tanimoto A. Gene expression profile of terminal end buds in rat mammary glands exposed to diethylstilbestrol in neonatal period. *Toxicol Lett* 2011;**205**:15–25
21. Hilakivi-Clarke L, Cho E, deAssis S, Olivo S, Ealley E, Bouker KB, Welch JN, Khan G, Clarke R, Cabanes A. Maternal and prepubertal diet, mammary development and breast cancer risk. *J Nutr* 2001;**131**:154S–7S,.
22. Latendresse JR, Bucci TJ, Olson G, Mellick P, Weis CC, Thorn B, Newbold RR, Delclos KB. Genistein and ethinyl estradiol dietary exposure in multigenerational and chronic studies induce similar proliferative lesions in mammary gland of male Sprague-Dawley rats. *Reprod Toxicol* 2009;**28**:342–53
23. Wang XJ, Bartolucci-Page E, Fenton SE, You L. Altered mammary gland development in male rats exposed to genistein and methoxychlor. *Toxicol Sci* 2006;**91**:93–103
24. You L, Sar M, Bartolucci EJ, McIntyre BS, Sriperumbudur R. Modulation of mammary gland development in prepubertal male rats exposed to genistein and methoxychlor. *Toxicol Sci* 2002;**66**:216–25
25. Colditz GA. Relationship between estrogen levels, use of hormone replacement therapy, and breast cancer. *J Natl Cancer Inst* 1998;**90**:814–23
26. Eliassen AH, Missmer SA, Tworoger SS, Spiegelman D, Barbieri RL, Dowsett M, Hankinson SE. Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. *J Natl Cancer Inst* 2006;**98**:1406–15
27. Key T, Appleby P, Barnes I, Reeves G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst* 2002;**94**:606–16
28. Ewertz M, Holmberg L, Tretli S, Pedersen BV, Kristensen A. Risk factors for male breast cancer – A case-control study from Scandinavia. *Acta Oncol* 2001;**40**:467–71
29. Hsing AW, McLaughlin JK, Cocco P, Co Chien HT, Fraumeni JF Jr. Risk factors for male breast cancer (United States). *Cancer Cause Control* 1998;**9**:269–75
30. Johnson KC, Pan S, Mao Y. Risk factors for male breast cancer in Canada, 1994–1998. *Eur J Cancer Prev* 2002;**11**:253–63
31. Cabanes A, Wang M, Olivo S, deAssis S, Gustafsson JA, Khan G, Hilakivi-Clarke L. Prepubertal estradiol and genistein exposures upregulate BRCA1 mRNA and reduce mammary tumorigenesis. *Carcinogenesis* 2004;**25**:741–8
32. Nagasawa H, Yanai R, Shodono M, Nakamura T, Tanabe Y. Effect of neonatally administered estrogen or prolactin on normal and neoplastic mammary growth and serum estradiol-17 beta level in rats. *Cancer Res* 1974;**34**:2643–6
33. Hilakivi-Clarke L, Onojafe I, Raygada M, Cho E, Skaar T, Russo I, Clarke R. Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. *Br J Cancer* 1999;**80**:1682–8
34. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* 1996;**17**:1451–7
35. Peng JH, Zhu JD, Mi MT, Li FJ, Cai L, Dong JZ, Zhang HX, Zhao Y, Xue RL. Prepubertal genistein exposure affects erbB2/Akt signal and reduces rat mammary tumorigenesis. *Eur J Cancer Prev* 2010;**19**:110–9
36. Chalabi N, Coxam V, Satih S, Davicco MJ, Lebecque P, Fontana L, Bignon YJ, Bernard-Gallon DJ. Gene signature of rat mammary glands: Influence of lifelong soy isoflavones consumption. *Mol Med Rep* 2010;**3**:75–81
37. Moral R, Wang R, Russo IH, Lamartiniere CA, Pereira J, Russo J. Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. *J Endocrinol* 2008;**196**:101–12
38. Moral R, Wang R, Russo IH, Mailo DA, Lamartiniere CA, Russo J. The plasticizer butyl benzyl phthalate induces genomic changes in rat mammary gland after neonatal/prepubertal exposure. *BMC Genomics* 2007;**8**:453
39. Su Y, Simmen FA, Xiao R, Simmen RC. Expression profiling of rat mammary epithelial cells reveals candidate signaling pathways in dietary protection from mammary tumors. *Physiol Genomics* 2007;**30**:8–16
40. Thomsen AR, Almstrup K, Nielsen JE, Sorensen IK, Petersen OW, Leffers H, Breinholt VM. Estrogenic effect of soy isoflavones on mammary gland morphogenesis and gene expression profile. *Toxicol Sci* 2006;**93**:357–68
41. You L, Bartolucci EJ. Gene expression profiles in mammary gland of male rats treated with genistein and methoxychlor. *Environ Toxicol Pharmacol* 2004;**18**:161–72
42. Schulze A, Downward J. Navigating gene expression using microarrays – A technology review. *Nat Cell Biol* 2001;**3**:E190–5
43. Suzuki A, Urushitani H, Watanabe H, Sato T, Iguchi T, Kobayashi T, Ohta Y. Comparison of estrogen responsive genes in the mouse uterus, vagina and mammary gland. *J Vet Med Sci* 2007;**69**: 725–31
44. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;**123**:1939–51
45. Singhal R, Badger TM, Ronis MJ. Reduction in 7,12-dimethylbenz[a]anthracene-induced hepatic cytochrome-P450 1A1 expression following soy consumption in female rats is mediated by degradation of the aryl hydrocarbon receptor. *J Nutr* 2007;**137**:19–24
