In Utero and Lactational Exposure to Flame Retardants Disrupts Rat Ovarian Follicular Development and Advances Puberty

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ABSTRACT

Brominated flame retardants (BFRs), including polybrominated diphenyl ethers and hexabromocyclododecane, leach out from consumer products into the environment. Exposure to BFRs has been associated with effects on endocrine homeostasis. To test the hypothesis that *in utero* and lactational exposure to BFRs may affect the reproductive system of female offspring, adult female Sprague Dawley rats were fed diets formulated to deliver nominal doses (0, 0.06, 20, or 60 mg/kg/day) of a BFR dietary mixture mimicking the relative congener levels in house dust from prior to mating until weaning. Vaginal opening and the day of first estrus occurred at a significantly earlier age among offspring from the 20 mg/kg/day BFR group, indicating that the onset of puberty was advanced. Histological analysis of ovaries from postnatal day 46 offspring revealed an increase in the incidence of abnormal follicles. A toxicogenomic analysis of ovarian gene expression identified upstream regulators, including HIF1A, CREB1, EGF, the β-estradiol, and PPARA pathways, predicted to be downregulated in the 20 or 60 mg/kg/day group and to contribute to the gene expression patterns observed. Thus, perinatal exposure to BFRs dysregulated ovarian folliculogenesis and signaling pathways that are fundamental for ovarian function in the adult.

Key words: brominated flame retardants; ovary; folliculogenesis; endocrine disruptors; estrogens.

Ovarian follicles are produced from a pool of primordial germ cells in the developing gonad early in gestation (Grive and Freiman, 2015). These primordial germ cells first form clusters (or "cysts") that later undergo a breakdown during which they become surrounded by layers of somatic granulosa cells, forming the follicles that represent the entire ovarian reserve (Hirshfield and Midgley, 1978). In the rat, follicular histogenesis is initiated in the hours following birth and spreads throughout the gonad in subsequent days (Hirshfield and DeSanti, 1995) under the control of signaling mechanisms between oocytes and their surroundings (Grive and Freiman, 2015). Hence, primordial follicle assembly during the fetal and neonatal periods determines the long-term reproductive capacity of female mammals and constitutes a sensitive window of exposure to chemical insults.

Brominated flame retardants (BFRs) are added to a wide variety of consumer products, such as furniture, textiles, plastics, and electrical circuits, to slow down the spread of flames in the event of a fire (Camino et al., 1991; Stapleton et al., 2012). Because BFRs are usually not covalently bound to these products (de Wit, 2002; Webster et al., 2009), they can leach out and are found ubiquitously in our home environments. Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDDs), among the most prominent BFRs, are

present in high concentrations in domestic dust in the majority of houses sampled in North America (reviewed in Malliari and Kalantzi [2017]). As a consequence, humans are continuously exposed to BFRs by inhalation and by ingestion (reviewed in Malliari and Kalantzi [2017]). Exposure is estimated to be higher early in life, in newborns and children, compared with adults (reviewed in Dufour and Charlier [2017]), due to the ingestion of house dust from hand-to-mouth contact and the consumption of contaminated breast milk (Jones-Otazo et al., 2005; Schecter et al., 2003; Stapleton et al., 2008b). Because they are lipophilic, BFRs bioaccumulate (Akortia et al., 2016; Darnerud, 2003; Segev et al., 2009) and are found in a multitude of human tissues and related matrices (Alaee, 2003; Poon et al., 2014), including follicular fluid (Johnson et al., 2012), breast milk (Marchitti et al., 2013; Meironyté et al., 1999), hair (Albert et al., 2018; Goodyer et al., 2017; Poon et al., 2014), and serum (Shin et al., 2016; Zota et al., 2013).

Over the past few decades, BFRs have been established to act as endocrine disrupting compounds (EDCs), affecting the activities of androgenic, estrogenic, and thyroid hormones (Darnerud, 2008; Linares et al., 2015; Lyche et al., 2015). Despite this, relatively few studies have examined their association with effects on female reproduction and fertility. Women with high PBDE levels in serum or follicular fluid have longer times to conception (Harley et al., 2010), decreased fertilization rates, embryo implantation failure (Johnson et al., 2012), and an increased risk of preterm birth (Peltier et al., 2015). High serum PBDE concentrations have also been associated with a younger age at menarche in adolescent girls (Chen et al., 2011).

Several animal studies have reported effects of exposure to a single PBDE congener on the female reproductive system. The exposure of rats to BDE-99 during gestation decreased the numbers of follicles in the ovaries of female offspring and delayed the onset of puberty (Lilienthal et al., 2006). Gestational exposure to BDE-47 was also reported to decrease follicle counts in female offspring; this effect was accompanied by a decrease in serum estradiol concentrations (Talsness et al., 2008). Previously, we demonstrated that the exposure of adult female rats prior to and during pregnancy to an environmentally relevant mixture of BFRs, formulated to mimic the BFR congener levels found in domestic dust (Allen et al., 2008; Stapleton et al., 2008a), adversely affected ovarian steroidogenesis and folliculogenesis (Lefèvre et al., 2016). Our goal in this study was to investigate the consequences of in utero and lactational exposure to this environmentally relevant mixture of PBDEs and HBCDDs on the onset of puberty and ovarian development in the F1 generation, the exposed female offspring.

MATERIALS AND METHODS

BFR mixture formulation. The median levels of the PBDEs and HBCDDs observed in the Boston house dust studies (Allen et al., 2008; Stapleton et al., 2008a) were used to develop a BFR mixture that was subsequently incorporated into an isoflavone-free diet formulated to reduce phytoestrogens (Teklad Global 2019 diet; Harlan Laboratories, Madison, Wisconsin), as described previously (Berger et al., 2014; Ernest et al., 2012; Lefèvre et al., 2016). Diets were prepared using 3 technical PBDE mixtures, DE-71 (52.1%), DE-79 (0.4%), and BDE-209 (44.2%), and 1 HBCDD mixture (3.3%) to give 0 (control), 0.75, 250, or 750 mg of BFR mixture per kg of diet, with target nominal doses of 0, 0.06, 20, and 60 mg/kg of body weight per day based on a daily food consumption of 80 g/kg body weight per day. The lowest dose reflects maximum human exposure based on a 100 mg/day dust ingestion rate in a

child (16.5 kg), taking into account the surface ratio of the human body compared with that of the rat (1:6.9).

Experimental animals and treatments. The details of animal treatment and breeding have been described previously (Tung et al., 2016). Briefly, virgin female Sprague Dawley rats aged 6–7 weeks were purchased from Charles River Laboratories (St-Constant, Quebec, Canada) and acclimated for 2 weeks on control diet. All animals were housed under controlled light (12L: 12D), humidity (40%–70%), and temperature (20°C–24°C). All studies were completed in accordance with the guidelines of the Canadian Council on Animal Care; experimental procedures were approved by the Health Canada Animal Care Committee.

Females were randomized ($n = 26$ per treatment) to 1 of 4 BFR treatments (0, 0.06, 20, or 60 mg/kg body weight/day). Food and water were provided *ad libitum*. After a minimum of 1 week on these diets, estrous cycles were monitored daily by vaginal lavage; females observed to be in proestrus ($n = 22$–24 per treatment group) were housed overnight with proven fertile male Sprague Dawley rats. The presence of a sperm-positive vaginal swab the following morning indicated mating and this day was recorded as gestational day (GD) 0. All mated females were housed singly and allowed to deliver (this was denoted as postnatal day 0 or PND 0). The litters were reduced and balanced by sex to 8 pups each on PND 4. Dams were maintained on their respective diets throughout lactation until the pups were weaned onto the control diet on PND 21. Littermates of the same sex were cohoused and food consumption was monitored throughout development, as previously described (Tung et al., 2016, 2017).

