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Authors

Robinson, Joshua F
Kapidzic, Mirhan
Hamilton, Emily G
et al.

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Genomic Profiling of BDE-47 Effects on Human Placental Cytotrophoblasts

Joshua F. Robinson,[*,†,1] Mirhan Kapidzic,[*,†] Emily G. Hamilton,[*,†] Hao Chen,[*,†] Kenisha W. Puckett,[*,†] Yan Zhou,[*,†] Katherine Ona,[*,†] Emily Parry,[‡] Yunzhu Wang,[‡] June-Soo Park,[‡] Joseph F. Costello,[§] and Susan J. Fisher,[*,†]

[*]Department of Obstetrics, Gynecology, and Reproductive Sciences, Center for Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, California 94143; and [†]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, California 94143; [‡]Environmental Chemistry Laboratory, Department of Toxic Substances Control, California Environmental Protection Agency, Berkeley, California 94710; and [§]Department of Neurological Surgery, University of California, San Francisco (UCSF), San Francisco, California 94158

[*]To whom correspondence should be addressed at Department of Obstetrics, Gynecology, and Reproductive Sciences, Center for Reproductive Sciences, University of California, San Francisco (UCSF), Box: 0665, Bldg: 513 Parnassus Ave, San Francisco, CA 94143-0665. Fax: (415) 476-1635. E-mail: joshua.robinson@ucsf.edu.

ABSTRACT

Despite gradual legislative efforts to phase out flame retardants (FRs) from the marketplace, polybrominated diphenyl ethers (PBDEs) are still widely detected in human maternal and fetal tissues, eg, placenta, due to their continued global application in consumer goods and inherent biological persistence. Recent studies in rodents and human placental cell lines suggest that PBDEs directly cause placental toxicity. During pregnancy, trophoblasts play key roles in uterine invasion, vascular remodeling, and anchoring of the placenta-fetal unit to the mother. Thus, to study the potential consequences of PBDE exposures on human placental development, we used an *in vitro* model: primary villous cytotrophoblasts (CTBs). Following exposures, the endpoints that were evaluated included cytotoxicity, function (migration, invasion), the transcriptome, and the methylome. In a concentration-dependent manner, common PBDE congeners, BDE-47 and -99, significantly reduced cell viability and increased death. Upon exposures to sub-cytotoxic concentrations ($\leq 5\,\mu M$), we observed BDE-47 accumulation in CTBs with limited evidence of metabolism. At a functional level, BDE-47 hindered the ability of CTBs to migrate and invade. Transcriptomic analyses of BDE-47 effects suggested concentration-dependent changes in gene expression, involving stress pathways, eg, inflammation and lipid/cholesterol metabolism as well as processes underlying trophoblast fate, eg, differentiation, migration, and vascular morphogenesis. In parallel assessments, BDE-47 induced low-level global increases in methylation of CpG islands, including a subset that were proximal to genes with roles in cell adhesion/migration. Thus, using a primary human CTB model, we showed that PBDEs induced alterations at cellular and molecular levels, which could adversely impact placental development.

Key words: placenta; cytotrophoblast; transcriptomics; polybrominated diphenyl ether; BDE-47; human; invasion; migration; *in vitro*; methylation.

Despite efforts to the contrary, the continued use of polybrominated diphenyl ethers (PBDEs) as flame retardants (FRs) in consumer goods warrants great concern (Jinhui *et al.*, 2015). Due to their bio-persistence these compounds continue to be identified in human maternal and fetal tissues (Zota *et al.*, 2013). In particular, of the 209 unique PBDE congeners, BDE-47 is found at the highest levels in the majority of US mothers and/or their offspring (Frederiksen *et al.*, 2009; Woodruff *et al.*, 2011; Zota *et al.*, 2018).

Numerous toxicological (Dingemans *et al.*, 2011) and epidemiological (Cowell *et al.*, 2015; Herbstman and Mall 2014) studies indicate that exposures to PBDEs *in utero* may be harmful to the developing human fetus. The ramifications of their bioaccumulation remain largely unknown. In human cell lines and/or animal models, common PBDEs congeners elicit developmental toxicity through several mechanisms, eg, alterations in thyroid hormone (TH) signaling, inflammation, oxidative stress, and epigenetic modifications (Costa *et al.*, 2014). Further investigations are needed to clarify targeted tissues/cell populations and windows of sensitivity during human pregnancy.

The placenta is essential for normal fetal development, and pregnancy complications linked with abnormal placentation are negatively associated with neonatal, child, and adult health (Vinnars *et al.*, 2014). Data generated in rodent models and human cell lines (Kalkunte *et al.*, 2017; Rajakumar *et al.*, 2015; Yang *et al.*, 2006) suggest that environmental compounds are toxic to the placenta. In humans, the placenta is in direct contact with maternal blood in which these chemicals are routinely detected. Thus, it is not surprising that PBDEs are regularly identified in human placentas (Leonetti *et al.*, 2016). While the consequences associated with exposures *in utero* are unknown, recent studies in mammalian models suggest that BDE-47 may alter placental development, causing oxidative stress and inflammation (Park *et al.*, 2014; park and Loch-Caruso, 2014) as well as perturbing hormone signaling (park and Loch-Caruso, 2015).

During pregnancy, the placenta undergoes numerous morphological and molecular transformations to support the growing demands of the developing fetus. Specialized placental cells known as cytotrophoblasts (CTBs) are critical for: (1) initiating and maintaining the physical anchor between the fetal and maternal units; (2) penetration and invasion into the uterine wall; and (3) remodeling of the uterine vascular architecture to reroute maternal blood flow to the placenta (Red-Horse *et al.*, 2004, 2005). Perturbations in CTB development may underlie several pregnancy complications, including: preeclampsia (PE; Fisher, 2015), intrauterine growth restriction (IUGR), preterm birth (Romero *et al.*, 2014) and over aggressive CTB invasion (Jauniaux and Jurkovic, 2012). *In vitro*, isolated cultured primary human villous CTBs (Fisher *et al.*, 1989; Hunkapiller and Fisher, 2008; Kliman *et al.*, 1986) have been proposed as a model of placentation.

CTB invasion is a complicated process with many components, some of which are unique. The cells must exit the placenta, crossing over to the uterus, where they attach to its surface before they deeply invade the parenchyma. This process is accompanied by a series of molecular transformations, involving relevant pathways, including cell-cell adhesion, migration/invasion, and vascular remodeling (Maltepe and Fisher, 2015). Upon isolation and culture, villous CTBs differentiate along the invasive pathway, modulating the same sets of molecules that mediate this process *in vivo* (Robinson *et al.*, 2017).

Genomic profiling of isolated CTBs revealed dramatic shifts at transcriptomic and epigenomic levels across gestation including many key molecules with known functional roles in placental development and disease (Roadmap Epigenomics *et al.*, 2015). In assessing environmental chemical impacts during development, the incorporation of genomic-based approaches in toxicological studies, ie, toxicogenomics, provides a global analysis of molecular response to environmental exposures (Robinson and Piersma, 2013). Given the unusual dynamics of the CTB genome, the potential for environmental chemicals to influence chromatin architecture/gene expression, and the links between placental health and successful birth outcomes (Burton *et al.*, 2016; Kovo *et al.*, 2012), investigations are needed to address potential interactions between these important variables.

Here, we used a cell culture model to study the effects of PBDEs on CTB differentiation and invasion. Acute exposures resulted in accumulation of the parent compound and minimal metabolism. Subcytotoxic concentrations of BDE-47 impaired the ability of CTBs to migrate and invade. These functional alterations were accompanied by alterations at transcriptomic and epigenomic levels. Together these data suggest that PBDE exposures could impact process and pathways that are integral to the placenta's role in governing pregnancy outcome.

MATERIALS AND METHODS

Tissue collection. All methods were initially approved by the UCSF Institutional Review Board. Informed consent was obtained from all donors. Second trimester placentas intended for cell isolations were collected immediately following elective terminations and placed in cytowash medium, consisting of DME/H-21 (Gibco), 12.5% fetal bovine serum (Hyclone), 1% glutamine plus (Atlanta Biologicals), 1% penicillin/streptomycin (Invitrogen), and 0.1% gentamicin (Gibco). Tissue samples were placed on ice prior to dissection.

Human primary villous cytotrophoblast isolation. CTBs were isolated from second trimester human placentas as described previously (Robinson *et al.*, 2017). Single cells were counted using a hemacytometer and immediately transferred to a Matrigel (BD biosciences)-coated 12-well plate. CTBs were cultured at a density of 500 000 CTBs/well in 1.5 ml medium containing DME/H-21, 2% Nutridoma (Roche), 1% sodium pyruvate (Sigma), 1% Hepes buffer (Invitrogen), 1% glutamate plus (Atlanta Biologicals), and 1% penicillin/streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO_2/95% air. We immunostained with anti-cytokeratin (CK; anti-CK rat polyclonal; 1:100, Damsky *et al.*, 1992), a marker regularly used to detect for relative trophoblast purity (~ 80–90% CTBs) in our cultures across experiments. Cell preparations not meeting this criteria were not used for downstream analyses.

Chemicals. PBDE congeners, BDE-47 (2,2′4,4′-tetrabromodiphenyl ether, >99%, CAS #5436-43-1, AccuStandard) and BDE-99 (2,2′4,4′,5-pentabromodiphenyl ether, >99%, CAS #60348-60-9, AccuStandard), bisphenol A (BPA; >99%, #80-05-7, Sigma), and perfluorooctanoic acid (PFOA, >96%; #335-67-1, Sigma) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to make stock solutions and serial dilutions for all experimental studies. Chemical exposures were introduced at a 1:1000 (vol/vol) media dilution for all assessments.

