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The effects of *in utero* bisphenol A exposure on the ovaries in multiple generations of mice

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Abstract

Bisphenol A is used in polycarbonate plastics and epoxy resins. Previous studies show that *in utero* BPA exposure inhibits germ cell nest breakdown in the F1 generation of mice, but its effects on germ cell nest breakdown and on the ovary in the F2–F3 generations were unknown. Thus, we tested the hypothesis that BPA has transgenerational effects on the ovary. Mice were exposed to BPA *in utero* (BPA 0.5, 20, or 50 µg/kg/day), and ovaries were collected at postnatal days (PND) 4 and 21 from the F1–F3 generations and subjected to histological evaluation and gene expression analyses. *In utero* BPA exposure did not have transgenerational effects on germ cell nest breakdown and gene expression on PND 4, but it caused transgenerational changes in expression in multiple genes on PND 21. Collectively, these data indicate that *in utero* BPA exposure has some transgenerational effects in mice.

Keywords

bisphenol A; germ cell nest breakdown; oxidative stress; apoptosis; autophagy; mouse; steroidogenesis; transgenerational

1. INTRODUCTION

Bisphenol A (BPA) is a synthetic compound used as a plasticizer in the manufacturing process of polycarbonate plastics and epoxy resins. It can be found in a variety of different products, such as plastic food and beverage containers, baby bottles and formula packaging, and the linings of canned food containers [1]. The potential threat to humans from use of these products stems from the ability of BPA to leach out of plastics and into the food and beverages they contain, under conditions such as ultraviolet light, heat, acidic conditions, and microwave use. Humans are commonly subjected to oral exposure of this chemical, with

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

studies showing BPA is present in human blood, urine, ovarian follicular fluid, breast milk, and the placenta [2–4].

Previous studies have shown that *in utero* exposure to BPA has a negative impact on ovarian development and function in mice, and that it leads to reduced fertility [5–7]. One mechanism by which BPA may impair fertility is by disruption of the normal process of germ cell nest breakdown. In mice, germ cells migrate to the genital ridge around embryonic day 10.5, where they proliferate quickly and form clusters. A layer of proliferating somatic cells surrounds these clusters and helps form germ cell nests. After birth, these nests are broken apart, resulting in individual oocytes. These oocytes are then surrounded by a layer of somatic cells to form primordial follicles. This is generally finished by postnatal days (PND) 4–6 in mice, and leaves a finite number of primordial follicles available to the female for the remainder of her life [8]. This process of germ cell nest breakdown is controlled by the drop in estrogen at birth that results from the pups leaving the mother who had high levels of estrogen [9]. Additionally, germ cell nest breakdown depends on the occurrence of apoptosis, a type of programmed cell death [10]. Since BPA is an estrogenic compound, exposure during this critical stage inhibits the natural apoptosis needed to release oocytes from their germ cell nests [5].

Many previous studies have shown that BPA has overall negative effects on the reproductive development and function of ovaries [7, 11, 12], but the extent of these effects on future generations is unclear. Specifically, the effect of *in utero* BPA exposure on the process of germ cell nest breakdown in multiple generations has yet to be explored. Further, the mechanisms by which BPA affects germ cell nest breakdown are relatively unknown. Our previous study showed that *in utero* BPA exposure increases expression of anti-apoptotic factors and decreases expression of pro-apoptotic factors and that these gene expression changes coincide with decreases in germ cell nest breakdown in the F1 generation [5]. Thus, in the current study, we examined whether *in utero* BPA exposure causes changes in gene expression of apoptotic factors (i.e., B cell leukemia/lymphoma 2 (*Bcl2*) and Bcl2-associated X protein (*Bax*)) in the F2–F3 generations. Further, we explored whether BPA exposure alters expression of other ovarian genes that regulate oxidative stress and autophagy in the F1–F3 generations.

We focused on genes that regulate oxidative stress because oxidative stress is thought to play a role in apoptosis and this might help regulate germ cell nest breakdown [8]. Oxidative stress occurs when an over-abundance of reactive oxygen species (ROS) is present in the body. Under normal conditions, ROS play an integral role in cell signaling and homeostasis, as they are formed as a natural by-product of oxygen metabolism. In response, the body produces anti-oxidant enzymes (i.e., superoxide dismutase 1 (*Sod1*), catalase (*Cat*), and glutathione peroxidase (*Gpx*)) to clear the ROS. However, an imbalance in this process can lead to oxidative stress-related damage in vital tissues in the body. In the case of the ovary, oxidative stress can interfere with apoptosis, which could affect germ cell nest breakdown and reproductive function [13].

We also focused on genes that regulate autophagy because autophagy plays an important role in regulating cell growth and development, especially during times of environmental

stress [14]. Depending on the circumstances, autophagy can lead to cell death or can help to promote cell survival [15]. During the process of germ cell nest breakdown, autophagy helps to maintain energy homeostasis, utilizing both autophagy related 7 (*Atg7*) and beclin 1, autophagy related (*Becn1*) to initiate the process. In this case, autophagy has a pro-survival role in the regulation of germ cell numbers before the formation of the primordial follicle pool [16]. Autophagy also can have protective effects against cell death due to oxidative stress [17]. One study correlates an increase in *Bcl2* with the disruption of the (autophagy related 12 - autophagy related 3 (*Atg12* - *Atg3*)) complex in embryonic fibroblast cells of mice, causing a resistance to cell death by mitochondrial pathways [18], whereas another study finds that *Atg12* alone has pro-apoptotic functions in human embryonic kidney cells [19].

Our previous study did not examine the effects of *in utero* BPA exposure on ovarian morphology at time points later than PND 4. After the breakdown of germ cell nests shortly following birth and the creation of the primordial follicle pool, BPA can continue to have effects on follicle type distribution in the ovary. In certain cases, toxicants can increase or decrease follicle recruitment into more advanced stages of development. With this in mind, we determined if *in utero* BPA exposure affects follicle numbers on PND 21, which is a later time-point than PND 4 and is just prior to puberty. We examined changes in gene expression on PND 21 in response to BPA exposure as well. In addition to the apoptotic factors and oxidative stress genes examined on PND 4, we also examined genes in the insulin-like growth factor (*Igf*) family, hormone receptors, and steroidogenesis-related genes.