46. Singhal R, Shankar K, Badger TM, Ronis MJ. Hepatic gene expression following consumption of soy protein isolate in female Sprague-Dawley rats differs from that produced by 17{beta}-estradiol treatment. *J Endocrinol* 2009;**202**:141–52
47. Irizarry RA, Ooi SL, Wu Z and Boeke JD. Use of mixture models in a microarray-based screening procedure for detecting differentially represented yeast mutants. *Stat Appl Genet Mol Biol* 2: Article1, 2003
48. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: An information aesthetic for comparative genomics. *Genome Res* 2009;**19**:1639–45
49. Singhal R, Shankar K, Badger TM, Ronis MJ. Estrogenic status modulates aryl hydrocarbon receptor–mediated hepatic gene expression and carcinogenicity. *Carcinogenesis* 2008;**29**:227–36
50. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**RESEARCH0034)
51. Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc* 1955;**50**:1096–121
52. Winer BJ. *Statistical Principles in Experimental Design*. New York, NY: McGraw-Hill, 1971:201–4
53. Russo J, Russo IH. Experimentally induced mammary tumors in rats. *Breast Cancer Res Treat* 1996;**39**:7–20
54. Ciarloni L, Mallepell S, Brisken C. Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development. *Proc Natl Acad Sci U S A* 2007;**104**:5455–60
55. Shyamala G, Schneider W, Schott D. Developmental regulation of murine mammary progesterone receptor gene expression. *Endocrinology* 1990;**126**:2882–9
56. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB. Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Mol Endocrinol* 1988;**2**:1157–62
57. Grober OM, Mutarelli M, Giurato G, Ravo M, Cicatiello L, De Filippo MR, Ferraro L, Nassa G, Papa MF, Paris O, Tarallo R, Luo S, Schroth GP, Benes V, Weisz A. Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for target gene regulation. *BMC Genomics* 2011;**12**:36
58. Schomberg DW. Steroidal modulation of steroid secretion in vitro: an experimental approach to intra-follicular regulatory mechanisms. *Adv Exp Med Biol* 1979;**112**:155
59. Kwon S, Stedman DB, Elswick BA, Cattley RC, Welsch F. Pubertal development and reproductive functions of Crl: CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. *Toxicol Sci* 2000;**55**:399–406

60. Lassurguère J, Livera G, Habert R, Jégou B. Time-and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. *Toxicol Sci* 2003;**73**:160–9
61. Chinoy NJ, Rao MV. Effect of estradiol benzoate on hormonal profile and steroidogenic organs of immature and adult male rats. *Endokrinologie* 1982;**80**:287
62. Docke F, Rohde W, Freitag J, Dorner G. Sex-specific postnatal development of negative oestrogen feedback in rats. *Exp Clin Endocrinol* 1988;**91**:301–10
63. Loke DF, Ratnam SS, Goh HH. Long-term oestrogen priming on basal and oestradiol-induced release of luteinising hormone secretion in male rats. *Ann Acad Med Singapore* 1992;**21**:765–8
64. de Assis S, Khan G, Hilakivi-Clarke L. High birth weight increases mammary tumorigenesis in rats. *Int J Cancer* 2006;**119**:1537–46
65. Moral R, Santucci-Pereira J, Wang R, Russo IH, Lamartiniere CA, Russo J. In utero exposure to butyl benzyl phthalate induces modifications in the morphology and the gene expression profile of the mammary gland: An experimental study in rats. *Environ Health* 2011;**10**:5
66. Horwitz KB, McGuire WL. Estrogen control of progesterone receptor induction in human breast cancer: role of nuclear estrogen receptor. *Adv Exp Med Biol* 1979;**117**:95–110
67. Mohla S, Clem-Jackson N, Hunter JB. Estrogen receptors and estrogen-induced gene expression in the rat mammary glands and uteri during pregnancy and lactation: changes in progesterone receptor and RNA polymerase activity. *J Steroid Biochem* 1981;**14**:501–8
68. Russo J, Ao X, Grill C, Russo IH. Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res Treat* 1999;**53**:217–27
69. Wang S, Counterman LJ, Haslam SZ. Progesterone action in normal mouse mammary gland. *Endocrinology* 1990;**127**:2183–9
70. Chen JR, Lazarenko OP, Shankar K, Blackburn ML, Lumpkin CK, Badger TM, Ronis MJ. Inhibition of NADPH oxidases prevents chronic ethanol-induced bone loss in female rats. *J Pharmacol Exp Ther* 2011;**336**:734–42
71. Liu YH, Huang Y, Shao X. Effects of estrogen on genioglossal muscle contractile properties and fiber-type distribution in chronic intermittent hypoxia rats. *Eur J Oral Sci* 2009;**117**:685–90
72. Velders M, Solzbacher M, Schleipen B, Laudenbach U, Fritzemeier KH, Diel P. Estradiol and genistein antagonize the ovariectomy effects on skeletal muscle myosin heavy chain expression via ER-beta mediated pathways. *J Steroid Biochem Mol Biol* 2010;**120**:53–9
73. Hatsumi T, Yamamuro Y. Downregulation of estrogen receptor gene expression by exogenous 17beta-estradiol in the mammary glands of lactating mice. *Exp Biol Med (Maywood)* 2006;**231**:311–6

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