Cytotoxicity assessments. Freshly isolated CTBs were cultured for 15 h ($t_{15\ h}$) and supplied with new media containing: media only (control), DMSO (vehicle control; 0.1%), BDE-47 (0.1, 1.0, 10, 25 μM), BDE-99 (0.1, 1.0, 10, 25 μM), BPA (0.1, 1.0, 10, 100 μM), or PFOA (1, 10, 25, 100, 250, 1000 μM). At $t_{15\ h}$, cultured CTBs are actively aggregating, one of the initial steps in differentiation/invasion. We evaluated cytotoxicity after a 24-h exposure due to previous studies suggesting that this time point was optimal to detect significant changes, at cellular and molecular levels, in developing *in vitro* systems (Costa *et al.*, 2015; park and Loch-Caruso, 2014). We measured cell viability using the neutral red (NR; 40 ng/ml, VWR) lysosomal uptake assay (Borenfreund and Puerner, 1985). Briefly, after 24 h, the media was removed and wells were gently washed with PBS to eliminate residual compound. New media containing NR was added to each plate and

incubated for 2 h at 37°C. Cells were washed with PBS and a 50% ethanol/1% acetic acid solution was added to release the NR dye, which was measured at an absorbance of 540 nM via a spectrophotometer (Biotek Epoch). In parallel, in exposed and control CTB cultures, we evaluated lactate dehydrogenase (LDH) activity—a marker of cell death—within the supernatant using the Cytotoxicity Detection Kit (Roche) following the recommended manufacturer protocol. Absorbance readings for LDH were acquired at 490 nM. For both cytotoxicity endpoints, within each experiment, background absorbance readings were subtracted from mean absorbance values. Adjusted values were normalized as compared with the control (= 100%). Average relative percentages and corresponding standard error (SEM) were computed across the independent experiments ($n \geq 3$). Additional pilot investigations were completed where the initial exposure occurred after 3 h ($t_{3\ h}$) post-plating, before CTB aggregation occurs. No differences in CTB sensitivity to PBDE-induced cytotoxicity were apparent in regards to when the exposure was initiated ($t_{3\ h}$ vs $t_{15\ h}$). We calculated benchmark concentrations (BMCs) for 50% cell viability or 200% LDH activity via asymmetrical dose-response modeling (Graphad Prism 7.0; Giraldo *et al.*, 2002).

PBDE accumulation and metabolism in CTBs. We tested for PBDE concentrations in cell and media fractions of CTB cultures exposed to 0.1% DMSO, BDE-47 1 or 5 μM after 24 h. We evaluated samples for BDE-47, related metabolites, ie, 5-OH-BDE-47 and 6-OH-BDE-47, as well as major PBDE congeners, eg, BDE-17, -28, -66, -85, -99, -100, -153, -154, -183, -196, -197, -201, -203, -206, -207, -209. PBDE concentrations were measured using gas chromatography/high resolution double-focusing sector mass spectrometry (GC-HRMS, DFS, Thermo Fisher, Bremen, Germany) at the Department of Toxic Substances Control (DTSC; Berkeley, California) as described previously (Zota *et al.*, 2013). Average concentrations in cell and media fractions were determined across three independent experiments ($n = 3$). Mass estimates of BDE-47 in cell/media fractions were calculated using chemical concentrations (μg/μl) and the molecular weight (489.79 g/mol) of BDE-47. Percent recovery was estimated to be within ~10% of the expected total amount (media + cell fraction).

Migration of PBDE-exposed CTBs. We evaluated the effect(s) of BDE-47 exposures on CTB migration/aggregation as described previously (Robinson *et al.*, 2017). In brief, we exposed cells with 1% DMSO, 1 or 5 μM of BDE-47 after early attachment ($t_{1\ h}$). Controls containing media only were also ran in parallel. At 5 or 15 h post-exposure, the culture media was removed and CTBs were washed once with PBS to remove residual chemical. Next, CTBs were fixed with 4% paraformaldehyde (PFA, 20 min). Cultures were washed again with PBS (2×) and stored (in PBS) at 4°C until further processing. Cold methanol was added to permeabilize the CTBs. Cultures were washed with PBS (3×) and a PBS-Hoechst 33342 (Life Technologies, 1:2500) solution was added. After 10 min, the liquid was removed and cultures were placed in PBS. For each well, 42 tethered fluorescent images consisting of a 5 mm^2 area were captured using a Leica inverted microscope with a 10× objective and the tiles can function (Leica Application Suite Advanced Fluorescence). Within each image, we identified all cells or "objects" via detection of Hoescht (nuclear-binding dye) using Volocity software (PerkinElmer; version 6.3). We applied automated erosion and division operational functions to enable improved measurements of aggregated cell populations. Objects that intersected with the border of the image or that were initially <20 μm^2 were eliminated from the analysis to limit potential artifacts. Images containing irregularities, eg, cell debris, high background, blurry features, were also removed (<5% of images). We evaluated ~20 000 cells per well. The minimum distance between nuclei (centroid to centroid) was evaluated using an automated process which measured all possible distances between objects within each image. We identified the average cell number per image in each independent experiment and removed images outside the normal range (mean ±1.5 SD; ~5% of images) to minimize the influence of density as a factor of cell-cell proximity. Within each experiment, we calculated the difference in the average minimum distance among cells exposed to DMSO or BDE-47 versus the media only control. The standard error (SE) to the mean was computed across the average of the six independent experiments. We applied ANOVA and pairwise Student paired *t*-tests to determine significant differences in migration between controls and BDE-47 exposed CTBs ($p < .05$; JMP 13.0).

Invasion of PBDE-exposed CTBs. As described previously (Hromatka *et al.*, 2013), we evaluated the influence of PBDE exposures on the ability of CTBs to invade. We used transwell inserts (8 μm pore size, 24-well plate, Corning Costar) pre-coated with 8 μl of diluted Matrigel™ (3:1 v/v in serum-free medium BD Biosciences) for 20 min at 37°C. CTBs at a density of 250 000 cells in 250 μl media were added into the upper compartment of inserts and placed in 24-well plates containing 800 μl of media per well. After attachment (~$t_{1\ h}$), CTBs were exposed to media only (control), 0.1% DMSO, or BDE-47 (1, 5 μM). After an additional 40 h at 37°C, cells were fixed with 3% PFA (30 min), washed in PBS (2×), and stored in PBS at 4°C until further processing. We quantified CTB projections using immunofluorescence, microscopy, and high-content analysis (Volocity). After PBS removal, cold methanol was added to permeabilize the CTBs (5 min). Cells were washed with PBS (3×) and 5% bovine serum albumin (BSA) (Hyclone)/PBS was added to block nonspecific reactivity. After 1 h, the solution was removed and the primary antibody, anti-CK (Fisher_001-clone7D3, RRID: AB_2631235, rat monoclonal; 1:100 [Damsky *et al.*, 1992]) in 5% BSA was added and incubated overnight at 4°C. The next day, cultures were washed with PBS (3×), inserts were cut using a razor blade, probed with Vectashield containing Dapi (Vector Bio-Labs), and cover slipped. Images were acquired using a Leica inverted microscope (20×) per slide. Invading CTBs and/or significant cell protrusions that reached the underside of the filter were counted using Volocity software (PerkinElmer; version 6.3) and preset optimized criteria (area > 4000 μm^2; max pixel intensity > 75; shape factor > 0.75). All images and generated counts were double checked manually to assure proper identification. Within each independent experiment ($n = 3$), we determined the total CTB protrusions per condition across three technical replicates (5 images per slide). Invasion was expressed as a ratio in CTB protrusions per image between each exposure group and the media only control (= 100%) per experiment. The standard error (SE) to the mean was computed across the average of the experiments. Significant effects were determined via ANOVA and Student *t*-tests between PBDE exposure groups and the vehicle control ($p < .05$; JMP 13.0).

RNA isolation from CTBs. We isolated RNA from CTBs exposed to BDE-47 and controls for downstream microarray or qRTPCR gene expression assessments. CTBs were cultured for 3 h ($t_{3\ h}$) or 15 h ($t_{15\ h}$) and supplied with new media containing: media only (control), DMSO (vehicle control), and/or BDE-47 (0.1–10 μM). Initiated exposure times in culture were selected due to our previous analyses (Robinson *et al.*, 2017), indicating that in unexposed cultures, single CTBs attach and begin to migrate at

~3 h in culture, and at ~15 h, aggregation and cell remodeling actively occur. We isolated RNA from CTBs for transcriptomic and qRT-PCR investigations. Samples used for transcriptomic studies consisted of cells (1) exposed to either DMSO or BDE-47 (1 μM); (2) from an independent placenta ($n = 3$); and (3) varied in age during the second trimester (gestational week [GW] 16.6, 19.3, or 21.6). In total, 12 samples were used for microarray analyses (Supplementary Table 1). Additional samples for qRT-PCR validation were also generated from individual placentas and ranged from GW 14–22. To isolate RNA, in brief, immediately following a 24 h exposure duration, the media was removed and RLT Lysis Buffer (Qiagen) was directly added to the culture dish. The lysate was collected and stored at −80°C. RNA was purified using the RNeasy Micro Kit (Qiagen). The RNA concentration and quality were estimated (absorbance 260 nm/280 nm = 1.9–2.1) by using a Nanodrop spectrometer (Thermo Scientific). Samples destined for microarray analyses were assessed for quality (RIN > 9) using the Agilent RNA 6000 Nano LabChip Kit and Bioanalyzer 2100 system.