We focused on whether BPA exposure affects members of the *Igf* family because they are involved in the regulation of the cell cycle and apoptosis in mice and thus, play a role in regulating folliculogenesis [20]. Insulin-like growth factor 1 (*Igf1*) is required for follicular development, and mice lacking this factor have immature ovaries with follicles arrested at the preantral and early antral stages, which causes infertility. It is only produced at low levels during embryonic development so it plays a larger role during postnatal growth and development. Insulin-like growth factor binding protein 2 (*Igfbp2*) is responsible for transporting *Igfs* in the bloodstream, and hence increases the half-life of *Igf* in the serum and prevents overstimulation of cell growth and excessive apoptosis [20].

We focused on whether BPA affects hormone receptors in the ovary because BPA is a known endocrine disrupter. Although it has been identified as having weak estrogenic activity and interfering with estrogen receptor alpha (*Esr1*), it also can have anti-androgenic activity as well. It has been shown to affect multiple steps of the activation and function of *Ar*, hence inhibiting the ability of natural androgens to bind to their receptor [21]. Further, ovaries lacking the androgen receptor have reduced fertility and abnormal ovarian function, likely because of the importance of *Ar* and androgens in enhancing follicle-stimulating hormone receptor (*Fshr*) action and the development of the proper cellular components and receptor activity needed for healthy follicles [22].

Finally, we focused on whether BPA exposure alters expression of genes that regulate steroidogenesis because the production of sex steroid hormones such as estrogens and androgens is needed for proper development and growth of follicles [23], and several

steroids play an important role in their synthesis. Steroidogenic acute regulatory protein (*Star*) is responsible for the transport of cholesterol, which is the starting material for the production of steroids. Cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*), and hydroxysteroid (17-beta) dehydrogenase 1 (*Hsd17b1*) are responsible for the conversion of progesterone and testosterone, respectively. After the stimulation of FSH, androstenedione can be converted to estrone and testosterone can be converted to 17 β -estradiol [24]. BPA has been shown to affect expression of these genes *in vitro* [25]. Thus, we examined whether it alters expression of these genes in the current study.

2. MATERIALS AND METHODS

2.1. Chemicals

Bisphenol A (99% purity, National Institute for Environmental Health Sciences) was dissolved into ethanol and then diluted in tocopherol-stripped corn oil. The final ethanol concentration in the tocopherol-stripped corn oil was less than 0.1%.

2.2. Animals

Inbred FVB mice were housed in conventional polysulfone cages at 25°C on 12L: 12D cycles. They had access to Teklad Rodent Diet 8604 (Harlan) and high purity water (reverse osmosis filtered) in glass bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and abide by the guidelines set forth by the National Institutes of Health for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.3. Study Design

Female mice (F0) were mated with control male mice at 12 weeks of age. Pregnancy was confirmed by the appearance of a vaginal sperm plug, and marked as gestational day (GD) 1. Once a vaginal sperm plug was present, females were removed from the males and individually caged. They were observed daily and their body weight gain was recorded to further confirm pregnancies. At GD 9, each pregnant female was randomly assigned to a treatment group. From GD 11 to birth, female mice were orally dosed once per day with tocopherol-stripped corn oil or BPA (0.5, 20, or 50 $\mu\text{g/kg/day}$) by placing a pipette tip with the dosing solution in the corner of their mouth, as described in a previous study [5].

BPA 0.5 $\mu\text{g/kg/day}$ was used to mimic the estimated human exposure from bottle feeding [26]. The BPA 20 $\mu\text{g/kg/day}$ dose has been shown to disrupt oocyte meiosis and cause aneuploidy in the eggs of mice [27]. BPA 50 $\mu\text{g/kg/day}$ is the reference dose for the Environmental Protection Agency (EPA) [28]. All dose selection was based on our previous study in F1 mice [5]. The doses were calculated and adjusted based on daily body weights, and delivered in 28–32 μl of corn oil. The chosen exposure window, GD 11 to birth, is a critical ovarian development window in the mouse. During the dosing period, mice were observed twice per day for abnormal behavior and clinical signs of toxicity. The mice were allowed to deliver naturally, and that day was designated as PND 0. On PND 4, the sizes of litters were standardized to 10 pups each if the litters consisted of more than 10 pups. This was done by random selection to yield 5 males and 5 females. If natural litters did not have

at least 10 pups, they were not standardized. At least one F1 female per litter was euthanized on PND 4 and PND 21 and the ovaries were used for histological and gene expression analysis as described below. Additionally, some F1 females were used to generate F2 females and F2 females were used to generate F3 females. In these experiments, control and exposed F1 and F2 females were mated with fertility confirmed non-exposed males to generate the next generation. On PND 4 and PND 21, F2 and F3 females were euthanized and their ovaries were used in the histological and gene expression analyses described below. After weaning the pups, F0 dams were euthanized and serum samples were collected for hormone analysis.

2.4. Histological evaluation

Ovaries were collected and fixed in 10% neutral buffered formalin and transferred to 70% ethanol after 24 hours until further processed. The fixed ovaries were embedded in paraffin, serially sectioned (5 μ m), placed on glass slides, and stained with Weigert's hematoxylin and picric acid-methyl blue. A sample consisting of every tenth section was used to count germ cells, primordial, primary, and pre-antral follicles according to published methods [29]. A germ cell was classified as being round in appearance and having a visible nucleus. Follicles were considered primordial if they had an intact oocyte with a visible nucleus surrounded by one layer of fusiform-shaped granulosa cells. Follicles were considered primary if they had an intact oocyte with a visible nucleus and a single layer of cuboidal granulosa cells. Follicles were considered preantral if they had an intact oocyte with a visible nucleus and 2–4 layers of granulosa cells with no antral space. Follicles were considered antral if they had three or more layers of granulosa cells and showed clearly defined antral space. When follicles were deemed as transitional, they were counted toward the more immature state. If follicles did not have a visible nucleus, they were not counted to avoid double counting. Sections were counted without knowledge of treatment.