One female pup from each litter was euthanized on PNDs 4, 21, and 46 (Figure 1). The ovaries were removed and weighed. One ovary from each female was fixed with 4% paraformaldehyde solution and the second was stored at $-80°C$ for gene expression analysis. Whole blood was recovered, allowed to clot, and centrifuged, and serum was aliquoted and stored at $-80°C$. Beginning on PND 32, all remaining female pups (1 or 2 pups per litter; 36 pups in 20 litters for Control; 21 in 11 litters in 0.06; 28 in 17 in 20 and 32 in 16 litters in 60 mg/kg) were examined daily (between 9:00 and 10:30 AM) for vaginal patency; once this was observed, vaginal cytology was sampled by lavage to determine the age at first estrus (FE).

Hormone measurements. Serum from 53 PND 46 females (Control $n = 16$; 0.06 mg/kg BFR = 10; 20 mg/kg = 13; 60 mg/kg = 14) was sent to OpAns (Durham, North Carolina) for a comprehensive steroid panel analysis (cortisol, corticosterone, 11-deoxycortisol, androstenedione, deoxycorticosterone, testosterone, OH-progesterone, progesterone, pregnenolone, estrone, and estradiol) using HPLC/MS/MS. With the exception of corticosterone and progesterone, hormonal levels in the vast majority of samples were below the lower limit of quantitation (Supplementary Figure 1A).

Follicle counts. Fixed ovaries were paraffin-embedded, serially sectioned (section thickness, 5 μm), and stained with hematoxylin-eosin. Sections were examined with a Leica microscope using ×200 or ×400 magnification. Ovarian follicles were counted and classified based on the morphology and thickness of the granulosa cell layer, using previously defined criteria (Hirshfield and Midgley, 1978; Lefèvre et al., 2016). A primordial follicle was defined by a single layer of flattened granulosa cells whereas primary follicles were characterized by a row of cuboid granulosa cells. The entire ovary was sectioned and screened
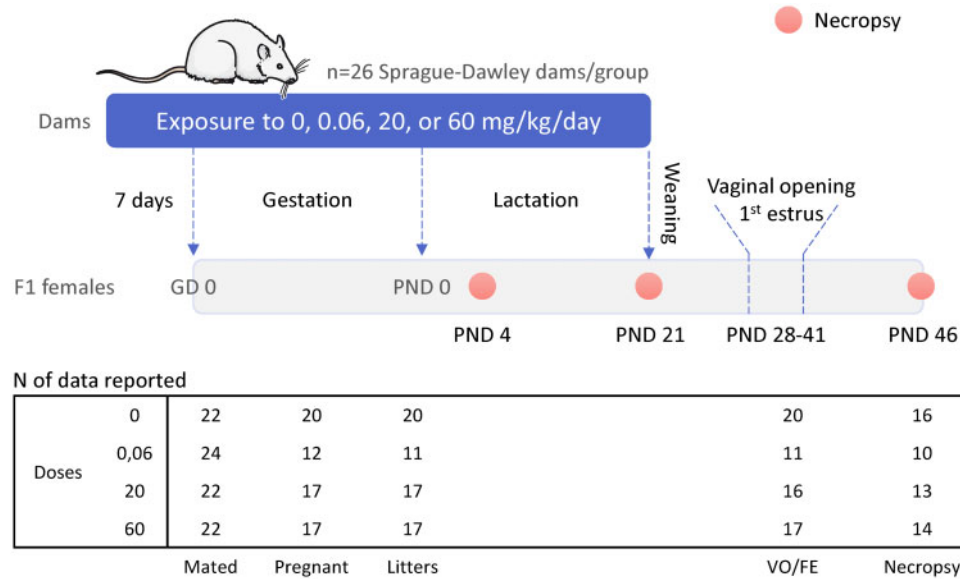
Figure 1. Experimental design. Sprague Dawley virgin females, divided into 4 treatment groups, received a brominated flame retardant (BFR) mixture in the diet, with target nominal doses of 0, 0.06, 20, and 60 mg/kg of body weight per day, from at least 1 week prior to mating until weaning at postnatal day (PND) 21. Female offspring were necropsied on PNDs 4, 21, and 46, and were monitored from PND 32 to PND 37 for vaginal opening (VO) and first estrus (FE). Values are means ± SEM; $n = 11$–20 females from independent litters.

for follicle counts on PND 4. To avoid counting the same follicle more than once on PND 46 primordial and primary follicles were counted only in every 4th section; the peripheral parts of the ovary were not screened. The total numbers of primordial and primary follicles were calculated and standardized to the whole ovary as $N = f \times NN \times 0.25$, where N is the total number of follicles, f is the fraction of ovarian sections sampled, NN is the total number of follicles at each stage counted in the sections sampled, and 0.25 is a correction factor for the 1 in 4 sections that were counted.

Secondary follicles were defined by the presence of 2 or more layers of granulosa cells and antral follicles by the formation of an antrum. All sections were examined at ×200 and secondary follicles scored as present only in sections where the nucleus was fully visible. The formula used here was $N = f \times NN$, where N is the total number of secondary or antral follicles, f is the fraction of ovarian sections sampled, and NN is the total number of follicles counted at each stage in the sampled sections.

Microarray analysis of RNA expression. RNA was extracted from the right ovaries from 1 female from separate litters (6 from control, 5 from 0.06, 5 from 20, and 6 from 60 mg/kg BFR treatment groups) using the Qiagen RNEasy Plus Mini Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions. RNA quality was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Ottawa, Ontario, Canada), with 260/280 and 260/230 absorbance ratios comprised between 1.9–2.1 and 2.0–2.2, respectively. RNA was labeled with Cy3 using the Agilent One Color Low Input Quick Amp kit and hybridized on arrays using the SurePrint G3 Rat Gene Expression 8 × 60K microarray kit (Agilent Technologies, Mississauga, Ontario, Canada), as per the manufacturer's instructions. Microarray analyses were done for RNA from the ovaries of 5–6 individual animals from independent litters for each treatment group and read with an Agilent SureScan Microarray Scanner G2600D. The resulting data were analyzed using GeneSpring version 14.9 (Agilent Technologies). Data were normalized to the 75th percentile using percentile shift normalization. Following quality control using principal component scores on all arrays, 1 sample was removed in the 0.06 mg/kg/day group. Transcripts affected by more than 2-fold were determined using a moderated t test and Benjamini-Hochberg FDR correction for each treatment. The microarray data have been uploaded to Gene Expression Omnibus (accession number GSE113000). The biological relationships between transcripts were analyzed using Ingenuity Pathway Analysis (IPA) version 01.12.