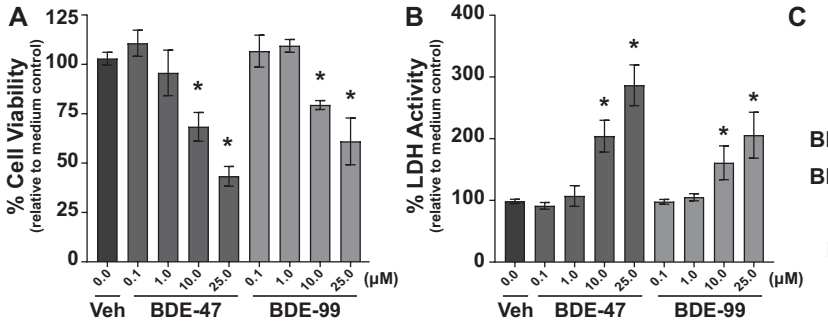
Gene expression profiling of BDE-47-exposed CTBs. We evaluated the effects of BDE-47 exposure on the global transcriptome in CTBs using the Affymetrix Human Gene 2.0 ST array platform. Sample processing and hybridization was performed by the UCSF Gladstone Institute as described previously (Winn *et al.*, 2007). Affymetrix CEL files were processed using the Affymetrix Expression Console and Transcriptome Analysis Console (TAC) software packages. Raw values were normalized via the robust multi-array average (RMA) algorithm. Raw and normalized data were deposited in the Gene Expression Omnibus (GEO; GSE104896). We analyzed probes with median intensities >20% of the total distribution and discarded duplicates probes representing the same transcript by using the most variable probe within the full sample set. In total, we examined 27 729 unique genes using this approach. A multivariate ANOVA model was applied [y(expression) = B_1x(exposure) + B_2x(time) + B_3x(placenta)] to identify differentially expressed (DE) genes due to BDE-47 or time, while adjusting for differences across genetically unique placental cell preparations, ie, batch effects. Average fold change (FC) values were determined by calculating the average ratio of the difference of log 2 intensities between BDE-47 (1 μM) and the respective vehicle control within each experiment. We defined DE genes due to BDE-47 exposure (BDE-47 DE Genes) by applying a cutoff of $p \leq .025$ (unadjusted) and an absolute average FC ≥ 1.25 between BDE-47 and DMSO for both of the two exposure scenarios, ie, initiated either at $t_{3\ h}$ or $t_{15\ h}$. Approaches used to control for false positives (eg, Bonferonni) were not applied because a limited number of genes passed standard criteria thresholds (FDR < 5%), a common observation in toxicological studies using primary human *in vitro* systems. Thus, downstream analyses focused on validated targets and changes in related enriched functional pathways. Hierarchical clustering of FC values was completed by using average linkage and Euclidean distance (TIGR MEV; Saeed *et al.*, 2006). We conducted functional enrichment of Gene Ontology (GO) Biological Processes (Level 4) of BDE-47 DE Genes defined using the Official Gene Symbol (OGS) using DAVID (Huang *et al.*, 2007). GO terms containing ≥ 7 DE Genes and an enrichment significance of $p \leq .01$, were selected as significantly overrepresented. Corresponding enrichment scores, ie, p-values, were also determined for up- and downregulated gene clusters. We grouped terms based on GO classification (Gene Ontology Consortium, 2015) and identified parent GO terms to define themes.

Transcription factor binding site enrichment analysis of BDE-47 DE genes. We identified potential upstream regulators, ie, TFs, of genes found to be DE due to BDE-47 exposure in our transcriptomic analyses using OPOSSUM. Enriched TFBS motifs were defined as (1) -2000 bases upstream of the transcription start site (TSS); (2) an identification score of ≥ 0.4; and (3) an enrichment score of $Z \geq 7$, which indicates the over-occurrence of the TFBS in BDE-47 genes/sequences as compared with the background total genome. Within this subset of related TFs, we explored transcript abundance in CTBs isolated from second trimester versus term human placentas using an RNA-seq dataset previously generated in our laboratory (Roadmap Epigenomics *et al.*, 2015). Expression data was processed as described previously (Robinson *et al.*, 2016). Abundance and significance of differential expression between second trimester versus term was determined using DESeq2 and Wald's test (Chen *et al.*, 2011).

Targeted validation of BDE-47 DE genes in CTBs. Using CTBs from independent placentas ($n \geq 3$) in addition to samples employed in the microarray analyses, we investigated expression levels of target genes in control and BDE-47 (1 μM, initiated at $t_{3\ h}$; 0.01–10 μM, initiated at $t_{15\ h}$) exposed-samples at 24 h to validate microarray analyses and further interrogate the concentration-response of CTBs to BDE-47, including at more physiologically relevant levels. Using purified samples, we converted RNA to cDNA using ISCRIPT Universal TaqMan (Bio-Rad), and performed qRT-PCR via TaqMan primers for FABP4, FABP7, GPR34, GREM1, HMGCS1, IL6, MMP1, NEUROD2, PLAC4, and SCD (Supplementary Table 2) mixed with TaqMan Universal Master Mix II, no UNG (Life Technologies). Reactions were carried out for 40 cycles. A minimum of 3 technical replicates were analyzed for all comparisons. Differential expression between PBDE-exposed and controls was calculated via the ΔΔCT method: (1) normalized to geometric mean of housekeeping genes, GAPDH and ACTB; and (2) adjusted to the vehicle control (DMSO) for each experiment. Housekeeping genes were selected due to their common application in experiments employing reproductive tissues/cells (Arenas-Hernandez and Vega-Sanchez 2013) and the fact that our microarray data showed that their expression was not significantly altered by BDE-47 exposures (not shown). To determine significant changes across concentrations BDE-47, we employed ANOVA (JMP). FC values were expressed as average log 2 ratios between each exposure group and the vehicle control.

DNA isolation of BDE-47-exposed CTBs. Following exposure to BDE-47 (1 μM) or vehicle for 24 h (initiated at $t_{3\ h}$ or $t_{15\ h}$), CTBs ($n = 3$ independent experiments) were washed with PBS and collected using a cell scraper. Cells (~1 million per sample) were suspended in 10 ml of PBS, pelleted (800 rpm, 5 min), washed 2× with PBS and stored at −80 C. To extract genomic DNA, CTBs were digested with 1 mg/ml proteinase K in lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS) overnight at 55°C. Following RNase treatment, DNA was isolated using the Phenol-Chloroform Isoamyl Alcohol (PCI) method, followed by precipitation with ethanol, and resuspended in TE. DNA quality was evaluated via Nanodrop and the Agilent RNA 6000 Nano LabChip Kit and Bioanalyzer 2100 system.

Methylation profiling of BDE-47-exposed CTBs. Downstream processing of genomic DNA bisulfite conversion was performed using the EZ DNA Methylation Kit (ZymoResearch) and Infinium HumanMethylation450 bead arrays (Illumina) following the manufacturer's protocols. Methylation data was processed via



	BMC_{50} Viability	BMC_{200} LDH
BDE-47	18.1μM	12.5μM
BDE-99	33.5μM	24.6μM
BPA	116.5μM	96.6μM
PFOA	363.8μM	432.9μM

Figure 1. Concentration-dependent CTB cytotoxicity induced by PBDEs. (A) CTB viability or (B) LDH activity at 24 h in cultures that contained PBDEs or 0.1% DMSO (vehicle control, VEH) relative to medium with no additives. The standard error (SE; black bars) of the mean was computed across experiments ($n \geq 4$). Asterisks (*) indicate significant differences between tested concentrations of PBDEs and vehicle control ($p \leq .05$). (C) Benchmark chemical concentrations (BMCs) corresponding to a 50% loss of viability or a 200% increase in LDH activity.

Illumina standard background subtraction and control probe normalization, and converted to M values, using the minfi package and BRB Array Tools (Simon *et al.*, 2007). Probes that mapped to regions of sex chromosomes were eliminated from the analysis. Similar to the model used for transcriptomic analyses, we utilized a fixed effect multivariate model (using M-value = ratio of methylated probe vs unmethylated probe intensities) to identify differentially methylated (DM) CpGs due to exposure or time, while controlling for baseline differences across CTBs from genetically unique placentas. Beta values, which reflect the proportion of methlyated probes (ranging from 0 to 1) were determined via logit transformation ($\log_2(M/(M+1))$ of M-values (Du *et al.*, 2010). The change in methylation was determined by subtracting β values between BDE-47 (1 μM) and the respective vehicle control within each experiment. Significant DM CpGs were identified using a $p \leq .005$ (unadjusted) and average absolute $\beta \geq 0.025$ between BDE-47 and DMSO for both exposure windows, ie, initiated either at $t_{3\ h}$ or $t_{15\ h}$. We evaluated enrichment of DM CpGs by chromosome location (Fisher's exact test). We interrogated genes in proximity to DM CpGs for functional relevance using DAVID (Biological Level 4). These analyses were also conducted for the subset of DM CpGs located proximal to promoter regions of genes, defined to be located <1500 b upstream of the TSS, 5′ untranslated region (UTR), or first Exon. Raw and normalized data were deposited in the NCBI GEO repository GSE115399.

We examined correlations between mRNA expression and DM CpGs by aligning the datasets based on associated OGS annotation. For the subset of DM CpGs in proximity of promoter regions, we assessed the influence of BDE-47 exposures on expression in comparison with the change in methylation at each independent CpG island.

RESULTS

Concentration-Dependent PBDE-Induced Cytotoxicity in CTBs

After 24 h, we evaluated the effects of BDE-47 or -99 (0.1–25 μM) exposures on CTB viability and death using the neutral red and LDH activity assays, respectively. In a concentration-dependent manner, both PBDE congeners significantly reduced cell viability (ANOVA, $p < .05$; Figure 1A) and increased LDH activity (Figure 1B). In general, the two congeners displayed similar potencies ($p \geq .05$). *Post-hoc* analyses (*t*-test) comparing cultures exposed to PBDE versus vehicle control (1% DMSO) revealed concentrations ≥ 10 μM of BDE-47 or -99 to be significantly cytotoxic. In pilot investigations, sensitivity was not dependent on exposure window (initiated at $t_{3\ h}$ vs $t_{15\ h}$; not shown). Comparisons of BMCs associated with a 50% loss in cell viability or 200% increase in LDH activity, suggested that CTBs were more sensitive to PBDEs versus other common endocrine disruptors (BPA or PFOA) *in vitro* (Figure 1C). Overall, our analyses indicated that PBDEs were relatively potent EDCs that induce concentration-dependent cytotoxicity in CTBs. Due to similarities in cytotoxic profiles between BDE-47 and -99 and previous studies indicating BDE-47 to be the highest detected congener in human placentas (Zota *et al.*, 2018), we focused our efforts on BDE-47 effects, at functional and molecular levels, in CTBs.