2.5. Gene Expression Analysis

RNA from snap frozen ovaries was extracted using a miRNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. Due to the limited amount of RNA in PND 4 ovaries, 2–3 ovaries per litter were pooled for each sample. For all endpoints n = number of completely separate litters from different dams. The samples were treated with DNase (Qiagen, Inc., Valencia, CA) during the process. Messenger RNA (mRNA; 100 ng) was reversed transcribed to cDNA and subjected to quantitative real time PCR (qPCR) using the CFX96 real-Time PCR Detection System (Bio-Rad Inc.) and accompanying software (CFX Manager Software). The initial incubation temperature was 95°C for 1 minute and 10 seconds, and an annealing temp of 60°C, which was run 40 times. The temperature was 72°C for 5 minutes, 65°C for 5 seconds, 95°C for 5 seconds, and 72°C for 5 minutes. The standard curve, amplification, melting curve, and melting temperature graphs were generated for each run. Primer sequences for each gene are listed in Table 1. The data from each generation were normalized to the corresponding value of actin, beta (*ActB*) from that generation, and relative fold changes were calculated as a ratio to the control according to the Pfaffl method [30]. All samples were run in triplicate.

2.6. Measurement of estradiol levels

Blood was drawn from the mice immediately after euthanasia, and the serum was subjected to enzyme-linked immunosorbent assays (ELISAs) in one run without knowledge of treatment group at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (n = 3–7/group, except BPA 0.5 and 20 µg/kg/day had n = 2; coefficient of variation < 20%).

2.7. Statistical Analysis

For follicle count, gene expression, and hormone data, a standard error of the mean (SEM) was generated. P values less than 0.05 were considered statistically significant. Tests used depended on the normality and homogeneity of variance of the samples. For normally distributed, homogenous data, analysis of variance (ANOVA) was performed and was followed by the LSD post hoc test. For data that was not normally distributed or homogenous, the independent samples Kruskal-Wallis H and Mann-Whitney test were used along with the Games Howell post hoc test.

3. RESULTS

3.1. The effects of BPA on germ cell nest breakdown on PND 4 in the F2 and F3 generations

Previously, we showed that *in utero* BPA exposure inhibits germ cell nest breakdown and increases the percentage of primordial follicles on PND 4 ovaries in the F1 generation of mice [5]. The current study expanded on these findings by determining the effects of BPA exposure on germ cell nest breakdown in the F2 and F3 generations of these mice. The data indicate that BPA exposure does not alter the percentage of germ cells or numbers of primordial follicles in F2 and F3 generations compared to control (Fig. 1A, B, C, and D). Further, BPA at any dose did not alter total oocyte numbers compared to controls (data not shown).

3.2. The effects of BPA on apoptotic factors in the ovary on PND 4 in the F2–F3 generations

Our previous work showed that *in utero* BPA exposure inhibits apoptosis by altering the expression of certain apoptotic factors in the ovaries from F1 PND 4 mice [5]. Specifically, BPA at 50 µg/kg/day increased expression of the anti-apoptotic factor *Bcl2* and BPA at 0.5 µg/kg/day and 20 µg/kg/day decreased expression of the pro-apoptotic *Bax* compared to control [5]. In the current study, we expanded the analyses of these factors into the F2 and F3 generations (Fig. 2). In the F2 generation, BPA at 0.5 µg/kg/day significantly decreased expression of *Bcl2* (Fig. 2A), but it did not significantly affect the expression of *Bax* compared to control (Fig. 2C). BPA also did not significantly affect the ratios of these two factors in the F2 ovaries. In the F3 generation, BPA exposure did not significantly affect levels of expression of *Bcl2*, *Bax*, or their ratio compared to control (Fig. 2B, D).

3.3. The effects of BPA on anti-oxidant genes in the ovary on PND 4 in the F1–F3 generations

Oxidative stress is thought to play a role in the process of germ cell nest breakdown [5] and oxidative stress is regulated in part by anti-oxidant factors. Thus, gene expression levels of the anti-oxidant factors *Sod1*, *Cat*, and *Gpx* were compared in control and BPA treated ovaries. In the F1 generation, the lowest dose of BPA (0.5 µg/kg/day) significantly increased the expression of *Cat* (Fig. 3D) and *Gpx* (Fig. 3G) compared to the control. The middle dose of BPA (20 µg/kg/day) significantly increased expression of *Sod1* (Fig. 3A), *Cat* (Fig. 3D), and *Gpx* (Fig. 3G) compared to the control. Additionally, the highest dose of BPA (50 µg/kg/day) significantly increased both *Cat* (Fig. 3D) and *Gpx* expression (Fig. 3G) compared to the control. In the F2 generation, the middle dose of BPA (20 µg/kg/day) continued to increase expression of *Sod1* (Fig. 3A) and *Cat* (Fig. 3E) compared to control. Further, the highest dose of BPA significantly increased *Cat* (Fig. 3D) compared to control. In the F3 generation, BPA did not significantly affect anti-oxidant gene expression compared to control (Fig. 3C, F, I).

3.4. The effects of BPA on autophagy-related genes in the ovary on PND 4 in the F1–F3 generations

The effects of BPA on levels of expression of several autophagy-related genes such as *Atg7*, *Atg12*, and *Becn1* were compared in control and BPA treated ovaries (Fig. 4). In the F1 generation, BPA at 0.5 µg/kg/day increased expression of *Atg7* compared to control (Fig. 4A). BPA at 20 µg/kg/day also increased expression of *Atg7*, *Atg12*, and *Becn1* compared to control (Fig. 4A, D, G). Additionally, the highest dose of BPA (50 µg/kg/day) significantly increased the expression of *Atg7* and *Atg12* compared to the control (Fig. 4 A, D). In the F2 generation, only the highest dose of BPA (50 µg/kg/day) increased the expression of *Atg7* compared to the control (Fig. 4B). In the F3 generation, BPA did not significantly alter expression of any of the selected autophagy-related genes compared to the control (Fig. 4C, F, I).