RT-qPCR. Seven genes of interest for validation were identified based on the microarray data (Supplementary Table 1). RNA from whole ovaries was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), as per the manufacturer's instructions. Gene expression was quantified using the QuantiTect SYBR Green PCR Kit (Qiagen) with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, California) under the following thermal conditions: initial heat activation for 15 min at 95°C, followed by 50 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C. Serial dilutions of whole ovary RNA pooled from animals from all treatment groups were used as an internal reference and to create standard curves for primer efficiency and template concentration optimization. Each gene was amplified in triplicate from 6 independent samples for each treatment group. Relative levels of gene amplification compared with the control group and housekeeping gene, *Ppia*, were calculated using StepOnePlus Software (version 2.1). A melt curve was systematically generated to ensure the specificity of the PCR.

Statistical analyses. Statistical analyses were done using the Prism software (version 6.07, GraphPad Software, Inc, La Jolla, California). Progeny outcome data were systematically expressed as average ± SEM per litter, and histological observations were done on females from independent litters. Ovary
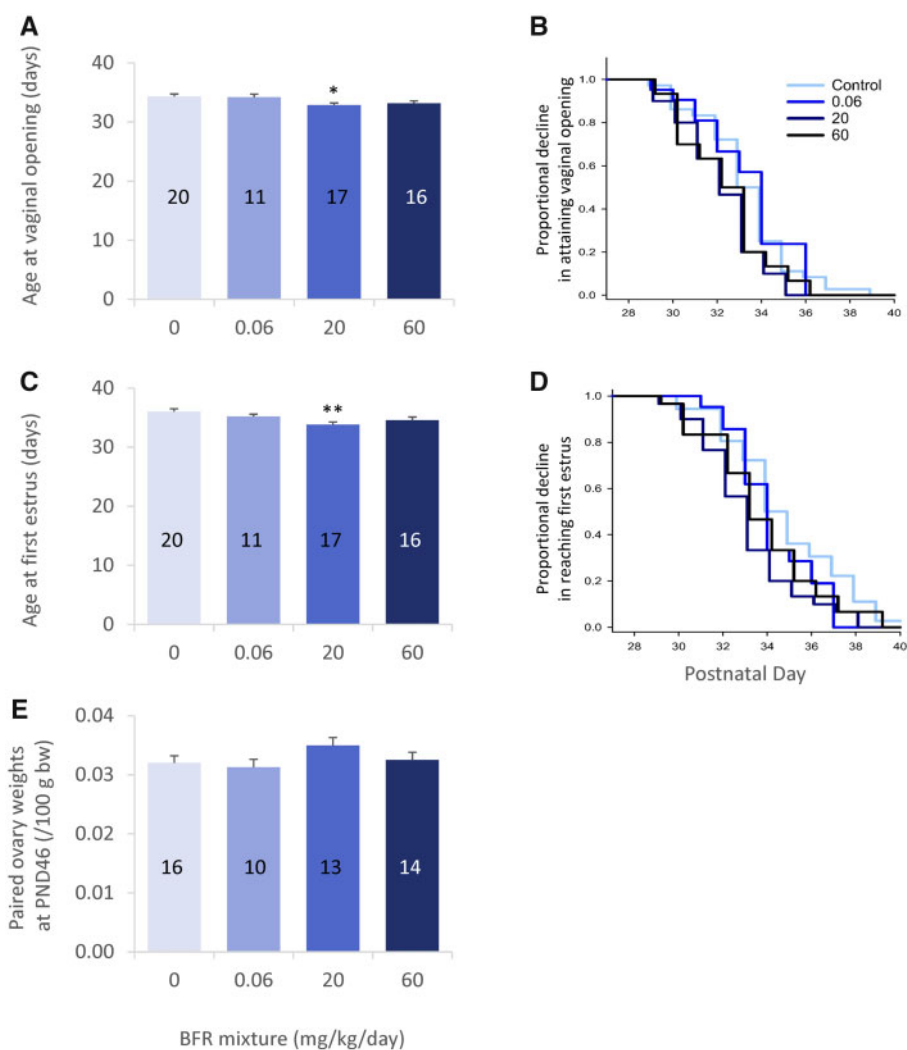
Figure 2. Pubertal onset in female offspring after *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant (BFR) mixture. Female offspring were monitored for vaginal opening (VO; A, B) and first estrus (FE; C, D) from postnatal day (PND) 27 to PND 41 for 2 pups per litter, and their ovaries were weighed on PND 46 (E). Mean ± SEM of litter average age at VO and FE are depicted (A and C, respectively) with the number of representative litters indicated on each bar. Data for all pups are depicted for VO and FE as survival plots in B and D, respectively. $*p < .05$ and $**p < .01$.

weights, vaginal opening (VO), FE, and RT-qPCR data were analyzed using 1-way ANOVA followed by a post hoc Dunnett's multiple comparison test. Follicle count data were analyzed with a Kruskal-Wallis nonparametric test followed by a post hoc Dunnett's multiple comparison test. The level of significance for all statistical tests was set to $p < .05$.

RESULTS

Ovarian Development and Puberty
The effects of BFR exposure on mating, pregnancy, litter sizes, and pup growth rates have been reported elsewhere (Tung *et al.*, 2016). Female offspring were weighed and necropsied on PNDs 4, 21, and 46 to assess the impact of exposure to BFRs on a variety of developmental landmarks (most described previously: Tung *et al.*, 2016, 2017) and ovarian development (Figure 1). Previously published data showed no significant differences in body weight in female offspring on PND 4 or 21 (Tung *et al.*, 2016), whereas there was a significant reduction in weight on PND 46 in the 60 mg/kg/day BFR treatment group. VO and FE were monitored to assess the effects of exposure to BFRs on

pubertal development. Both VO and the day of FE occurred at a significantly earlier age in progeny from the 20 mg/kg/day BFR treatment group, whereas the apparent delay in these parameters observed in the 60 mg/kg/day treatment group did not reach statistical significance ($p = .14$ for VO and 0.052 for FE) (Figs. 2A, B and 2C, D, respectively). There were no significant differences in paired ovary weights between control and treated animals on PND 21 (data not shown) or PND 46 (Figure 2E).

Steroid Hormone Measurements
Serum from PND 46 females underwent a comprehensive steroid panel analysis. The concentrations of progesterone and corticosterone (Supplementary Figs. 1B and 1C) were not significantly affected by exposure to BFR mixtures in PND 46 females in diestrus.