Partitioning of BDE-47 in Isolated CTBs and Their Culture Medium

Using gas chromatography-mass spectrometry, we evaluated: (1) BDE-47; (2) hydroxylated metabolites of the parent species; and (3) other PBDE co-contaminants, in CTBs and their culture medium, which contained subcytotoxic concentrations of BDE-47 or vehicle (0.1% DMSO). First, we quantified BDE-47 in the medium after 24 h of exposure to 1 or 5 μM of this compound. In both cases, the measured amounts were less than the input: 0.7 ± 0.3 μM or 1.2 ± 0.2 μM, respectively (Figure 2A). Second, we plotted the mass distribution (in μg) of BDE-47 in the medium and cells versus the expected recovery (Figure 2B) also at 24 h. Quantification of BDE-47 in the exposed cultures revealed 0.5 ± 0.2 μg (1 μM) and 0.9 ± 0.1 μg (5 μM) in the medium, and 0.3 ± 0.2 μg (1 μM) and 3.1 ± 0.3 μg (5 μM) in the cells. At the concentrations tested, total recovery (medium + cells) was within 10% of expected estimates (black bars). This suggested an ∼10× increase in CTB accumulation of BDE-47 at the 5 μM versus the 1 μM exposure. Overall, our results suggested bioaccumulation of BDE-47 in the cells and that the uptake rate was nonlinear and concentration dependent.

In general, hydroxylated metabolites of BDE-47 (5-OH, 6-OH) were not detected at appreciable levels. The metabolite 6-OH-BDE-47 was identified in one of the three CTB samples that were analyzed, which was 0.0003% of the ∑BDE-47 recovered. In addition to BDE-47, four of the eighteen PBDE congeners evaluated, ie, -17, 28, -85, -99, were detected at low levels in the majority of all samples, which was <0.4% of the ∑PBDEs (Supplementary Table 3). PBDE concentrations were below the minimum detection level (MDL) in all samples exposed to the vehicle control, evidence that congeners other than BDE-47, which were detected in the exposed CTBs, were contaminants of the chemical stock. These results suggested that CTBs had little to no hydroxylated metabolism of the parent compound during 24 h of exposure.

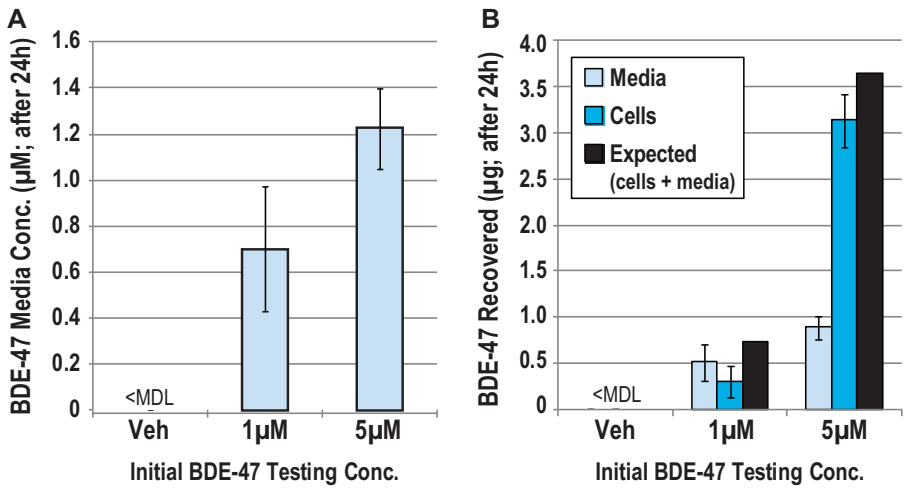


Figure 2. Bioaccumulation of BDE-47 in CTBs. A, CTBs were cultured for 24 h in BDE-47 (1 or 5 µM) or DMSO (0.1%). Gas chromatography-mass spectrometry enabled measurements of the compound amount in each conditioned medium sample. B, Distribution of BDE-47 in the medium (light shading) or cells (dark shading) versus the expected recovery in both compartments (black bars). In general, the hydroxylated metabolites (6-OH-BDE-47 and 5-OH-BDE-47) were not detected. MDL, Minimum level of detection.

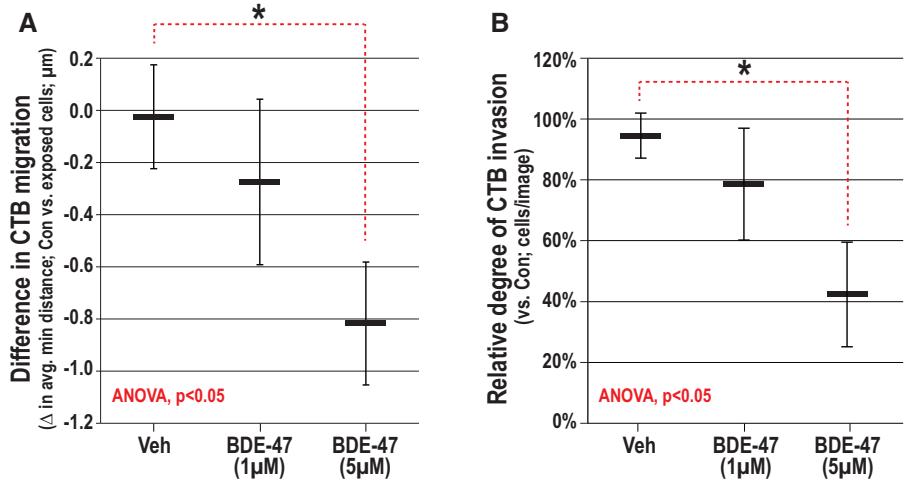


Figure 3. Concentration-dependent effects of BDE-47 on CTB migration and invasion. A, The minimum distance between CTBs exposed to BDE-47 (1 or 5 µM) or vehicle (0.1% DMSO) following 5 h exposure durations. B, The average number of identified projections per image in BDE-47 exposed and control cultures after 40 h. Asterisks signify significant effects between tested concentrations and vehicle control (*t*-test, $p \leq .05$). The standard error (SE) of the mean was computed across independent experiments ($n \geq 4$).

BDE-47 Effects on CTB Migration and Invasion

Under control conditions, CTBs plated on Matrigel™ migrate toward one another at a rate of ~0.3 µm/h, forming multicellular aggregates during the first 15 h of culture. Significant inhibition of migration/aggregation was observed with BDE-47 after 5 h exposure (ANOVA, $p < .05$). Pairwise comparisons between specific concentrations of BDE-47 and vehicle control indicated that 5 µM significantly reduced migration (Δ in average minimum distance = 0.8 µm; $p < .05$), while 1 µM caused only a modest reduction in inhibition (Figure 3A). At 15 h, differences in CTB migration were not observed across groups (not shown), suggesting the ability of CTBs to compensate for the differences that were initially observed. In addition, using a transwell culture system, we assessed BDE-47 effects on the cells' ability to invade. After 40 h exposure, with 1 µM BDE-47, there was no difference between the levels in experimental versus control cultures. In contrast, 5 µM significantly impaired invasion (42% of media only control; $p < .05$, Figure 3B). Our results suggested that BDE-47 perturbs CTB migration and invasion in a concentration-dependent manner.

Global Expression Profiling of BDE-47 in CTBs

CTBs were plated for 3 or 15 h before they were exposed for 24 h to BDE-47 (1 µM) or vehicle (0.1% DMSO) after which global gene expression profiling was performed. We applied a fixed effects linear model (ANOVA) to identify BDE-47 responsive transcripts. In total, the expression of 276 genes was significantly altered ($p \leq .025$, absolute FC $\geq$ 1.25). Hierarchical clustering showed that 159 genes were upregulated and 117 genes were

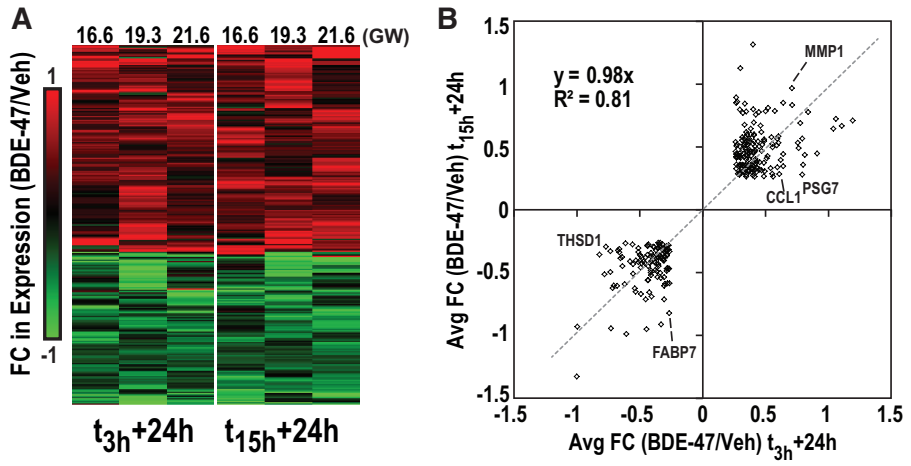


Figure 4. Gene expression profiling of BDE-47-exposed CTBs. A, Cultures were exposed to 1 μM BDE-47 for 24 h starting either 3 or 15 h after they were plated. Global transcriptional profiling showed that 276 genes were DE as compared to CTBs that were cultured in 0.1% DMSO ($p \leq .025$, absolute FC ≥ 1.25). Hierarchical clustering demonstrated similar responses among the CTB cultures and between the two exposure windows. FC ratios are displayed as the log 2 difference between BDE-47 (1 μM) and the vehicle control for each experiment. B, Correlations between the average gene expression FC responses for the different exposure windows. Red (upregulated genes); green (downregulated genes); GW gestational week.

downregulated (Figure 4A). On average, responses to BDE-47 were independent of when exposures were initiated ($y = 0.98x$, $R^2 = 0.81$; Figure 4B). Thus, BDE-47 induced significant transcriptomic alterations in cultured CTBs and these changes occurred irrespective of the exposure window.