3.5. The effects of BPA on estradiol levels in the ovary on PND 4 in the F1–F3 generations

The process of germ cell nest breakdown in mice is driven by a drop in estrogen levels around the time of birth [10]. For this reason, the levels of estradiol were examined in the pups from the F1, F2, and F3 generations. In the F0 dams, BPA did not significantly affect estradiol levels compared to control (control: 2.18 ± 0.13 ; BPA 0.5 µg/kg/day: 2.23 ± 0.48 ; BPA 20 µg/kg/day: 2.40 ± 0.13 ; BPA 50 µg/kg/day: 2.28 ± 0.34 ; pg/mL, n = 3–6). In the F1 mice, BPA 20 µg/kg/day significantly increased estradiol levels compared to control (control: 5.65 ± 0.50 ; BPA 0.5 µg/kg/day: 6.59 ± 0.14 ; BPA 20 µg/kg/day: 7.30 ± 0.61 ; BPA 50 µg/kg/day: 7.09 ± 0.57 ; pg/mL, n = 3–7, except BPA 0.5 µg/kg/day had n = 2, p = 0.05). In the F2 mice, BPA did not significantly affect estradiol levels compared to control (control: 11.84 ± 0.79 ; BPA 0.5 µg/kg/day: 13.21 ± 1.8 ; BPA 20 µg/kg/day: 12.19 ± 1.47 ; BPA 50 µg/kg/day: 13.93 ± 0.80 ; pg/mL, n = 3–7, except BPA 0.5 µg/kg/day had n = 2, p = 0.05). In the F3 mice, BPA did not significantly affect estradiol levels compared to control (control: 5.75 ± 0.57 ; BPA 0.5 µg/kg/day: 6.11 ± 0.77 ; BPA 20 µg/kg/day: 6.85 ± 1.12 ; BPA 50 µg/kg/day: 7.16 ± 0.30 ; pg/mL, n = 3–4, except BPA 0.5 and 20 µg/kg/day had n = 2).

3.6. The effects of BPA on follicle numbers in ovaries on PND 21 in the F1–F3 generations

Our previous study did not examine whether *in utero* BPA exposure affects follicle numbers at a time later than PND 4. Thus, in the current study, the numbers of primordial, primary, preantral, and antral follicles were compared in control and BPA treated ovaries collected on PND 21 (Fig. 5). In the F1 PND 21 ovaries, BPA at 0.5 µg/kg/day significantly decreased the percentage of primordial follicles and increased the percentage of primary follicles compared to control (Fig. 5A). BPA at 50 µg/kg/day also increased the percentage of preantral follicles compared to control (Fig. 5A). In the F2 mice at this time point, BPA at 0.5 µg/kg/day significantly decreased primary and increased preantral follicle percentages compared to control (Fig. 5B). BPA at 20 µg/kg/day decreased primordial and increased preantral follicles compared to control (Fig. 5B). BPA at 50 µg/kg/day decreased the percentage of primordial follicles compared to control (Fig. 5B). In the F3 generation, BPA did not significantly affect follicle percentage compared to control (Fig. 5C). Further, BPA at any dose did not affect in total oocyte numbers compared to controls (data not shown).

3.7. The effects of BPA on apoptotic factors in ovaries on PND 21 in the F1–F3 generations

The same apoptotic factors that were examined on PND 4 (*Bcl2* and *Bax*), along with caspase 8 (*Casp8*), were examined on PND 21 to determine the effect of BPA on gene expression of apoptotic factors at a later time point (Fig. 6). In the F1 generation, BPA did not significantly change expression of any of selected genes (Fig. 6A, D, G). In the F2 generation, BPA at the highest dose (50 µg/kg/day) increased expression of *Bcl2* (Fig. 6B) and *Casp8* (Fig. 6H) compared to control. In the F3 generation, BPA at the 0.5 µg/kg/day dose decreased the expression of *Bcl2* (Fig. 6C) and *Casp8* (Fig. 6I) compared to control. In contrast, BPA at the 20 µg/kg/day dose increased expression of *Casp8* compared to control (Fig. 6I). Further, BPA at the highest dose (50 µg/kg/day) significantly increased the levels of *Bax* compared to control (Fig. 6F). BPA did not significantly affect the ratios of *Bcl2/Bax* in any generation at any doses compared to the control.

3.8. The effects of BPA on anti-oxidant genes in ovaries on PND 21 in the F1–F3 generations

Due to the BPA induced changes in expression of anti-oxidant genes in the ovaries at PND 4, the same gene levels were analyzed in the PND 21 mice (Fig. 7). In the F1 generation, BPA did not significantly change expression of any of the selected anti-oxidant genes compared to control (Fig. 7A, D, G). In the F2 generation, BPA significantly increased expression of *Sod1* (Fig. 7B), *Cat* (Fig. 7E) and *Gpx* (Fig. 7H) at both the 20 µg/kg/day and 50 µg/kg/day doses compared to the control. In the F3 generation, the 20 µg/kg/day dose of BPA continued to increase expression of *Sod1* (Fig. 7C) and the 50 µg/kg/day dose increased the expression of *Sod1* (Fig. 7C) and *Gpx* (Fig. 7I) compared to the control.

3.9. The effects of BPA on factors in the *Igf* family in ovaries on PND 21 in the F1–F3 generations

The effects of BPA on the expression levels of *Igf1* and *Igfbp2* were examined because the *Igf* family is involved in regulating the cell cycle and apoptosis [20]. In the F1 generation, BPA did not significantly change expression of *Igf1* (Fig. 8A) and *Igfbp2* (Fig. 8D)

compared to control. In the F2 generation, the lowest dose of BPA (0.5 µg/kg/day) increased expression of *Igf1* (Fig. 8B), and the highest dose (50 µg/kg/day) increased both *Igfbp2* (Fig. 8E) and *Igf1* (Fig. 8B) compared to control. In the F3 generation, BPA at 0.5 µg/kg/day significantly decreased the expression of *Igfbp2* compared to the control (Fig. 8F). The middle dose of BPA (20 µg/kg/day) significantly increased the expression of both *Igf1* (Fig. 8C) and *Igfbp2* (Fig. 8F) compared to the control. BPA at the highest dose (50 µg/kg/day) significantly increased the expression of *Igfbp2* compared to the control (Fig. 8F).