Ovarian Folliculogenesis
To assess the effects of *in utero* and lactational exposure to BFRs on follicular histogenesis and progression, we counted ovarian follicles at PNDs 4 (Supplementary Figure 2) and 46 (Figure 3). No significant differences in the numbers of primordial and
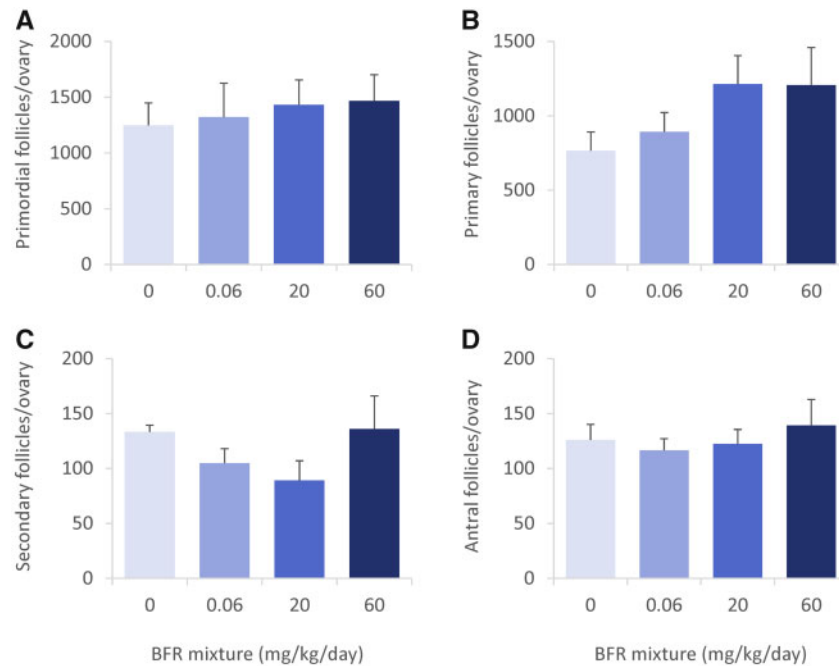
Figure 3. Ovarian follicular counts in postnatal day 46 female offspring after *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant (BFR) mixture. Primordial (A), primary (B), secondary (C), and antral (D) follicles were counted in all available sections and the total numbers calculated for the whole ovary. Values are expressed as means ± SEM; based on assessment of 1 female (left ovary) per litter for 6 litters from control, 5 from 0.06, 5 from 20, and 6 from 60 mg/kg treatment groups; there were no significant differences following a Kruskal-Wallis test and post hoc Dunnett's multiple comparison test.

primary follicles were observed at PND 4 among any of the treatment groups (Supplementary Figs. 2A and 2B). However, we did observe an apparent increase in secondary follicles in the treated groups (Supplementary Figure 2C); this suggests a precocity in ovarian development in association with BFR treatment.

On PND 46 we observed a tendency toward an increase in primordial and primary follicles (Figs. 3A and 3B) that appeared dose-dependent and toward a decrease in the number of secondary follicles in the 20 mg/kg/day BFR treatment group ($p = .19$) (Figure 3C). The number of antral follicles did not differ from the control in treated animals (Figure 3D). Exposure to BFRs did not affect the total number of follicles on either PND.

Interestingly, the histological analysis of follicles on PND 46 revealed the presence of 2 abnormal phenotypes: multioocyte follicles (MOFs) (Figure 4) and follicles with disorganized granulosa cell layers (Figure 5). We observed multioocyte primordial (Figure 4A), primary (Figure 4C), secondary (Figure 4E), and antral (Figure 4G) follicles in PND 46 ovaries. Although most MOFs contained 2 oocytes, follicles with 3 oocytes were also observed (Supplementary Figure 3A). Some follicles contained what appeared to be a dividing oocyte (Supplementary Figure 3B) whereas others appeared to contain follicles that were fusing (Supplementary Figure 3C). The numbers of primordial MOFs increased 8- and 7-fold, respectively, among offspring from 20 to 60 mg/kg/day BFRs mixture treatment groups (Figure 4B). Similarly, exposure to the highest dose of the BFR mixture significantly increased the number of primary MOFs by 15-fold (Figure 4D). Very few secondary MOFs were observed and there were no significant differences among the control and BFR exposed animals (Figure 4F). However, there was a dose-dependent increase in the numbers of viable and atretic antral MOFs that reached significance in the 60 mg/kg/day BFR treatment group (Figure 4H). In this study, atretic follicles were

defined by the presence of a degenerating oocyte. MOFs were also counted on PND 4 to investigate their origin. Occasional MOFs (Supplementary Figure 2E) were observed in the 0.06 and 60 mg/kg/day BFR treatment groups on PND 4, but no treatment group differed significantly from control.

The second abnormal phenotype that was observed was the presence of follicles with absent or disorganized granulosa cell layers (Figure 5A). A dose-dependent increase in the numbers of disorganized secondary and antral follicles was observed (Figs. 5B and 5C).

Ovarian Gene Expression

The impact of perinatal exposure to BFRs on ovarian gene expression was assessed to provide additional insights into their possible biological effects and to investigate mechanisms of action that may contribute to the impairment of folliculogenesis that was observed. Ovarian gene expression was analyzed in 4–6 independent PND 46 females per treatment group using single-color microarrays. The numbers of significantly differentially expressed transcripts (with a fold-change threshold $\geq$ 2) between the control and individual treatment groups are depicted in Figure 6A. In the low-dose BFR mixture exposure group (0.06 mg/kg/day), 14 transcripts were significantly upregulated and 4 were downregulated. Thirteen transcripts were significantly upregulated and 11 were downregulated in the 20 mg/kg/day BFR treatment group. Finally, exposure to 60 mg/kg/day altered the expression of a total of 91 genes: 28 were upregulated and 63 downregulated. The list of these transcripts is provided in Supplementary Table 2.

The extent to which the significantly altered transcript sets for each of the treatment doses overlap is depicted in the Venn diagram in Figure 6B. The transcripts affected in the 3 BFR treatment groups had minimal overlap, with only the epidermal growth factor (EGF)-like repeats and discoidin domain 3 (*Edil3*),
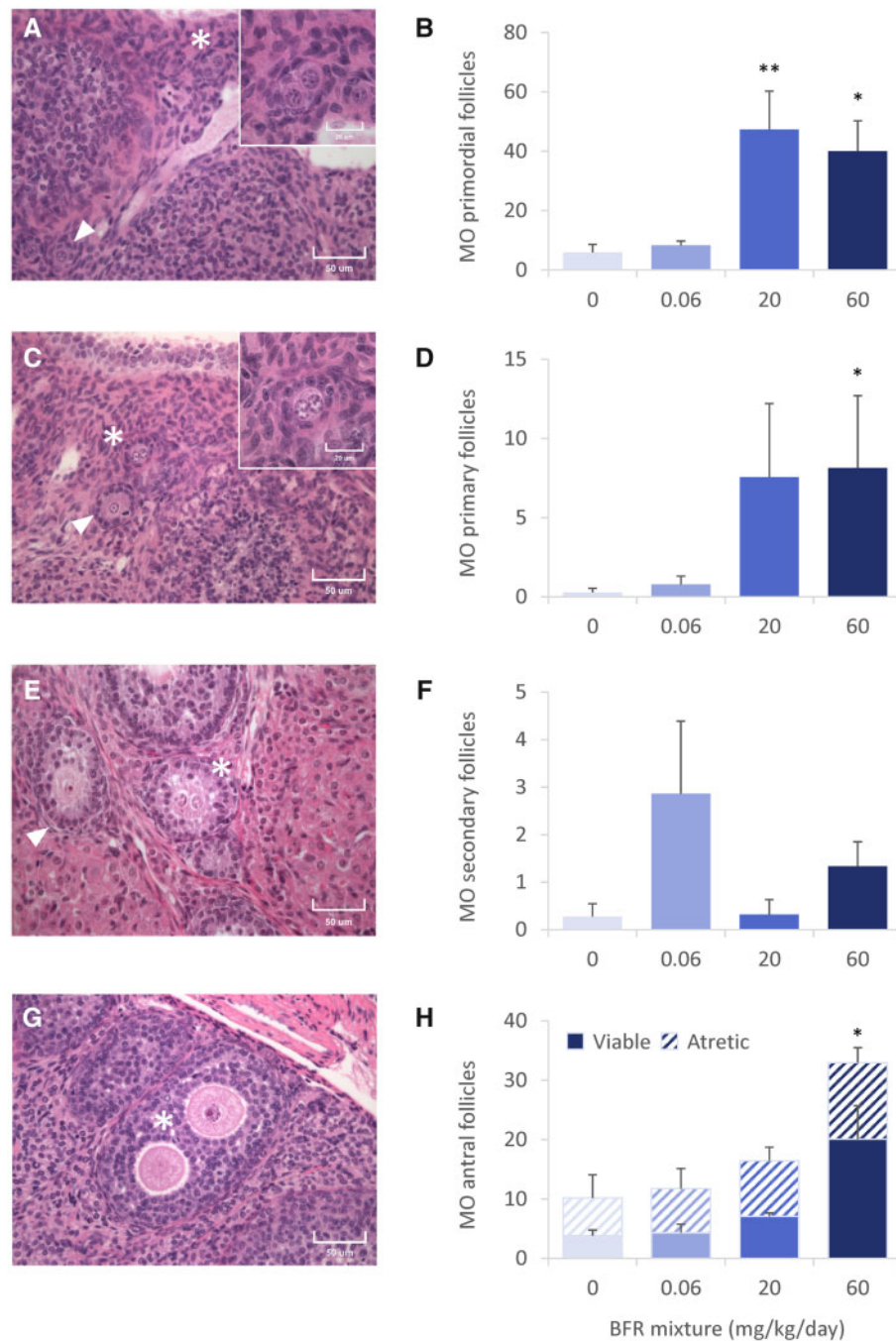
Figure 4. Multioocyte (MO) follicles in ovaries from postnatal day 46 females after *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant (BFR) mixture. High-quality photomicrographs and associated counts of multioocyte primordial (A, B), primary (C, D), secondary (E, F), and antral (G, H) follicles were calculated for the whole ovary. Follicles presenting with normal morphology are indicated with white arrowheads; abnormal follicles are indicated with an asterisk. Values are expressed as means ± SEM; based on the assessment of 1 female (left ovary) per litter from 6 litters for control, 5 from 0.06, 5 from 20, and 6 from 60 mg/kg treatment groups; $*p < .05$ and $**p < .01$ following a Kruskal-Wallis test and post hoc Dunnett's multiple comparison test.