GO Analyzes of BDE-47 Responsive Genes

Genes whose expression was modulated by BDE-47 were mapped into GO terms. The majority of enriched biological processes were driven by upregulated transcripts. They included morphogenesis, vasculature development, cell differentiation, cell migration, signal transduction, inflammatory response, protein metabolism, and regulation of biosynthetic process-related terms ($p \leq .01$; # of genes changed within each GO term ≥ 7; Figure 5A). Fewer enriched biological processes were driven by downregulated genes. In general, these GO terms were related to lipid and steroid metabolism.

Next, we clustered genes belonging to GO terms related to important aspects of CTB biology and placental toxicology: morphogenesis, vasculature development, cell migration, inflammatory responses, and cellular lipid metabolic processes (Figure 5B). Forty-one genes were included in this subset. This analysis highlighted BDE-47-induced perturbations in the expression of genes involved in: (1) trophoblast differentiation (eg, MMP1, MMP8, TEK, NODAL, BMP2); (2) inflammatory pathways (eg, CCL13, IL1A, IL6, IL1RN, CCL1); and (3) lipid/steroid metabolism (eg, FABP4, FABP7, FASN, INSIG1, HMGCS1). From this group we selected seven genes (in italics) and an additional two targets (GPR34, PLAC4) to further interrogate the concentration-response relationship of BDE-47 exposures (0.1–10 μM) and mRNA expression. We observed significant effects for the 9 targets, 8 of which had monotonic relationships with BDE-47 exposures (ANOVA, $p \leq .05$). Specifically, IL6, MMP1, GREM1, and PLAC4 were significantly upregulated; GPR34, SCD, HMGCS1, and FABP7 were significantly downregulated (Figure 5C). Minimal changes were observed at concentrations ≤0.1 μM. Trends in response to BDE-47 (1 μM) were similar for the two exposure windows initiated at 3 or 15 h. The qRT-PCR results positively correlated with the microarray data ($R^2 = 0.74$; Supplementary Figure 1). Thus, our findings suggested specific pathways with known roles in trophoblast development and/or PBDE-induced toxicity were significantly altered by exposure to this compound.

Transcription Factor Binding Site (TFBS) Enrichment Analysis of BDE-47 Responsive Genes

Given the important role of transcription factors in regulating gene expression and chemical responses, we analyzed the TFBSs of genes that encoded mRNAs whose abundance changed due to BDE-47 exposure. Ten of these motifs for human TFs were enriched in this gene subset ($Z \geq 7.0$; Table 1). Based on our RNA-seq data for second trimester and term CTBs (GEO accession number), nine were among the most highly expressed transcripts (>33% percentile of RNA counts); four (PBX1, RORA, NFKB, TCFCP2L1) were significantly DE between second trimester and term ($p \leq .05$, highlighted). NFYA (Vaiman *et al.*, 2013), NFKB (Vaiman *et al.*, 2013), EBF1 (Buckberry *et al.*, 2017), RELA (Minekawa *et al.*, 2007), and RORA (Qiu *et al.*, 2015) are implicated in placental development/disease; NFYA (Jin *et al.*, 2001), NFKB (Puscheck *et al.*, 2015), and RELA (Yamamoto *et al.*, 2017) are associated with environmental-induced stressors. These analyses suggested that BDE-47 exposure may impact specific transcriptional responses of CTBs.

Global CpG Methylation Analysis of BDE-47-Exposed CTBs

In parallel with the transcriptomic analyses, we profiled global CpG methylation in BDE-47 (1 μM) and vehicle-exposed CTBs following a 24 h duration (initiated at 3 h or 15 h post-plating). Using a fixed effects linear model, we identified 758 CpGs as DM CpGs associated with BDE-47 exposure ($p \leq .005$; absolute average $\Delta\beta \geq 0.025$). Overall, exposure significantly increased global CpG methylation (0.8 ± 0.2%; Figure 6A). A further increase was observed when the analysis was constrained to BDE-47 DM CpGs (3.4 ± 0.3%; Figure 6A). This was consistent with data from individual CpGs; 93% had increased methylation (708/758 total) and 7% had decreased methylation (50/758 total; Figure 6B). On average, methylation changes due to BDE-47 were similar whether exposures were initiated at 3 h or 15 h ($y = 0.90x$, $R^2 = 0.69$; Figs. 6A–C).

Furthermore, within the BDE-47 DM CpG subset, we asked whether there was enrichment based on gene features. Intergenic sequences were overrepresented and those proximal

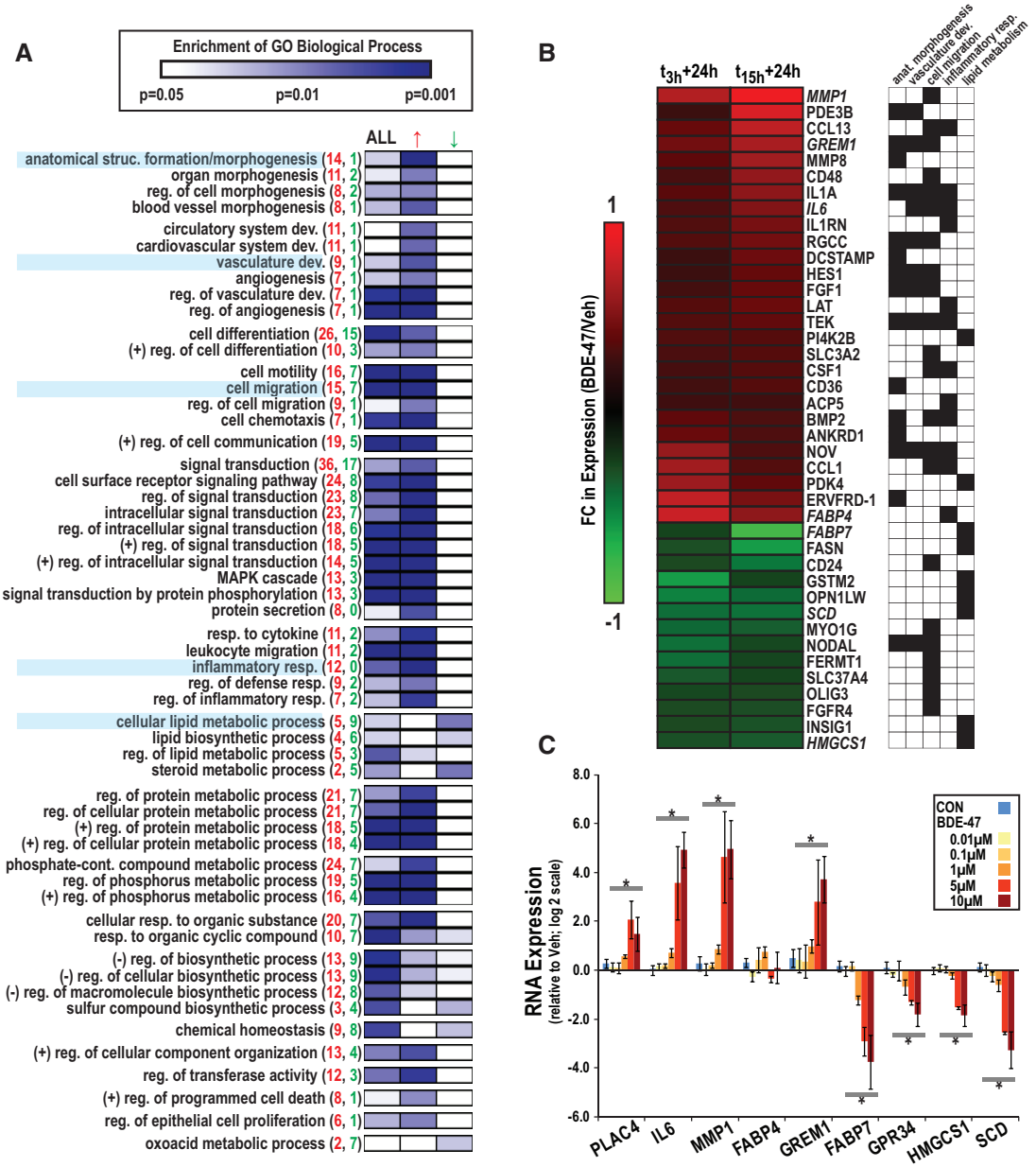


Figure 5. Functional analyses of genes that were DE due to BDE-47 exposures in CTBs. A, Enriched GO Biological Processes within BDE-47 DE Genes identified using DAVID (criteria: $p \leq .01$, number of DE genes associated with enriched term ≥ 7). GO enrichment scores (-log (p)) are displayed for all BDE-47 DE Genes and upregulated or downregulated gene subsets. The total number of DE Genes due to BDE-47 is located in parentheses (up, downregulated). B, Clustering of BDE-47 DE Genes associated with terms: anatomical structure formation involved in morphogenesis, vasculature development, cell migration, inflammatory response, and cellular lipid metabolic process. FC values represent average difference between BDE-47 and vehicle control. C, Concentration-dependent expression alterations in BDE-47 DE Genes using qRT-PCR. Expression values ($-\Delta\Delta CT$) were normalized to housekeeping genes (GAPDH, ACTB) and adjusted by the respective vehicle control. Asterisks indicate significance across all concentrations of BDE-47 and vehicle control (ANOVA, $p < .05$). All targets were observed to be significantly altered, with the exception of FABP4, which was significantly altered only with 1 μM in pairwise comparisons with the vehicle control.

to promoters (eg, TSS1500, TS200, first Exon) were underrepresented (Figure 6D). A relatively large number were also associated with gene bodies, although this feature did not achieve statistical significance because the identified regions did not exceed expectations. GO analyses of genes near BDE-47 DM CpGs revealed enrichment of processes and pathways that are involved in hormone response, cellular projection, signaling, response to oxygen-containing compounds, morphogenesis, and vesicle transport/localization (Figure 6E). Promoter proximal BDE-47 DM CpGs (178 total) were significantly associated with vesicle transport/localization and secretion-related terms.