3.10. The effects of BPA on sex steroid hormone receptors in ovaries on PND 21 in the F1–F3 generations

BPA has been shown to alter expression of sex steroid hormone receptors [21], and sex steroid hormone receptor levels play a role in regulating follicle numbers and growth [23]. Thus, to determine the effects of BPA on sex steroid receptors that are important in the ovary, the effects of BPA on the expression levels of *Esr1*, *Esr2*, and *Ar* were compared to control (Fig. 9). In the F1 generation, BPA did not significantly change expression of either *Esr1* or *Ar* compared to control (Fig. 9A, D). In the F2 generation, the highest dose of BPA (50 µg/kg/day) significantly increased expression of *Esr1* (Fig. 9B) and *Ar* (Fig. 9E) compared to control. In the F3 generation, BPA significantly decreased expression of *Esr1* at the lowest dose (0.5 µg/kg/day) compared to control (Fig. 9C). BPA did not significantly alter expression of *Esr2* in the F1–F3 generations (data not shown).

3.11. The effects of BPA on steroidogenesis-related genes in ovaries on PND 21 in the F1–F3 generations

Previous *in vitro* studies indicate that BPA inhibits expression of steroidogenic enzymes and steroidogenesis-related genes [25, 31]. Thus, we compared effects of BPA on expression of these genes in the F1–F3 generations (Fig. 10). In the F1 generation, BPA did not significantly affect the expression levels of *Fshr* and *Star* (Fig. 10A, J), but the highest dose (50 µg/kg/day) increased the levels of *Hsd17b1* (Fig. 10G) and *Cyp17a1* (Fig. 10D) compared to the control. In the F2 generation, BPA at the lowest dose (0.5 µg/kg/day) significantly increased the expression of *Hsd17b1* (Fig. 10H) and *Fshr* (Fig. 10B) compared to control. Additionally, BPA at the middle dose (20 µg/kg/day) increased *Fshr* (Fig. 10B), *Cyp17a1* (Fig. 10E), *Hsd17b1* (Fig. 10H), and *Star* (Fig. 10K) compared to control. In the F3 generation, the highest dose of BPA (50 µg/kg/day) increased expression of *Fshr* (Fig. 10C) and *Cyp17a1* (Fig. 10F) compared to control.

3.12. The effects of BPA on estradiol levels in mice on PND 21 in the F1–F3 generations

Given that *in utero* BPA exposure alters expression of several genes that regulate steroidogenesis (Fig. 10), we examined whether BPA exposure affects estradiol levels on PND 21 in the F1–F3 generations. In the F1 mice, BPA did not significantly affect estradiol levels compared to control (control: 3.58 ± 0.26 ; BPA 0.5 µg/kg/day: 3.45 ± 0.07 ; BPA 20 µg/kg/day: 3.55 ± 0.30 ; BPA 50 µg/kg/day: 3.98 ± 0.17 ; pg/mL, $n = 3-4$, except BPA 0.5 µg/kg/day had $n = 2$). In the F2 mice, BPA did not significantly affect estradiol levels compared to control (control: 5.71 ± 0.89 ; BPA 0.5 µg/kg/day: 5.19 ± 0.35 ; BPA 20 µg/kg/day: 6.3 ± 0.45 ; BPA 50 µg/kg/day: 6.02 ± 2.39 ; pg/mL, $n = 4-6$). In the F3 mice, BPA did not significantly affect estradiol levels compared to control (control: 7.24 ± 0.58 ; BPA 0.5

µg/kg/day: 8.43 ± 0.78 ; BPA 20 µg/kg/day: 8.51 ± 0.70 ; BPA 50 µg/kg/day: 9.03 ± 0.61 ; pg/mL, n = 3–5).

4. DISCUSSION

Our study shows that *in utero* BPA exposure inhibits germ cell nest breakdown in PND 4 ovaries of the F1 generation, but not in the F2 or F3 generations. Our previous study showed that *in utero* BPA exposure caused more germ cells to remain in nests and less formation of primordial follicles at PND 4 in the F1 generation [5]. Additionally, BPA at the highest dose reduced litter size [5], which led to a low sample size in later generations in the current study. Further, our previous *in vitro* study showed that BPA increases the percentage of germ cells in nests and decreases the size of the primordial follicle pool at PND 4 and 8 [32]. Collectively, these data suggest that BPA directly targets the ovary to inhibit germ cell nest breakdown in the F1 generation, but the ability of BPA to inhibit the process of germ cell nest breakdown is not carried over into subsequent generations.

Our results also suggest that the ability of BPA to affect apoptotic gene expression in the PND 4 ovary is not transgenerational. In our previous study, BPA increased expression of anti-apoptotic factors and decreased expression of pro-apoptotic factors in PND 4 ovaries in the F1 generation [5]. These changes in expression, however, do not continue in the F2 and F3 generations. Specifically, BPA decreased expression of anti-apoptotic factors in the F2 generation, but not in the F3 generation. The finding that BPA decreased anti-apoptotic factors and increased pro-apoptotic factors in the F1 and F2 generations is consistent with results from an *in vitro* study [32]. Specifically, BPA exposure *in vitro* increased the anti-apoptotic factor *Bad* at PND 1, 2, and 4, increased pro-apoptotic factors at PND 2, and increased the ratio of anti-apoptotic to pro-apoptotic factors at PND 1, 2, and 4 [32]. Collectively, these data suggest that the ability of BPA to inhibit germ cell nest breakdown in the F1 generation may be due to its ability to inhibit apoptosis. In the F1 generation, we observed both decreased apoptosis and decreased germ cell nest breakdown. In the F2 generation, BPA-induced changes in apoptotic factors were less prevalent than in the F1 generation and BPA did not change apoptotic factors in the F3 generation. Interestingly, BPA also did not inhibit germ cell nest breakdown in the F2 and F3 generations. Although apoptosis may not be solely responsible for these changes, our data indicate that there is correlation between inhibition of apoptosis and reduced germ cell nest breakdown in the F1 generation.