the olfactory receptor *Orad4* and the patched domain *Ptchd1* being commonly affected by exposure to 0.06 and 60 mg/kg/day, and 5 transcripts (C-X-C motif chemokine ligand 14, *Cxc14*; protocadherin, *Pcdh8*; secretogranin, *Scg2*; sortilin-related VPS10 domain containing receptor 2, *Sorcs2*; and TNF receptor superfamily member 11 b, *Tnfrsf11b*) commonly affected in the 20 and 60 mg/kg/day treatment groups.

All transcripts that were differentially expressed with a fold-change ≥ 2 were imported into IPA software to investigate the

biological relationships underlying these gene expression changes. Although canonical pathways were not specifically predicted to be activated or inhibited (null z-scores), we identified potential upstream regulators that may contribute to the gene expression patterns that were observed in BFR-treated groups (Table 1; Figure 7). No upstream regulator was identified in the lowest dose group. Hypoxia inducible factor 1 alpha (HIF1A) was predicted to be downregulated in both the medium- and high-dose groups (z-scores of −1.98 and −1.99,
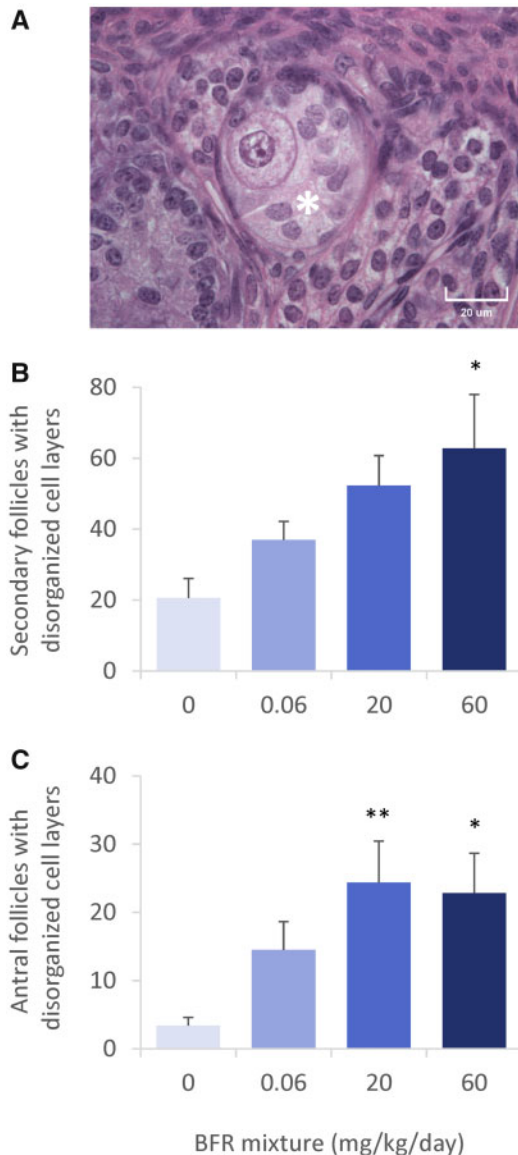
Figure 5. Follicles presenting with disorganized granulosa cell layers in ovaries from postnatal day 46 females after *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant (BFR) mixture. High-quality photomicrograph of a secondary follicle with disorganized granulosa cell layers (A, indicated with an asterisk). Counts for secondary (B) and antral (C) follicles with disorganized granulosa cell layers, as calculated for the whole ovary. Values are expressed as means ± SEM based on the assessment of 1 female (left ovary) per litter of 6 litters from control, 5 from 0.06, 5 from 20, and 6 from 60 mg/kg treatment groups; *$p < .05$ and **$p < .01$ following a Kruskal-Wallis test and post hoc Dunnett's multiple comparison test.

respectively), as was poly rI:rC RNA (z-scores of -1.97 and -2.00, respectively) (Table 1; Figs. 7A and 7B). Cyclic AMP responsive element binding protein 1 (CREB1; z-score = -2.20) and EGF (z-score = -2.00) were predicted to be downregulated in the 20 mg/kg/day BFR treatment group (Table 1; Figure 7A). Peroxisome proliferator-activated receptor alpha (PPARA; z-score = -2.20), β-estradiol (z-score = -2.20), and the Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor Alpha (NFKBIA; z-score = -2.0) were predicted to be downregulated in the 60 mg/kg/day treatment group (Table 1; Figure 7B).

Seven target genes of interest were selected for validation by RT-qPCR based on pathways analysis of the gene expression data (Figure 8). Because we did not find a housekeeping gene with stable expression in the high-dose group RT-qPCR, data are provided only for the 0, 0.06, and 20 mg/kg/day groups. Within the CREB1 and HIF1A pathways, we confirmed *Scg2* to be significantly downregulated in both 0.06 and 20 mg/kg/day BFR treatment groups (Figure 8A). Within the β-estradiol pathway, we confirmed the significant upregulation of *Cyp1b1* at the lowest dose (Figure 8B), and the significant downregulation of *Tnfrsf11b* and *Scg2* in both the 0.06 and 20 mg/kg/day treatment groups (Figs. 8A and 8C). This effect on *Tnfrsf11b* was also consistent with the predicted inactivation of NFKBIA; however, qRT-PCR did not confirm the significant downregulation of *Tnfaip3* (Figure 8D). Finally, within the PPARA pathway, we found *Hpx* to be significantly downregulated at 0.06 mg/kg/day and upregulated, although not significantly, in the 20 mg/kg/day group (Figure 8E). Other transcripts of interest included the protocadherin *Pcdh8* (Figure 8F) as well as the Ras-related protein *Rab3b* (Figure 8G), which were both confirmed to be significantly downregulated in the 0.06 and 20 mg/kg/day BFR treatment groups.