Next, we examined the chromosomal location of BDE-47 DM CpGs, which on the whole, showed a larger proportion of increased versus decreased methylated CpGs on each chromosome (Figure 7A). The results revealed enrichment on chromosomes 10 and 11 and a relative absence on chromosome 18. Figure 7B shows the chromosome 11 data, which had the highest significance of enrichment, by genomic location. This enabled identification of a BDE-47 DM CpG cluster on Ch. 11p15.5

Table 1. Transcription Factor Binding Site (TFBS) Enrichment Analysis of BDE-47 DE Genes

TF	JASPAR	TFBSs	Z	Genes	Fisher	Count (pct.)	Age, FC (Second /Term)
NFYA	MA0060.1	45	16.6	30 (16↑, 14↓)	3.4	73%	−0.07
AR	MA0007.1	3	10.8	3 (3↑, 0↓)	2.4	33%	−0.46
PBX1	MA0070.1	18	10.7	17 (12↑, 5↓)	4.9	85%	1.19**
EBF1	MA0154.1	94	10.6	52 (36↑, 16↓)	4.2	35%	−0.27
RORA (1)	MA0071.1	43	10.2	26 (17↑, 9↓)	1.4	47%	1.68**
GFI	MA0038.1	126	9.7	59 (37↑, 22↓)	3.4	13%	0.36
TBP	MA0108.2	64	9.0	34 (22↑, 12↓)	1.0	56%	0.07
NFKB	MA0061.1	48	7.8	29 (17↑, 12↓)	1.0	85%	0.36*
RELA	MA0107.1	35	7.5	25 (18↑, 7↓)	1.6	88%	−0.13
TCFCP2L1	MA0145.1	71	7.0	40 (28↑, 12↓)	1.2	89%	1.64**

Overrepresented motifs and associated transcription factors (TFs) in proximity of genes DE by BDE-47 in CTBs. Number of DE genes (up vs downregulated) with TF-binding sites are displayed in combination with corresponding enrichment values for TF binding sites (Z) and genes (Fisher). Abundance of RNA levels (percentile in top 20 000 genes) of TFs in freshly isolated second trimester and term CTBs as determined via post-hoc analysis of RNA-seq data. Significance of differences in expression between second trimester and term CTBs (*$p < .05$; **$p < .001$).

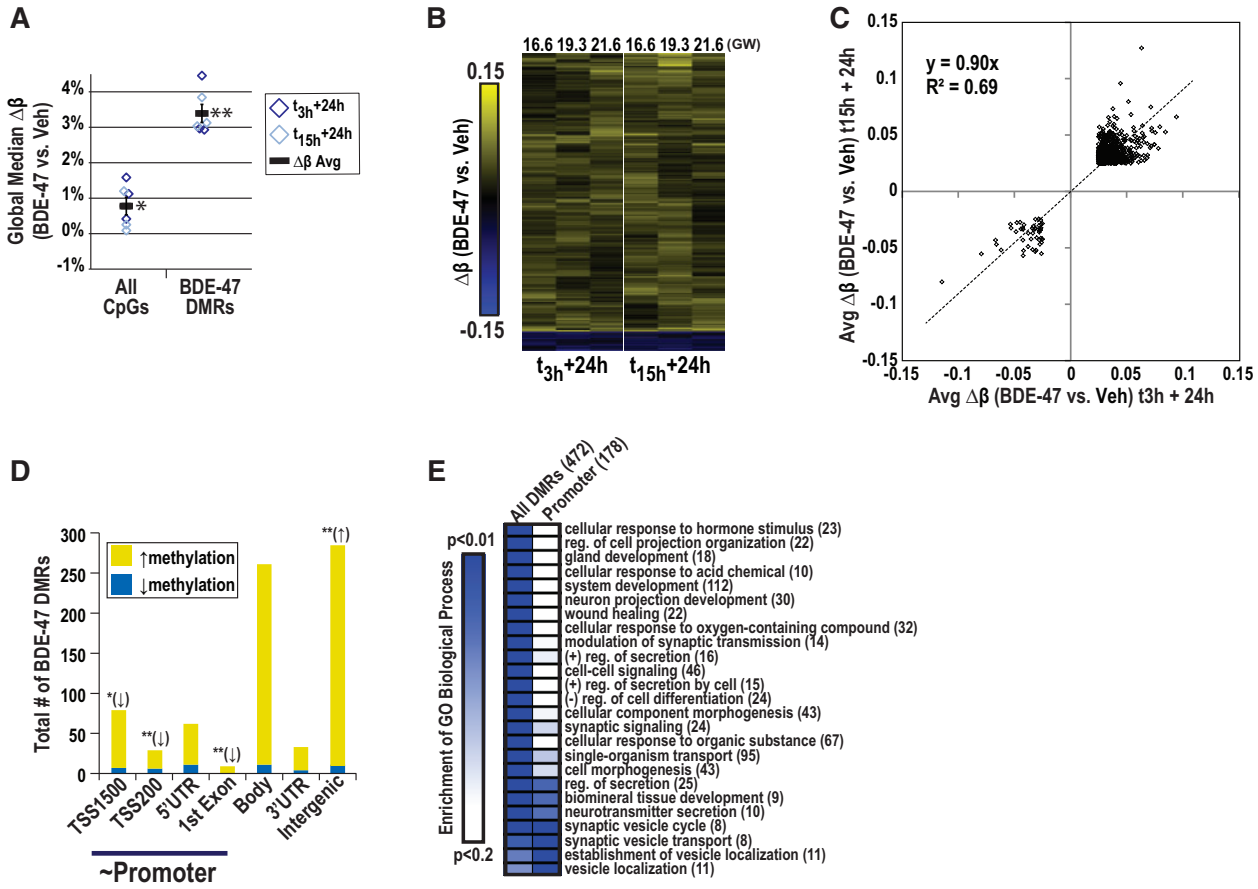


Figure 6. Profiling CpG methylation of BDE-47-exposed CTBs. Cultured CTBs were exposed to 1 μM BDE-47 or 0.1% DMSO for 24 h (initiated at 3 or 15 h post-plating). We identified 758 CpGs to be DM with BDE-47 versus 0.1% DMSO ($p \leq .005$; absolute average $\Delta\beta \geq 0.025$). A, Median difference in methylation levels ($\Delta\beta$) between BDE-47 exposed and vehicle control cultures in all evaluated CpGs and BDE-47 DM CpGs after 24 h. B, Hierarchical clustering of the changes in methylation in BDE-47 DM CpGs (BDE-47 vs concurrent vehicle control). C, Cross-scatter plot comparing average $\Delta\beta$ in BDE-47 response in CTBs exposed at $t_{3\ h}$ or $t_{15\ h} + 24$ h. D, Distribution of BDE-47 DM CpGs by regulatory region. Asterisks indicate over (↑) or under (↓) representation (Fisher's test; (*) $p \leq .05$, (**) $p \leq .001$)). E, Enrichment of GO biological processes within genes (# in parentheses) in proximity of BDE-47 DM CpGs.

(see arrow marked with an asterisk; genome location: 2000000–2300000). Interestingly, the mRNAs encoded by genes in this region (*H19*, *IGF2*, *INS-IGF2*, and *ASCL2*), whose products are among the most abundant in CTBs, play a critical role in gestational development (Smith *et al.*, 2007) and are responsive to multiple environmental exposures (LaRocca *et al.*, 2014; Wu *et al.*, 2004).

Correlating BDE-47-Induced Changes in DNA Methylation With Alterations at the mRNA Level

We identified 178 DM CpGs in gene promoters. A subset (156 of which 152 were unique) were correlated with mRNA expression levels. In total, four genes (3 unique mRNAs) whose promoters were DM had mRNA levels that were also responsive to BDE-47

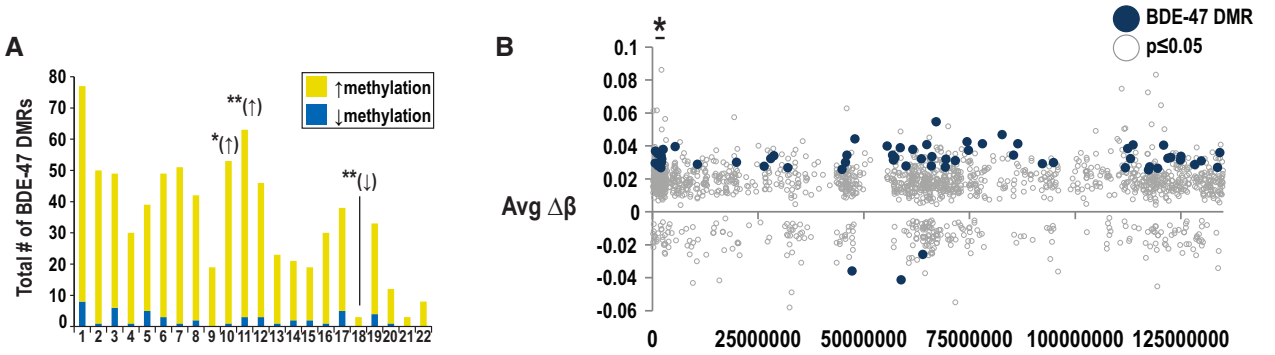


Figure 7. Chromosomal distribution of dysmethylated CpGs in BDE-47-exposed CTBs. A, Location of BDE-47 DM CpGs by chromosome. Asterisks indicate over (↑) or under (↓) representation (Fisher's test; $^{*}p \leq .05$, $^{**}p \leq .001$). B, Change in methylation ($\Delta\beta$) between BDE-47 exposed and vehicle control in CpGs across chromosome 11. Dark blue and gray dots signify significant BDE-47 DM CpGs and CpGs with $p \leq .05$, respectively. Asterisk indicates cluster of BDE-47 DM CpGs located in Ch. 11p15.5.