In addition to inhibition of apoptosis playing a role in BPA-induced inhibition of germ cell nest breakdown, it is likely that oxidative stress plays a role in regulating germ cell nest breakdown. Our results, however, suggest that the ability of BPA to affect oxidative stress in the PND 4 ovary is not transgenerational. In the F1 generation, BPA exposure increased expression of each examined antioxidant gene. Interestingly, this could lead to reduced oxidative stress, a scenario that could inhibit germ cell nest breakdown in the F1 generation. In the F2 generation, BPA still increased expression of anti-oxidant genes, but to a lesser extent than in the F1 generation. In the F3 generation, BPA did not significantly affect expression of anti-oxidant genes compared to control. Consistent with these findings, BPA did not inhibit germ cell nest breakdown in the F2 and F3 generations.

Our findings that BPA increases expression of anti-oxidant genes in the F1 generation *in vivo* differ from those we obtained *in vitro* [32]. Our previous *in vitro* study showed that BPA did not consistently affect oxidative stress-related genes compared to the control at PND 1, 2, and 4 [32]. The reasons for differences between *in vivo* and *in vitro* studies are unclear. It is possible that BPA does not increase the expression of anti-oxidant genes of the ovary directly, but it does so indirectly. Perhaps, BPA exposure *in vivo* alters the hypothalamic-pituitary-gonadal axis in a manner that leads to changes in the expression of anti-oxidant genes in the ovary. Although our study took place before the hypothalamic-pituitary-gonadal axis has finished its development, BPA could target this axis in the long term. It is also possible that metabolism of BPA differs *in vitro* and *in vivo* and that these metabolic differences lead to differences in expression of anti-oxidant genes.

Our results suggest that BPA may also inhibit germ cell nest breakdown by inhibiting autophagy and that the ability of BPA to affect autophagy genes in PND 4 ovaries is not transgenerational. BPA increased the expression levels of all genes that prevent autophagy in the ovaries from the F1 generation, but only increased expression of *Atg7* in the F2 generation, and did not change expression in the F3 generation compared to the control. As genes in the *Atg* family prevent autophagy, and autophagy plays a role in the process of germ cell nest breakdown [33], the F1 generation data are consistent with BPA inhibiting autophagy, leading to inhibition of germ cell nest breakdown. In the F2 generation, BPA only increased expression of one autophagy gene, similar to the effects of BPA on the apoptotic factors in this generation. Further, in the F3 generation, BPA did not change autophagy gene expression, which fits with the lack of inhibition of germ cell nest breakdown in these generations. The results from the F1 generation agree with previous studies that show that the *Atg* family can act to protect against cell death and oxidative stress in the ovary [16]. The degree to which autophagy plays in the process of preventing germ cell nest breakdown at PND 4 is unknown, but our data implicate that the examined genes are involved in this process to some extent.

Our results show that BPA increased estradiol levels at PND 4 in the F1 generation, but these effects are not transgenerational. Specifically, BPA increased the levels of estradiol in F1 generation, but not in the F2 and F3 generations. The increase in estradiol levels in the F1 generation correlates with the decrease in germ cell nest breakdown at the same time point [5]. Previous studies indicate that germ cell nest breakdown is initiated by a drop in estrogen at birth in mice [10]; thus, the BPA-induced change in estradiol levels could explain why BPA inhibits germ cell nest breakdown. The lack of effects of BPA on estradiol levels in the F2 and F3 generations agrees with our data that BPA does not inhibit germ cell nest breakdown in these generations.

Our study also shows that BPA may affect follicle numbers/percentages at PND 21, but these effects are not transgenerational. In the F1 generation, our data suggest that BPA increases follicle recruitment into the primordial to the primary stage of development. In the F2 generation, our data suggest that BPA increases follicle recruitment to the preantral stage. In the F3 generation, BPA did not change follicle numbers compared to the control. It is possible that the BPA-induced reduction in primordial follicles observed on PND 4 may be contributing to the decrease in primordial follicles observed at PND 21. However, these data

do not provide an explanation for the BPA-induced increases in preantral follicles observed in the F1 generation. These data may indicate that some of the effects of BPA exposure on the process of folliculogenesis are occurring after the process of germ cell nest breakdown is finished. It is important to note that BPA may also be inducing changes at time points not investigated in this study. Nevertheless, *in utero* BPA exposure is affecting the normal distribution of follicles in the F1 and F2 generations at PND 21.

Although gene expression changes at PND 4 did not appear to be transgenerational, our results on expression of apoptotic factors suggest that the ability of BPA to affect apoptotic factor gene expression in PND 21 ovaries may be transgenerational. In the F1 generation, BPA did not affect expression of apoptotic factors compared to control. In the F2 generation, however, BPA increased expression of anti-apoptotic factors without changing the expression of pro-apoptotic factors. In the F3 generation, BPA continued to change gene expression of selected apoptotic factors, as BPA decreased anti-apoptotic factors and increased pro-apoptotic factors compared to the control. Although BPA significantly changed follicle numbers at PND 21 in the F1 generation, the lack of significant effects of BPA on gene expression may indicate that BPA acts through other mechanisms than apoptosis in this generation. It is possible that the BPA-induced increases in anti-apoptotic factors correlate with increased follicle recruitment into the preantral stage of development in the F2 generation, as less apoptosis could lead to an increase in follicle numbers. In the F3 generation, BPA decreased expression of anti-apoptotic factors, but consistently affected pro-apoptotic factors at the different doses. It is unclear how these varied results fit with the follicle count data. Thus, further studies are required to examine this in more detail.

Our results also indicate that the ability of BPA to affect expression of anti-oxidant genes in PND 21 ovaries may be transgenerational. BPA exposure increased expression of all selected anti-oxidant genes in the F2 and F3 generations. Overall, the effects of BPA on anti-oxidant gene expression at PND 21 did not correlate with the observed changes in follicle numbers. Thus, it may be that oxidative stress does not play a large role in the effects of BPA on development of follicles at this time point.

Our findings on the effects of BPA on gene expression levels in the *Igf* family suggest that the ability of BPA to affect these genes in PND 21 ovaries may be transgenerational. In the F1 generation, BPA did not affect expression of *Igf* family members, but in the F2 generation, it increased expression of *Igf* family members. In the F3 generation, BPA decreased expression of *Igf* family members at the lowest dose and increased expression at the middle and highest doses of BPA. Since *Igfs* have shown to help prevent apoptosis [20], these observed increases in *Igf* expression correspond with the BPA-induced decrease in apoptosis and increase in anti-oxidant genes at this time point.