DISCUSSION

To the best of our knowledge, this study is the first to demonstrate that early life exposure to an environmentally relevant BFR mixture leads to disruption of ovarian development in post-pubertal female rats that is accompanied by accelerated puberty and abnormal follicular structure. Ovarian histological analysis revealed a significant elevation in the incidence of MOFs and a dose-dependent increase in the numbers of follicles with disorganized granulosa cell layers. Using a toxicogenomic approach, we found that exposure to BFRs significantly altered the expression of a number of genes in the ovary and identified potential upstream regulators that may be targeted; these included HIF1A, poly rI:rC RNA, β-estradiol, PPARA, NFKB1A, CREB1 and EGF.

A limited number of studies have investigated the effects of exposure to individual PBDE congeners on the onset of puberty. Previously, we reported that exposure to the environmentally relevant mixture of BFRs during gestation and lactation altered developmental programing and induced thyroid hormone dysfunction (Tung et al., 2016, 2017) in the offspring. Here, we observed the premature onset of puberty in female offspring after perinatal exposure to 20 mg/kg/day of this BFR mixture; the apparent delay observed in females exposed to a higher dose failed to reach statistical significance. These data corroborate epidemiological studies showing associations between exposure to PBDEs and lower age at menarche (Chen et al., 2011) or premature thelarche (Deodati et al., 2016). However, other studies have reported associations between PBDE exposures and a higher age at pubertal transition (Windham et al., 2015) or menarche (Harley et al., 2017) in girls. In an animal study, the exposure of pregnant Long-Evans dams to a single PBDE congener, BDE-99, from GD 10 to 18 produced a significant delay in the onset of puberty in female offspring (Lilienthal et al., 2006); a trend toward a delay was also observed in a second study (Ceccatelli et al., 2006). These discrepancies in human and animal studies highlight the need for a better understanding of the mechanisms of action that are at play and intricacies involved in interpreting the consequences of exposure to mixtures of endocrine disrupting chemicals.

Previous studies have reported decreases in the numbers of follicles in Long-Evans rats after treatment with a single BDE congener, BPE-99 (Lilienthal et al., 2006) or BDE-47 (Talsness
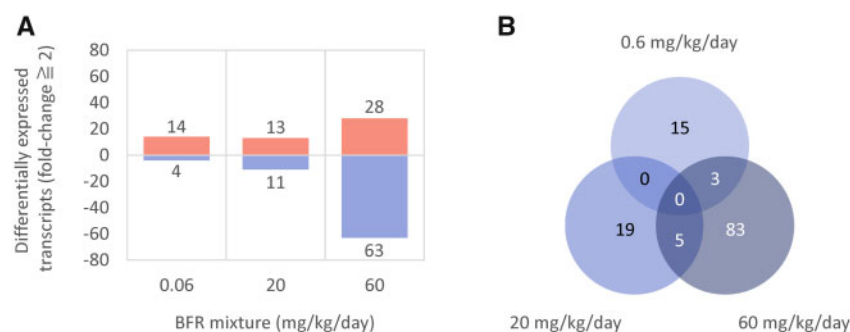
Figure 6. Ovarian gene expression in postnatal day 46 females following *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant (BFR) mixture. A, Number of uniquely mapped transcripts significantly up or downregulated by more than 2-fold determined by moderated t test and Benjamini-Hochberg FDR correction ($p > .05$). B, Venn diagram representing commonalities in differential gene expression between treatment groups. All data were generated using $n = 4$–6 female rats from independent litters.

Table 1. Upstream Regulators in PND 46 Female Offspring Ovaries That Are Predicted Using Ingenuity Pathway Analysis to be Significantly Affected by Exposure to 20 or 60 mg/kg/day of an Environmentally Relevant BFR Mixture

	Upstream Regulator	Predicted State	z-Score	p Value of Overlap	Target Molecules in Dataset
20 mg/kg/day	CREB1	Inhibited	−2.20	6.84E−02	*Cdh8, Hla-a, Ina, Nr4a3, Scg2*
	miR-423-5p	Inhibited	−2.00	2.11E−01	*Angpt4, C11orf87, Cacfd1, Slc22a12*
	EGF	Inhibited	−2.00	1.17E−01	*Cxcl14, Nr4a3, Scg2, Spp1*
	HIF1A	Inhibited	−1.98	6.22E−02	*Cxcr1, Nr4a3, Scg2, Tnfrsf11b*
	poly rI:rC RNA	Inhibited	−1.97	6.48E−02	*Hla-a, NR4A3, Spp1, Tnfrsf11b*
60 mg/kg/day	PPARA	Inhibited	−2.20	2.73E−02	*Apoa4, Fga, Hpd, Hpx, Pbld, Sord*
	ß-estradiol	Inhibited	−2.20	3.04E−01	*Apoa4, Cd274, Cox6a2, Cyp1b1, Gzmc, Scg2, Sord, Tmf1, Tnfaip3, Tnfrsf11b, Wrap73*
	NFKBIA	Inhibited	−2.00	1.95E−01	*Cyp1b1, Il2rA, Tnfaip3, Tnfrsf11b*
	poly rI:rC RNA	Inhibited	−2.00	1.52E−01	*Cd274, Il2rA, Tnfaip3, Tnfrsf11b*
	HIF1A	Inhibited	−1.99	1.46E−01	*Cd274, Cxcr1, Scg2, Tnfrsf11b*

et al., 2008). Here, we report that the overall numbers of ovarian follicles at PND 46 remained unaffected by exposure to BFRs, although there was a trend toward a dose-dependent increase in the number of primordial and primary follicles and a decrease in the number of secondary follicles in the 0.06 and 20 mg/kg/day groups.

In utero and lactational exposure to our environmentally relevant BFR mixture induced a dose-dependent increase in the number of follicles displaying 2 abnormal phenotypes: the presence of more than 1 oocyte in a single follicle (MOFs) and a disorganization of granulosa cell layers. MOFs are often found in rats during the prepubertal period but are uncommon in adulthood (Davis and Hall, 1950). However, MOFs have been reported in different species (rodents, sheep, caiman crocodiles, and primates) after exposure to endocrine-disrupting chemicals. Their presence may be due to the division of an oocyte within a follicle, perhaps because of mitotic slippage or dysregulation of the separation of germ cell clusters, commonly referred to as germinal cysts. Alternatively, there is speculation that MOFs can arise in juvenile rat ovaries through fusion of adjacent follicles (Gaytán et al., 2014). Our data do not allow us to distinguish among these possibilities because some follicles contained what appeared to be a dividing oocyte (Supplementary Figure 3B) whereas others appeared to contain follicles that were fusing (Supplementary Figure 3C). We also found no statistically significant differences in the numbers of follicles at any stage at PND 4, suggesting that the nondifferentiation of cysts is a possibility.