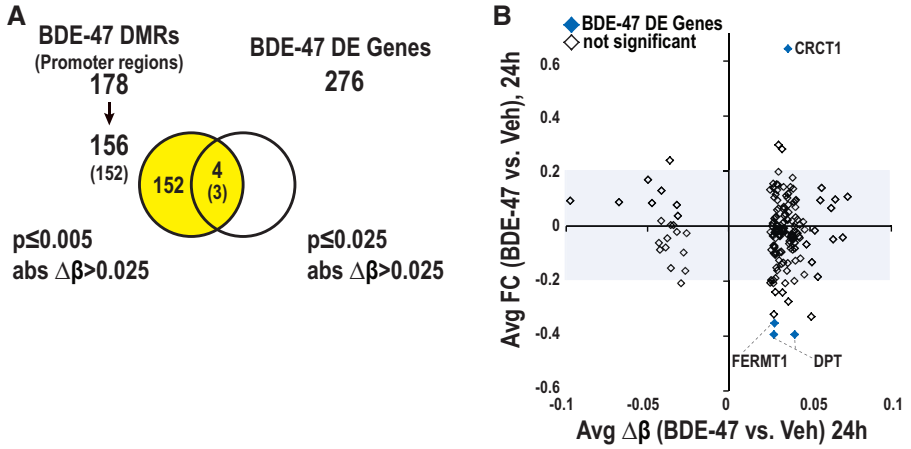


Figure 8. Correlation between BDE-47 altered methylated promoter regions and gene expression. A, The overlap of BDE-47 DM CpGs (associated genes located in promoter regions of DM CpGs) and BDE-47 DE Genes. B, Correlation of average FC in gene expression (*y*-axis) and Δβ (*x*-axis) associated with BDE-47 exposures in gene subset (shaded in Venn). DE Genes are shown as solid blue diamonds.

(Figure 8A). We plotted the average Δβ versus the average FC in expression between BDE-47 and vehicle control for the entire subset (Figure 8B). We identified three mRNA targets of BDE-47 (FERMT1, DPT; downregulated; CRCT1 upregulated) with increased CpG methylation in their promoters due to chemical exposure (Solid blue diamonds, quadrant IV). Our results suggest that BDE-47 exposures may alter the expression of specific genes by modifying CpG methylation within promoter regions.

DISCUSSION

Due to widespread identification of PBDEs in human placental tissues (Leonetti *et al.*, 2016; Zota *et al.*, 2018) and increasing evidence that these compounds cause placental toxicity in rodent and human cell lines (park and Loch-Caruso, 2014, 2015), we utilized a primary cell model to evaluate the effects of BDEs on human CTB differentiation. First, we surveyed a wide range of BDE-47 and -99 concentrations (0.1–25 μM) for cytotoxicity (Figure 1). Both congeners induced significant concentration-dependent effects on cell viability and death. CTBs displayed sensitivities in the range of other vulnerable human cells, eg, primary fetal human neural progenitor cells (Schreiber *et al.*, 2010). Furthermore, PBDEs were more potent in inducing cytotoxicity as compared to other common endocrine disruptors, BPA or PFOA, suggesting PBDEs are relatively potent toxicants in the CTB model.

In CTBs exposed to subcytotoxic concentrations of BDE-47 (Figure 2), we demonstrated acute cellular bioaccumulation of the unmetabolized form after 24 h. These results agree with previous toxicological studies in rodent *in vitro* or *in vivo* models that described cellular/tissue accumulation of PBDEs (Mundy *et al.*, 2004). Partitioning of BDE-47 between the media and cellular fractions was dependent on the initial testing concentration, with a greater proportion of BDE-47 distributed in the cells exposed to 5 μM versus 1 μM. This provided a possible basis for concentration-dependent, nonlinear responses to BDE-47 on the mRNA level. We did not detect a significant amount of the major hydroxylated metabolites of BDE-47 (5-OH or 6-OH). This finding and the observation that ~100% of the parent compound was recovered in the cell/media fractions suggested limited metabolism over 24 h. While the placenta may act as a barrier for several xenobiotics (Robins *et al.*, 2011), its capacity to metabolize PBDEs remains unknown. Other studies from our laboratory (Roadmap Epigenomics *et al.*, 2015) and other groups (Hakkola *et al.*, 1996), indicate that CYP2B6-the primary CYP p450 enzyme involved in BDE-47 metabolism (Feo *et al.*, 2013; Penell *et al.*, 2014)-is expressed at low levels in the placenta.

Here, we present data suggesting that CTBs are unable to breakdown BDE-47 *in vitro*. These mass spectrometry analyzes showed that other primary PBDE congeners (~0.4%) were also present in the mixture. Even though these co-contaminants were a minor component, they could contribute to the observed downstream effects. Future studies are needed to address whether CTB accumulation of PBDEs depends on the exposure duration (acute vs chronic) and the contribution of other placental cell types, eg, STBs, to the metabolism of these compounds.

We tested the ability of BDE-47 to alter CTB migration or invasion—inherent properties of CTBs that enable successful penetration of the decidua and the resident blood vessels. Furthermore, perturbations in CTB migration/invasion underlie pregnancy complications such as PE, PTL, and IUGR, contributing to the pathogenesis of adverse neonatal outcomes (Kaufmann *et al.*, 2003). Our results suggest that BDE-47 significantly disrupts the ability of CTBs to migrate and invade (Figure 3) in line with studies indicating other toxicological compounds, eg, arsenic (Li, and Loch-Caruso, 2007), cadmium (Alvarez, and Chakraborty 2011), impair trophoblast migration. Together these results provide a potential link between chemical exposures, trophoblast dysfunction, and placental-driven pregnancy complications.

Additionally, we conducted a comprehensive assessment of transcriptomic and methylomic changes which correlate with BDE-47 exposure in CTBs. We observed that a subcytotoxic concentration (1 μM) of BDE-47 induces perturbations on transcriptomic and methylomic levels in cultured CTBs isolated from three independent human placentas. We profiled the effects of BDE-47 following a 24 h exposure duration, which was either initiated at $t_{3\ h}$ and $t_{15\ h}$, corresponding with the (1) initial projection of CTB migration or (2) CTB aggregation, respectively (Robinson *et al.*, 2017). In general, significant genomic responses to BDE-47 were similar, irrespective of when exposures were introduced into the media. Overall, these results suggest a general conservation of BDE-47 response in our model system. Future studies which employ larger sample sizes will enable interrogation of factors which also may play a role in sensitivity to environmental exposures *in utero* such as sex, ethnicity, and maternal and gestational age.

As for effects on the transcriptome, we observed dysregulated expression of 276 genes with BDE-47 exposure (Figure 4). Our analyses highlight DE genes associated with (1) proposed mechanisms of PBDE-toxicity, eg, inflammation, Δ hormone response; and (2) pathways critical for placental and trophoblast development. Below, we discuss specific targets of BDE-47 in CTBs that were identified in this study, and their proposed roles in placental development and disease.

Interleukins and other inflammatory mediators drive signaling aspects of placental development and hyperexpression may signal or contribute to pregnancy complications (Tjoa *et al.*, 2003). Here, we provide evidence that BDE-47 alters the expression of several genes known to regulate inflammatory response (Figure 5). Genes dysregulated by BDE-47, included: IL-6 (↑), IL1A (↑), IL1RN (↑), CCL1 (↑), and CCL13 (↑). Interestingly, IL6 may play major roles in immune defense (Rose-John *et al.*, 2017), and altered expression may underlie placental (Prins *et al.*, 2012) and neurodevelopmental disease (Shen *et al.*, 2008; Sorokin *et al.*, 2014). Supporting these findings, in a human trophoblast cell line, in a concentration and time-dependent manner, BDE-47 co-induced IL-6, IL-8, and oxidative stress mediators (park and Loch-Caruso, 2014, 2015). Our results support the hypothesis that PBDE exposures cause inflammation in the placenta, which may contribute to toxicity.

Due to their inherent lipophilic properties and similarities in biochemistry as endogenous hormones, PBDEs and other EDCs may alter cholesterol and fatty acid metabolism pathways which influence the regulation/production of critical hormones for fetal growth. In rodent models, PBDE exposure leads to disruption of specific hormones in placenta (Zhu *et al.*, 2017a) and Leydig cells (Zhao *et al.*, 2011) as well as increased cholesterol serum levels in perinatally exposed juveniles (Tung *et al.*, 2017). Here, we propose specific mRNA targets such as regulatory binding proteins (FABP7 [Thumser *et al.*, 2014], INSIG1 [Dong, and Tang, 2010]) and metabolizing enzymes (FASN [Jones, and Infante, 2015], HMGCS1 [Vock *et al.*, 2008], SCD [Zhang *et al.*, 2005]) involved in lipid/cholesterol metabolism to be significantly downregulated with BDE-47. While these pathways during fetal development are clearly important, the specific role of these molecules in the context of chemical toxicity and/or trophoblast differentiation remains undefined. Our results suggest BDE-47 alters the expression of molecules regulating cholesterol/fatty acid biosynthesis, which may play upstream roles in hormonal regulation in the placenta.