Our results on sex steroid hormone receptors suggest that the ability of BPA to affect these receptors in PND21 ovaries may be transgenerational. BPA altered the expression levels of *Esr1* and *Ar* in the F2 and F3 generations, but not the F1 generation compared to control. These data are consistent with a previous *in vitro* study that showed that BPA decreased expression of *Esr1* after 24 hours of culture compared to control, but the expression returned to normal by 72 hours [34]. In the F2 generation, BPA increased expression of both *Esr1* and

Ar, but in the F3 generation, BPA decreased expression of *Esr1* in the ovary. As *Esr1* and *Ar* are important for the process of folliculogenesis [35, 36], the BPA-induced increases may play a role in the decreased primordial follicle counts observed in the F2 generation. The ability of BPA to decrease expression of the *Esr1* in the F3 generation may indicate that BPA affects receptors differently at that generation.

Our results on steroidogenesis suggest that the ability of BPA to affect steroidogenic genes in PND 21 ovaries may be transgenerational. BPA increased expression levels of steroidogenesis-related genes in the F1 through the F3 generation. In some cases, BPA exposure increased *Fshr*, *Cyp17a1*, *Star*, and *Hsd17b1* gene expression in 2–3 generations. *Fshr*, *Cyp17a1*, *Star*, and *Hsd17b1* are important for sex steroid hormone biosynthesis. Thus, we expected that we would observe changes in estradiol levels at the same time-point. However, that was not the case. It could be that changes in protein levels or activity of the enzymes are required for changes in sex steroid hormone levels *in vivo*. Additionally, the estradiol levels for the F2 generations at both time points were consistently higher than that of the other generations. The same laboratory ran all of the samples in a single run without knowledge of treatment group. In addition, preliminary tests indicated that the levels of BPA used in this study do not cross-react with estradiol levels in the hormone assays. Further, if cross-reactivity was an issue, it would also affect the F1 and F3 data as well, not just the F2 results. Thus, we are uncertain as to why F2 estradiol levels should be consistently higher than those in F1 and F3 animals. One possibility is that it is due to unmeasured endpoints in the current study. For example, it is possible that there are differences in the metabolism of estrogen in the F2 mice compared to the F1 and F3 mice, but this is something that would have to be explored in future studies. Interestingly, BPA has been shown to inhibit steroidogenic enzyme expression and sex steroid hormone levels *in vitro* [25, 34], and although this does not exactly translate to our *in vivo* results, it does give an indication of the mechanisms involved in the effects of BPA on steroidogenesis.

In conclusion, this study shows that *in utero* BPA exposure directly inhibits germ cell nest breakdown *in vivo* at PND 4, likely by inhibiting apoptosis, oxidative stress, and autophagy. These effects are not transgenerational. Further, this study shows that *in utero* BPA exposure alters follicle numbers and gene expression at PND 21, and that some BPA-induced changes in gene expression at PND 21 may be transgenerational. Since the environment of an individual plays a very important role in its development, the disruption of the endocrine system by a toxicant like BPA can have effects lasting into adulthood. In some cases, changes are mediated by changes to the epigenome, as opposed to that of normal genetic mutations. Changes in the germ line, particularly primordial cell migration, often predate these transgenerational effects [37]. A future study may benefit from a micro-array or RNA-seq to help elucidate more of the rationale for the changes observed in the follicle count data and previous studies.

Although most of the previous studies on BPA exposure focus on changes in the F1 generation, several studies suggest that BPA causes transgenerational effects by causing epigenetic reprogramming in the fetal germ cells of rodents exposed to BPA *in utero* and in the subsequent generations [38, 39]. Specifically, previous studies show that BPA decreases female fertility in the F1–F3 generations of mice [5, 40] and decreases the size of the

primordial follicle pool in the F3 generation of rats [37]. Although this study did not observe transgenerational changes in primordial follicle numbers, this may be due to differences in the exposure windows, dosing methods, or species. Further, BPA exposure *in utero* decreases fertility and impairs spermatogenesis in the F2 and F3 generations of rats [41]. BPA also has been documented to cause transgenerational effects on behavior [42, 43] and social recognition and activity through the F3 generation [44]. Significant data in the later generations with no apparent changes in earlier generations may indicate that changes have been made in the epigenome, but cannot be seen in the current study. With this in mind, examining the effects of *in utero* BPA exposure on the ovaries of multiple generations of mice provides additional insight into the changes in the ovary that might be occurring from one generation to another in response to BPA.

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Highlights

- *In utero* BPA does not inhibit germ cell nest breakdown in the F2-F3 generations
- *In utero* BPA affects ovarian apoptotic factor gene expression in 3 generations
- *In utero* BPA affects ovarian oxidative stress gene expression in 3 generations
- *In utero* BPA affects ovarian autophagy gene expression in 2 generations

Fig 1. Effect of *in utero* BPA exposure on germ cell nest breakdown. Ovaries were collected from mice on PND 4 and subjected to histological evaluation of germ cell and primordial follicle formation. Please note that the percentage of germ cells remaining in nest and the percentage of formed primordial follicles do not add up to 100% of germ cells, as a small percentage of germ cells were primary follicles. Data are presented as a mean $\pm$ SEM (n= 1–5 ovaries/treatment).

Fig 2.
Effect of *in utero* BPA exposure on the mRNA expression levels of selected apoptotic factors. Ovaries were collected from mice on PND 4 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Bcl2* in the (A) F2 generation and (B) F3 generation and *Bax* in the (C) F2 generation and (D) F3 generation. All data are presented as a mean $\pm$ SEM (n= 3–5 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 3. Effect of *in utero* BPA exposure on the mRNA expression levels of selected anti-oxidant genes. Ovaries were collected from mice on PND 4 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Sod1* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation; *Cat* in the (D) F1 generation, (E) F2 generation, and (F) F3 generation; and *Gpx* in the (G) F1 generation, (H) F2 generation and (I) F3 generation. All data are presented as a mean $\pm$ SEM (n= 3–5 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 4. Effect of *in utero* BPA exposure on the mRNA expression levels of selected autophagy-related genes. Ovaries were collected from mice on PND 4 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Atg7* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation; *Atg12* in the (D) F1 generation, (E) F2 generation and (F) F3 generation; and *Becn1* in the (G) F1 generation, (H) F2 generation and (I) F3 generation. All data are presented as a mean $\pm$ SEM (n= 3–5 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 5. Effect of *in utero* BPA exposure on follicle percentages. Ovaries were collected from mice on PND 21 and subjected to histological evaluation of primordial, primary, preantral and antral follicles. Data are presented as a mean $\pm$ SEM (n= 2–6 ovaries/treatment). Percentages of follicle types from the total counted in (A) F1 generation, (B) F2 generation, and (C) F3 generation. Asterisks (*) represent statistically significant differences from the vehicle control ($p < 0.05$).