The second abnormal phenotype that we observed was an increase in the numbers of follicles with disorganized granulosa cells. The connections between oocytes and granulosa cells are established by cytoplasmic extensions bearing communicating junctions formed by connexins. A deficiency in connexin-43 in granulosa cells at the preantral stage has been reported to induce incompetent oocytes (Kidder and Mhawi, 2002). Although microarray analyses revealed connexin-43 to be present in all samples, there was no difference in mRNA levels between any BFR treatment groups. Moreover, in rats irradiated *in utero* an imbalance between the number of oocytes and somatic cells prevented follicular growth, leading to a reduction in follicular stock (Mazaud Guittot et al., 2006). Such a phenomenon may be involved in the appearance of disorganized follicles after exposure to BFRs.

The consequences of either MOFs or follicles with disorganized structure for health and fertility are not clear. The lack of observed effects on hormone levels in this study suggests that these are not associated with major endocrine disruption. It is not clear if MOFs can produce fully viable oocytes but oocytes collected from MOFs of ovaries from mice perinatally exposed to diethylstilbestrol (Iguchi et al., 1991) or from patients in fertility clinics (Dandekar et al., 1988) can be fertilized; however, in both cases the resulting embryos develop more slowly than those from single ovular follicles. These observations demonstrate that MOFs produce mature oocytes via hormonal manipulation but do not necessarily reveal their fate under normal physiological conditions.
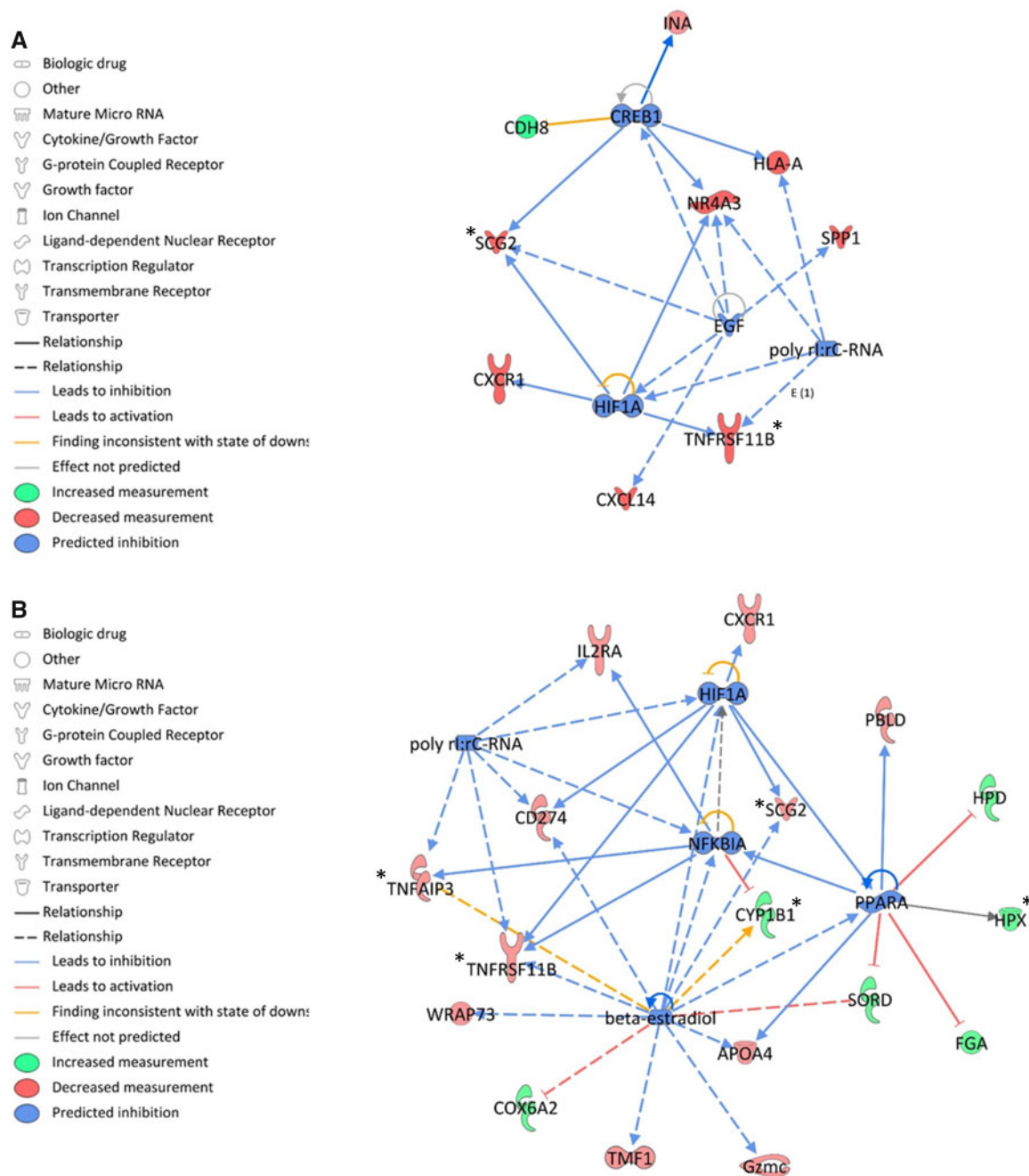
Figure 7. Ingenuity Pathway Analysis predictions of upstream regulators significantly affected by exposure to an environmentally relevant brominated flame retardant mixture. A, Upstream regulators predicted to be affected by exposure to 20 mg/kg/day of the BFR mixture. B, Upstream regulators predicted to be affected by exposure to 60 mg/kg/day of the BFR mixture. An asterisk indicates the transcripts associated with these pathways that were validated using qRT-PCR.

We identified several potential upstream regulators to be targeted by BFRs; these include HIF1A and poly rI:rC RNA (in ovaries from both the middle and high BFR treatment groups). HIF1A plays an important role in mammalian ovarian follicular development and ovulation (Wang et al., 2012; Zhang et al., 2012) and is expressed in both granulosa cells and mature oocytes (Takahashi et al., 2016; Wang et al., 2012). HIF1A has been implicated in the regulation of progesterone synthesis in luteinizing granulosa cells and is downregulated in atretic follicles (Fadhillah et al., 2017). Double-stranded RNA such as poly rI:rC RNA plays a key role in activating protein kinase R, a key innate

immune sensor (Hull and Bevilacqua, 2016). Double-stranded RNA is an upstream regulator linking damage sensing to multiple responses, including the insulin signaling pathway (Hage Hassan et al., 2016) and wound healing (Zhu et al., 2017). In the ovary, the administration of poly rI:rC RNA inhibited estradiol synthesis and induced ovarian granulosa cell apoptosis (Yan et al., 2015).

In the middle-dose group, CREB1 and EGF were also identified as potential upstream regulators. The CREB1 pathway plays a critical role in mediating the gonadotropin hormone responsiveness of granulosa cells (Mukherjee et al., 1996). The
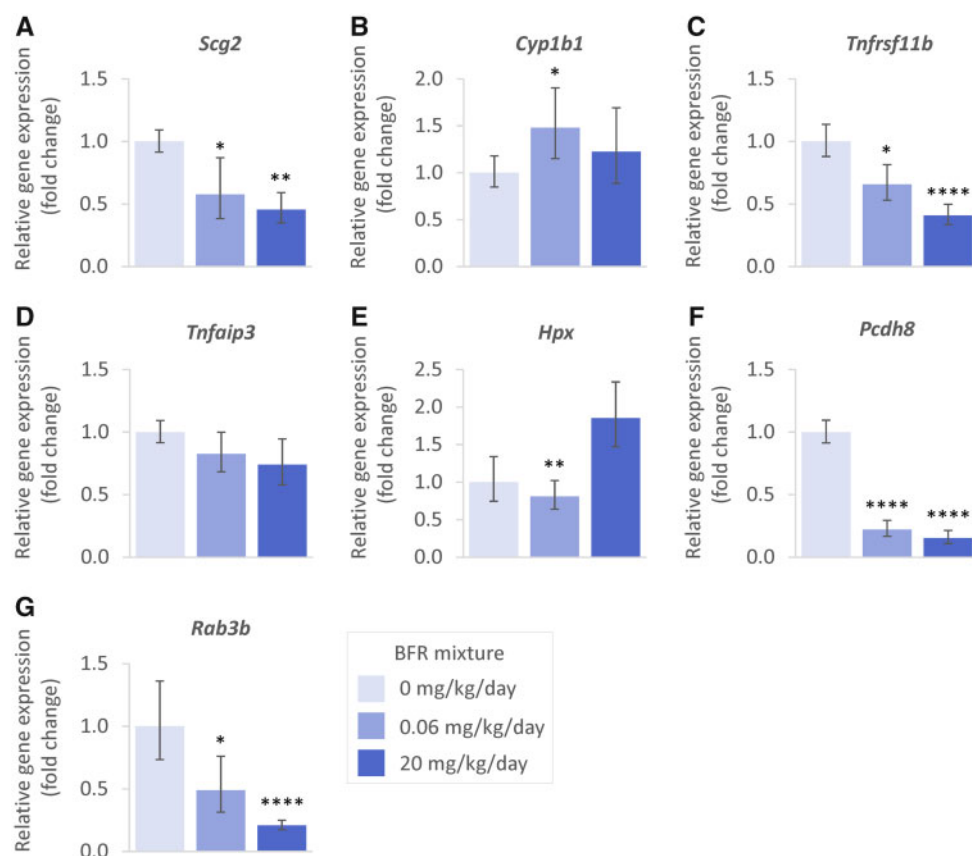
Figure 8. RT-qPCR analyses of the expression of selected transcripts following *in utero* and lactational exposure to 0, 0.06, 20, or 60 mg/kg/day of an environmentally relevant brominated flame retardant mixture. Relative levels of gene amplification compared with the 0 mg/kg/day group and housekeeping gene *Ppia* were calculated following the ΔΔCt method. Significance was determined by 1-way ANOVA corrected by Dunnett's multiple comparison test; $n = 6$ biological replicates plated in triplicate; $^*p < .05$; $^{**}p < .01$; $^{****}p < .0001$.

knockdown of CREB attenuates the induction of StAR expression and progesterone synthesis by proinflammatory cytokines in human granulosa-lutein cells (Dang *et al.*, 2017). EGF signaling also plays a crucial role in the ovary in regulating oocyte maturations and ovulation (Richani and Gilchrist, 2018). Pharmacological downregulation of the EGF pathway has been reported to suppress the induction of cumulus expansion and oocyte maturation (Shimada *et al.*, 2016).

Finally, in the high-dose group, NFKB1A, PPARA, and β-estradiol were identified as potential upstream regulators targeted by BFR exposure. NFKBIA encodes a protein that negatively regulates the NF-kappa-B pathways, and thus would be expected to affect expression of cytokines involved in inflammatory responses. To our knowledge, this pathway has not been specifically implicated in folliculogenesis in the ovary, but gene association studies have linked NFKBIA variants with some inflammatory diseases (Hong *et al.*, 2018).

The β-estradiol signaling pathway was predicted to be downregulated in ovaries from the high BFR dose group. Although serum estradiol concentrations were below the level of detection in our study (Supplementary Figure 1A), previous studies have reported a decrease in estradiol concentration after exposure to BDE-10 (Lilienthal *et al.*, 2006) and BDE-47 (Talsness *et al.*, 2008). However, developmental exposure to certain BDEs was reported to upregulate the expression of estrogen target genes in the uterus (Ceccatelli *et al.*, 2006; Dang *et al.*, 2017). It is consistent with this finding that several PBDE congeners, and their hydroxylated metabolites, have been reported to act as NR3A1 (ER

alpha) and NR3A2 (ER beta) receptor agonists (Meerts *et al.*, 2001; Mercado-Feliciano and Bigsby, 2008). Hydroxylated BDE-47 metabolites have also been reported to stimulate ovarian aromatase activity (Karpeta *et al.*, 2013). Thus, the mechanism by which exposure to the mixture of BFRs we have studied affects β-estradiol pathway endpoints is likely to be complex. Previously, our lab reported that exposure to a mixture of PBDEs similar to that in human follicular fluid reduced estradiol secretion in KGN cells, an immortalized human granulosa cell line (Lefevre *et al.*, 2016). Dysregulation of estradiol signaling may play an important role in dysregulating ovarian folliculogenesis. Treatment with diethylstilbestrol or estradiol has been reported to decrease the number of small antral follicles and induce MOF formation in the neonatal rodent ovary (Iguchi *et al.*, 1990; Kipp *et al.*, 2007). Increased follicular recruitment and MOFs have also been described after exposure to estrogenic compounds such as bisphenol A (Hunt *et al.*, 2012; Rodríguez *et al.*, 2010).

The PPARA pathway was also predicted to be significantly inhibited in the 60 mg/kg/day BFR exposure group; this pathway is involved in estradiol biosynthesis in granulosa cells (reviewed in Komar [2005]). Numerous studies have shown that PPARs are expressed throughout the female reproductive system (Vitti *et al.*, 2016) and are critical for normal ovarian function (Huang, 2008; Komar, 2005; Vélez *et al.*, 2013). Certain compounds, such as herbicides (eg, 2,4-dichlorophenoxyacetic acid, 2,4-D), plasticizers (eg, di-(2-ethylhexyl) phthalate, DEHP; di-(2-ethylhexyl) adipate, DEHA; and dibutyl phthalate, DBP), and perfluorooctanoic acid, as well as other hygiene components (hair spray and

solvent for perfumes) activate PPARs in the ovary and testis (Nakajima *et al.*, 2002), reviewed in Huang (2008) and Vitti *et al.* (2016), resulting in effects such as prolonged sexual cycle, suppressed ovulation, contracted preovulatory follicles, decreased estradiol levels, elevated FSH, and alterations in steroidogenesis. The effects of reduced activation of PPARA on ovarian function are not clear as female mice that lack a functional PPARA gene are fertile (Lee *et al.*, 1995; Yessoufou *et al.*, 2006) with a slightly increased rate of spontaneous abortion (Yessoufou *et al.*, 2006). Consequently, it is not clear what reduced PPARA activation might predict for ovarian health and fertility.

Using a rodent model, we have shown that *in utero* and lactational exposure to an environmentally relevant mixture of BFR congeners representative of those present in house dust produces several effects on the ovaries of female offspring. These include an early onset of puberty and an increase in the incidence of MOFs. Our data suggest that such effects may be a consequence of the downregulation of pathways that are fundamental for ovarian function; these include the HIF1A, CREB1, EGF, β-estradiol, and PPAR pathways, in addition to pathways that regulate inflammatory responses.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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