We observed several molecules with known critical functions in placental development, including: (1) maintenance of trophoblast progenitor populations, eg, BMP2 (↑, [Golos *et al.*, 2013]) and NODAL (↓, [Ma *et al.*, 2001]); (2) trophoblast invasion, eg, MMP1 (↑, [Cohen *et al.*, 2006]), MMP8 (↑, [Zhu *et al.*, 2012]); and (3) villi morphogenesis, eg, GREM1 (↑, [O'Connell *et al.*, 2013]), to be significantly altered by BDE-47 exposures (Figure 5B). In addition, our analyzes revealed targets with less defined roles in placentation with links with placental complications, eg, PLAC4 (↑, (Tuohey *et al.*, 2013]), and candidates yet to be studied in the context of placental development. For example, BDE-47-induced expression of GPR34 (Figure 5C), which controls aspects of migration in cancer cells (Jin *et al.*, 2015), and may be required for sufficient immune response to pathogens (Liebscher *et al.*, 2011). Our findings, which complement our observations of BDE-47 functional impairment, provide specific pathways with known roles in trophoblast/placental development to be significantly altered with exposure.

We evaluated for potential upstream regulators of BDE-47 DE Genes by conducting enrichment analysis of TF-related motifs in promoter regions of the gene subset (**Table 1**). In previous studies, similar approaches have been applied to propose key regulatory nodes of developmental toxicity (Robinson *et al.*, 2011). Based on these analyses, we identified TFs involved in PBDE-induced response such as NFKB, a highly recognized TF involved in regulating inflammation signaling pathways in the placenta, and environmental stress-responsive gene expression networks (Simmons *et al.*, 2009). Interestingly, other TFs identified through this analysis, eg, RORA (Qiu *et al.*, 2015), RELA (Minekawa *et al.*, 2007), were also found to differ in expression levels between second trimester and term CTBs and have postulated roles in placenta development, suggesting that these TFs may regulate PBDE-response networks in the context of altered trophoblast development.

While the mechanisms remain poorly understood, diverse environmental exposures, including PBDEs (Byun *et al.*, 2015; Kappil *et al.*, 2016; Sales, and Joca, 2016; Woods *et al.*, 2012), are associated with epigenetic modifications which may regulate transient or irreversible genomic changes leading to transgenerational inheritance of altered phenotypes (Bernal, and Jirtle, 2010; Skinner, and Guerrero-Bosagna, 2009). Here, in parallel with transcriptomic assessments, we provide evidence of BDE-47 exposures to produce subtle, but significant, global changes on the methylome following a 24 h exposure duration (Figs. 6

and 7). Within the subset of BDE-47 DM CpGs, the majority of CpG sites were increased in methylation with BDE-47 (average $\Delta\beta = 3.4\%\uparrow$; 93% CpGs were increased, $\beta > 0$), and predominately located in nonpromoter regions (ie, intergenic positions, gene body). Interestingly, in general, these observations did not correlate with a global reduction in transcription (Figure 8) or modified expression of DNA methyltransferases (DNMTs)/TET family enzyme members (not shown). *In vitro*, upon plating, proliferating CTBs exit the cell cycle, and do not propagate in culture. At each autosomal CpG site, methylation is expected to be 0, 50, or 100% methylated. Thus, the subtle changes in methylation observed with BDE-47 may represent an active binary shift (methylated vs not methylated) in a subset of cells in culture. While increased DNA methylation within promoter regions is a recognized mechanism in silencing mRNA transcription, the role of methylation within nonpromoter regions remains undefined and complex (Szyf, 2011). For instance, increased global methylation within gene-body regions may actually promote gene expression by suppressing cryptic promoters, eg, antisense targets, which compete with RNA Polymerase II-directed transcription (Gagnon-Kugler *et al.*, 2009). Furthermore, global changes in methylation due to environmental exposures are of particular interest in the context of the placenta due to its unique DNA methylation and chromatin state. As compared with adult somatic cells and tissues, the CTB and placental genome is (1) globally hypomethylated; (2) contains a higher proportion of variably methylated CpG sites (β = 20–80% methylated); and (3) age-dependent in its specificity (global methylation higher at term versus second trimester [Roadmap Epigenomics *et al.*, 2015]). Therefore, our results which suggest environmental-induced changes on a global methylation level could have dramatic long-term impact(s) on regulatory molecular/cellular functions of the dynamically changing placenta.

Examining the localized distribution of DM CpGs across the genome, we identified a vulnerable region located on Ch. 11p15.5, which included five DM CpGs (all $\uparrow$) due to BDE-47 exposure (Figure 7B, asterisk). This region of Ch. 11 has previously been identified to contain a cluster of imprinted genes important for fetal growth, which have significant associations with several gestational diseases (Smith *et al.*, 2007). Interestingly, genes within this cluster, H19, IGF2, and INS-IGF2, are in the top 99% of abundant transcripts in trophoblast cell populations based on analyzes of second and term trophoblasts in our laboratory (not shown), further suggesting a high level of importance of these molecules in CTB development. Recently, investigators have summarized growing evidence linking alterations in genomic imprinting of this region and adverse placental, ie, PE, and neurobehavioral disease outcomes (Nomura *et al.*, 2017)-key outcomes of interest in regards to PBDEs and other chemical exposures which occur during human pregnancy. Our study along with other environmental investigations in rodent (Ouko *et al.*, 2009; Susiarjo *et al.*, 2013; Wu *et al.*, 2004) and humans (LaRocca *et al.*, 2014) suggest associations between environmental exposures and epigenetic alterations within this particular gene cluster to have high sensitivity and importance in developmental toxicology.

Limited overlap in genes linked with BDE-47 DM CpGs (located in promoter regions) and BDE-47 DE Genes were identified (4 total, 3 unique genes; Figure 8). Closer examination of the influence of BDE-47 on methylation within DM CpGs and RNA expression, in general, suggests a lack of correlation between Δ in methylation and expression due to BDE-47 at 24 h. Poor correlation could be due to the lack of temporal data. Our comparative analyses indicate that two candidates may be epigenetically controlled in response to BDE-47. Both targets, DPT and FERMT1, were identified to have increased methylation in the promoter region associated with downregulation of expression with BDE-47 exposure. These molecules have relatively unknown functions in placentation, but recent evidence implies roles in adhesion and cell migration (Liu *et al.*, 2013) as well as links to regulatory pathways known to be important for trophoblast development (DPT: TFG-β signaling [Jones *et al.*, 2006; Okamoto *et al.*, 1999]); FERMT1: epithelial-mesenchymal transition/Wnt-signaling (Knofler, and Pollheimer, 2013; Liu *et al.*, 2017). Future mechanistic studies may explore the relationships between methylation, RNA expression, and impaired outcomes in CTBs as related to BDE-47 exposures.

We tested 0.01–25 μM of BDE-47, which includes concentrations proposed as physiologically relevant for human exposures and which cause deleterious effects in human embryonic/fetal cells (Park *et al.*, 2014; Schreiber *et al.*, 2010). In general, toxicological responses to BDE-47 were observed at concentrations of $\geq 1\,\mu$M or higher which are ~3 orders of magnitude higher than the geometric mean concentrations ($\pm$standard deviation) recently reported in maternal serum (0.17 $\pm$ 1.97 ng/g), cord serum (0.22 $\pm$ 1.75 ng/g), or placenta (0.15 $\pm$ 1.96 ng/g) (Zota *et al.*, 2018). However, extrapolating exposure levels between *in vitro* and *in utero* is challenging due to the numerous factors that influence sensitivity, including: (1) absorption, metabolism, and excretion; (2) length of exposure (chronic vs acute); (3) life-style factors; (4) nutritional status; and (5) life-stage (Doucet *et al.*, 2009; Grandjean, 1992), which are not addressed in cell culture models. Accounting for factors that underlie variable exposures as well as the lipid content of the placenta, *in vitro* concentrations as high as 8 μM are estimated to be relevant to human exposures (Park *et al.*, 2014). Furthermore, during pregnancy, the fetal/placental unit is exposed to multiple PBDEs and other environmental compounds that may act through similar mechanisms, additively or synergistically (Eriksson *et al.*, 2006; Tagliaferri *et al.*, 2010). Future experiments could use the CTB model to evaluate developmental toxicity in the context of other FRs, including emerging alternatives and complex chemical mixtures.

In this study, we demonstrate the application of a primary human villous CTB model to examine potential environmental interactions which occur during placentation using PBDEs as a relevant model chemical toxicant. Primary human placental cells offer multiple advantages as a toxicological model as compared with transformed human cell lines (Bilban *et al.*, 2010) or rodent (Silva, and Serakides, 2016) due to suspected differences in the underlying cellular and molecular components that regulate placental development. Moving forward, larger sample sizes may be incorporated to improve detection of environmental interactions and control for genetic diversity, gestational age, and sex. Future investigations may also add complimentary investigations of other placental cell types, eg, STBs, which may improve resolution of mechanisms proposed to play roles, eg, Δ hormone levels, (Zhu *et al.*, 2017a,b), in PBDE-induced placental toxicity in studies examining the complete placental unit. In summary, we provide evidence that PBDEs induce toxicity in human primary placental cells and alter levels of expression of specific transcripts and epigenetically controlled regions. Perturbations may be evaluated as biomarkers *in vitro* or *in vivo* to determine correlations between PBDEs and adverse developmental/pregnancy outcomes.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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