Fig 6. Effect of *in utero* BPA exposure on the mRNA expression levels of selected apoptotic factors. Ovaries were collected from mice on PND 21 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Bcl2* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation; *Bax* in the (D) F1 generation, (E) F2 generation and (F) F3 generation; and *Casp8* in the (G) F1 generation, (H) F2 generation and (I) F3 generation. All data are presented as a mean $\pm$ SEM (n= 4–6 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 7.

Effect of *in utero* BPA exposure on the mRNA expression levels of selected anti-oxidant genes. Ovaries were collected from mice on PND 21 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Sod1* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation; *Cat* in the (D) F1 generation, (E) F2 generation and (F) F3 generation; and *Gpx* in the (G) F1 generation, (H) F2 generation and (I) F3 generation. All data are presented as a mean $\pm$ SEM (n= 4–6 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 8.

Effect of *in utero* BPA exposure on the mRNA expression levels of selected genes in the *Igf* family. Ovaries were collected from mice on PND 21 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Igf1* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation and *Igfbp2* in the (D) F1 generation, (E) F2 generation and (F) F3 generation. All data are presented as a mean $\pm$ SEM (n= 4–6 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 9.

Effect of *in utero* BPA exposure on the mRNA expression levels of selected sex hormone receptors. Ovaries were collected from mice on PND 21 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Esr1* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation and *Ar* in the (D) F1 generation, (E) F2 generation and (F) F3 generation. All data are presented as a mean $\pm$ SEM (n= 4–6 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Fig 10.

Effect of *in utero* BPA exposure on the mRNA expression levels of selected steroidogenic-related genes. Ovaries were collected from mice on PND 21 and subjected to measurement of gene expression by real-time PCR analysis. Relative fold changes normalized to *Actb* are shown for *Fshr* in the (A) F1 generation, (B) F2 generation, and (C) F3 generation; *Cyp17a1* in the (D) F1 generation, (E) F2 generation and (F) F3 generation; *Hsd17b1* in the (G) F1 generation, (H) F2 generation and (I) F3 generation; and *Star* in the (J) F1 generation, (K) F2 generation and (L) F3 generation. All data are presented as a mean $\pm$ SEM (n= 4–6 ovaries/treatment). Asterisks (*) represent statistically significant differences from the vehicle control (p < 0.05).

Table 1

Sequences of primer sets used for gene expression analyses.

Gene name	Abbreviation	Forward (5' → 3')	Reverse (5' → 3')
Actin, beta	<i>ActB</i>	GGGCACAGTGTGGGTGAC	CTGGCACACACCTTCTAC
Superoxide dismutase 1	<i>Sod1</i>	TTCCGTCCGTCGGCTTCTCGT	CGCACACCGCTTTCATCGCC
Catalase	<i>Cat</i>	GCAGATACCTGTGAACTGTC	GTAGAATGTCCGCACCTGAG
Glutathione peroxidase	<i>Gpx</i>	CCTCAAGTACGTCCGACCTG	CAATGTCGTTGCGGCACACC
B cell leukemia/lymphoma 2	<i>Bcl2</i>	ATGCCTTTGTGGAATATATGGC	GGTATGCACCCAGAGTGATGC
BCL2-associated X protein	<i>Bax</i>	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
Autophagy related 7	<i>Atg7</i>	GTTGAGCGGCGACAGCATTAG	ATGGCAGGAAAGCAGTGTGG
Autophagy related 12	<i>Atg12</i>	TAAACTGGTGGCCTCGGAAC	CCATCACTGCCAAAACACTCA
Beclin 1, autophagy related	<i>Becn1</i>	ACTCACAGCTCCATTACTTACCA	CACCATCCTGGCGAGTTTCA
Caspase 8	<i>Casp8</i>	CGAGAGGAGATGGTGAGAGAGC	CAGGCTCAAGTCATCTTCCAGC
Insulin-like growth factor 1	<i>Igf1</i>	ATCCCAAGCCCTGTTTGTT	TGCCCCCAGTGTGTTGAAGT
Insulin-like growth factor binding protein 2	<i>Igfbp2</i>	CTCTACTCCCTGCACATCCC	TTCAGAGACATCTTGCACTGCT
Aldehyde dehydrogenase family 1, subfamily A1	<i>Aldh1a1</i>	GGTGAGGAGGACTAGTTGTGAC	TCACAACACCTGGGGAACAG
Estrogen receptor 1 (alpha)	<i>Esr1</i>	CCGTGTGCAATGACTATGCC	GTGCTTCAACATTCTCCCTCCTC
Estrogen receptor 2 (beta)	<i>Esr2</i>	GGAATCTCTTCCCAGCAGCA	GGGACCACATTTTGCACCTT
Androgen receptor	<i>Ar</i>	GGCGGTCCTTCACTAATGTCAACT	GAGACTTGTGCATGCGGTACTCAT
Follicle stimulating hormone receptor	<i>Fshr</i>	AGCAAGTTTGGCTGTTATGAGG	GTTCTGGACTGAATGATTTAGAGG
Cytochrome P450, family 17, subfamily a, polypeptide 1	<i>Cyp17a1</i>	CCAGGACCCAAGTGTGTTCT	CCTGATACGAAGCACTTCTCG
Hydroxysteroid (17- beta) dehydrogenase 1	<i>Hsd17b1</i>	ACTGTGCCAGCAAGTTTGCG	AAGCGGTTTCGTGGAGAAGTAG
Steroidogenic acute regulatory protein	<i>Star</i>	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG