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Effects of endocrine disrupting chemicals on *in vitro* global DNA methylation and adipocyte differentiation

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ABSTRACT

Recent studies suggest that endocrine disrupting chemicals (EDCs) may form a risk factor for obesity by altering energy metabolism through epigenetic gene regulation. The goal of this study is to investigate the effects of a range of EDCs with putative obesogenic properties on global DNA methylation and adipocyte differentiation *in vitro*. Murine N2A and human SK-N-AS neuroblastoma cells and murine preadipocyte fibroblasts (3T3-L1) were exposed to tributyltin (TBT), diethylstilbestrol (DES), bisphenol A (BPA), 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin (TCDD), 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153), hexachlorobenzene (HCB), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47) , perfluorinated octyl acid (PFOA) and perfluorinated octyl sulfonate (PFOS). A modest decrease in global DNA methylation was observed in N2A cells exposed to 10 μM DES, BPA, TCDD, BDE-47, PCB-153 and 1 μM HCB, but no changes were found in the human SK-N-AS cells. We reveal for the first time that BDE-47 increases adipocyte differentiation in a dose-dependent manner (2.5–25 μM). Adipocyte differentiation was also enhanced by TBT ($\geqslant$10 nM) and BPA (>10 μM) and inhibited by TCDD ($\geqslant$0.1 nM). The other chemicals showed either modest or no effects on adipocyte differentiation at the concentrations tested (PFOA, PFOS and HBCD at 10 μM; PCB-153, 3.4 μM and HCB, 1 μM). This study demonstrates that selected EDCs can induce functional changes in murine adipocyte differentiation *in vitro* which are accompanied by decreased global DNA methylation.

1. Introduction

Obesity is a worldwide public health issue with at least 500 million adults clinically obese (WHO, 2012). The main causes of obesity are decreased physical activity and increased food intake (McAllister et al., 2009), but evidence is accumulating that factors in early development such as exposure to environmental contaminants may increase susceptibility to obesity later in life (Heindel and vom Saal, 2009). Support for this "obesogen hypothesis" (Grun and Blumberg, 2006) comes from epidemiological as well as animal studies (reviewed in Legler et al., 2011). For example, epidemiological studies have demonstrated associations between *in utero* exposure to polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) and the development of obesity later in life (Smink et al., 2008; Valvi et al., 2012). In addition, a number of animal studies indicate that *in utero* or neonatal exposure to environmental chemicals may lead to weight gain in later life. For instance, low concentrations of diethylstilbestrol (DES) and bisphenol A (BPA) during neonatal life in mice have been shown to cause a significant increase in body weight as adults (Newbold et al., 2008; Van Esterik et al., 2011). Similary, *in utero* exposure of mice to tributyltin (TBT) has demonstrated elevated adipose mass in adult animals (Grun et al., 2006) while developmental exposure of mice to perfluorooctanoic acid (PFOA) has been shown to result in adult weight gain (Hines et al., 2009).

Obesogenic chemicals may exert their effects at the molecular level by disturbing pathways related to energy homeostasis such as lipid metabolism, adipogenesis or appetite regulation (Crews and McLachlan, 2006). Endocrine disrupting chemicals (EDCs) may disturb these pathways through multiple mechanisms, such as stimulating or antagonizing nuclear receptors, inhibiting aromatases, activating expression of the P450 enzymes and affecting release of brain-produced substances by alteration of neuronal synapse formation (reviewed in Elobeid and Allison, 2008). Several studies have shown that exposure to EDCs such as TBT, BPA and

Abbreviations: EDCs, endocrine disrupting chemicals; TBT, tributyltin; DES, diethylstilbestrol; BPA, bisphenol A; TCDD, 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin; PCB-153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; HCB, hexachlorobenzene; HBCD, hexabromocyclododecane; BDE-47, 2,2′,4,4′-tetrabrominated diphenyl ether; PFOA, perfluorinated octyl acid; PFOS, perfluorinated octyl sulfonate.

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butylparaben induces 3T3-L1 embryonic murine preadipocyte fibroblast cells to differentiate into adipocytes (Grun et al., 2006; Masuno et al., 2002; Taxvig et al., 2012).

An additional and novel mechanism which may underlie the developmental origins of obesity is altered epigenetic gene regulation (Godfrey et al., 2011; Tobi et al., 2009). Epigenetic modifications control gene expression by regulating the accessibility of chromatin without altering the DNA sequence itself. The most well studied epigenetic mechanism is DNA methylation. Abnormal epigenetic programming through DNA methylation can lead to aberrant genomic responses and ultimately altered cell function (Bombail et al., 2004). Animal studies have shown that epigenetic alterations caused by chemical exposure during early development may lead to increased susceptibility to disease later in life (reviewed in Jirtle and Skinner, 2007). Examples of developmental exposure to EDCs linked to increased body weight in adult rodents and permanent alteration of DNA methylation in animal models are DES (Alworth et al., 2002) and BPA (Dolinoy et al., 2007). Furthermore, methylation of specific CpG sites was suggested to contribute to regulating leptin gene expression during adipocyte differentiation (Yokomori et al., 2002). Sakamoto et al. (2008) also demonstrated that during adipocyte differentiation changes in genome-wide DNA methylation status take place.

Given the evidence that chemicals can alter the developmental programming of obesity, with changes in epigenetic gene regulation as a possible underlying mechanism, the aim of this study was to examine the effects of a range of putative obesogenic EDCs on *in vitro* DNA methylation and adipocyte differentiation. Since many different target genes are involved in the development of obesity, the effects of EDCs on global DNA methylation status were assessed to provide a first indication of epigenetic effects of EDC exposure on a genome-wide level. To this end, *in vitro* models were selected which represent important tissues involved in energy metabolism, namely neurons and adipocytes. Murine N2A and human SK-N-AS neuroblastoma cells and the murine embryonic fibroblasts 3T3-L1 adipocyte differentiation model were exposed to representative EDCs from five major classes suggested previously to be implicated in obesity and related disorders in either human or animals (reviewed in Legler et al., 2011). These chemicals included 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin (TCDD) representing the group of dioxin-like polyhalogenated hydrocarbons (PHAHs); 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153) representing non dioxin-like PHAHs; HCB representing organochlorine pesticides; hexabromocyclododecane (HBCD) and 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47) representing brominated flame retardants and perfluorinated octyl acid (PFOA) and perfluorinated octyl sulfonate (PFOS) representing perfluoroalkyl substances. In addition, TBT, DES and BPA were included as reference compounds shown previously to be obesogenic in animal studies as stated above. Our *in vitro* studies reveal that several compounds decrease global DNA methylation which is concurrent with enhanced adipocyte differentiation.

2. Materials and methods

2.1. Chemicals

TBT chloride (96%), DES (97%), 5-azacytidine (5-AC, >98%), BPA (99%) and troglitazone (TRO, >98%) were obtained from Sigma–Aldrich, Munich, Germany. HCB (>99%) and PFOS (98%) were obtained from Fluka, Munich, Germany. PFOA (96%) and dimethylsulfoxide (DMSO, 99.9%) were purchased from Acros, Geel, Belgium. BDE-47 and HBCD (>99%; purified on charcoal column to remove possible impurities of brominated dibenzofurans and dioxins) were kindly provided by Dr. Marsh and Prof. Bergman (Stockholm University, Sweden). PCB-153 was purchased from Neosyn Inc., Connecticut, USA and purified to remove dioxin-like impurities (Hamers et al., 2011). TCDD (99%) was obtained as a 50 μg/ml stock solution in DMSO from Cambridge Isotope Laboratories, Andover, MA, USA. All chemicals were dissolved in DMSO and stored at 4 °C as 1000× concentrated stock solutions.

2.2. Cell culture

Neuro-2A cells (N2A) were kindly provided by C. Margadant (Netherlands Cancer Institute, The Netherlands) and SK-N-AS cells were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained in DMEM (Gibco, Paisley, United Kingdom) supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco). 3T3-L1 preadipocytes from the mouse *Mus musculus* were kindly provided by Dr. Leo van der Ven (RIVM, The Netherlands) at passage 9 and were maintained in high glucose DMEM/F12 (Gibco) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% non essential amino acids (Gibco). All cell cultures were maintained at 37 °C in an incubator with 5% (v/v) CO_2.

2.3. Cytotoxicity

All compounds were tested for cytotoxic effects in the neuroblastoma and 3T3-L1 cell lines with a combined lactate dehydrogenase leakage (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell lines were seeded in their specific culture medium in 96-well plates (Greiner, Frickenhausen, Germany) at a density of 2500 cells/well. After 24 h cells were exposed to 100 μl medium containing increasing concentrations of the chemicals (0.1% DMSO) with 10 μM as the maximum concentration tested ($n = 6$). After exposure, LDH was measured directly in the medium by transferring 50 μl of medium from each exposure to a 96 well plate. Directly after adding a mixture of sodium pyruvate (Sigma) and NADH (Applichem, Darmstadt, Germany) to the medium, the NADH decrease was measured kinetically using a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA, USA) at 340 nm. For the MTT measurement, cells were given MTT at a concentration of 5 mg/ml. After 1 h of incubation at 37 °C, the MTT medium was discarded with an aspirator and cells were lysed in 100 μl of DMSO. Formazan formation was measured using the Spectramax 340PC plate reader at 562 nm. Cytotoxicity was assessed at 24 h, 48 h and 72 h after exposure. Only concentrations of chemicals with no significant differences in both LDH and MTT to the vehicle control (0.1% DMSO) were used for subsequent exposures. 3T3-L1 cells were tested for cytotoxicity as stated above in undifferentiated state and checked for cytotoxicity microscopically by checking for cell shape alterations, membrane blebbing and vacuolization during the 8 day differentiation period. As exposure to DES in 3T3-L1 cell lines resulted in cytotoxicity, this chemical was excluded from experimentation.

2.4. Neuroblastoma cell line exposure

N2A and SK-N-AS cells were seeded in 6-well plates (Greiner) at a density of 50,000 cells/well. At 24 h after seeding, cells were exposed to 0.1 μM TBT or TCDD, 1 μM 5-AC or HCB, or 10 μM BPA, DES, PFOA, PFOS, HBCD, PCB-153, or BDE-47 for 48 h (N2A) or 72 h (SK-N-AS). DMSO (0.1%) was included in all experiments as a vehicle control. Exposure time was selected to cover at least two cycles of cell division. In the case of N2A cells, in addition to investigating global methylation with HPLC, arbitrary primed PCR (AP-PCR) was also applied following both 2 and 7 day exposure to 5-AC (1 μM) and DES (10 μM).

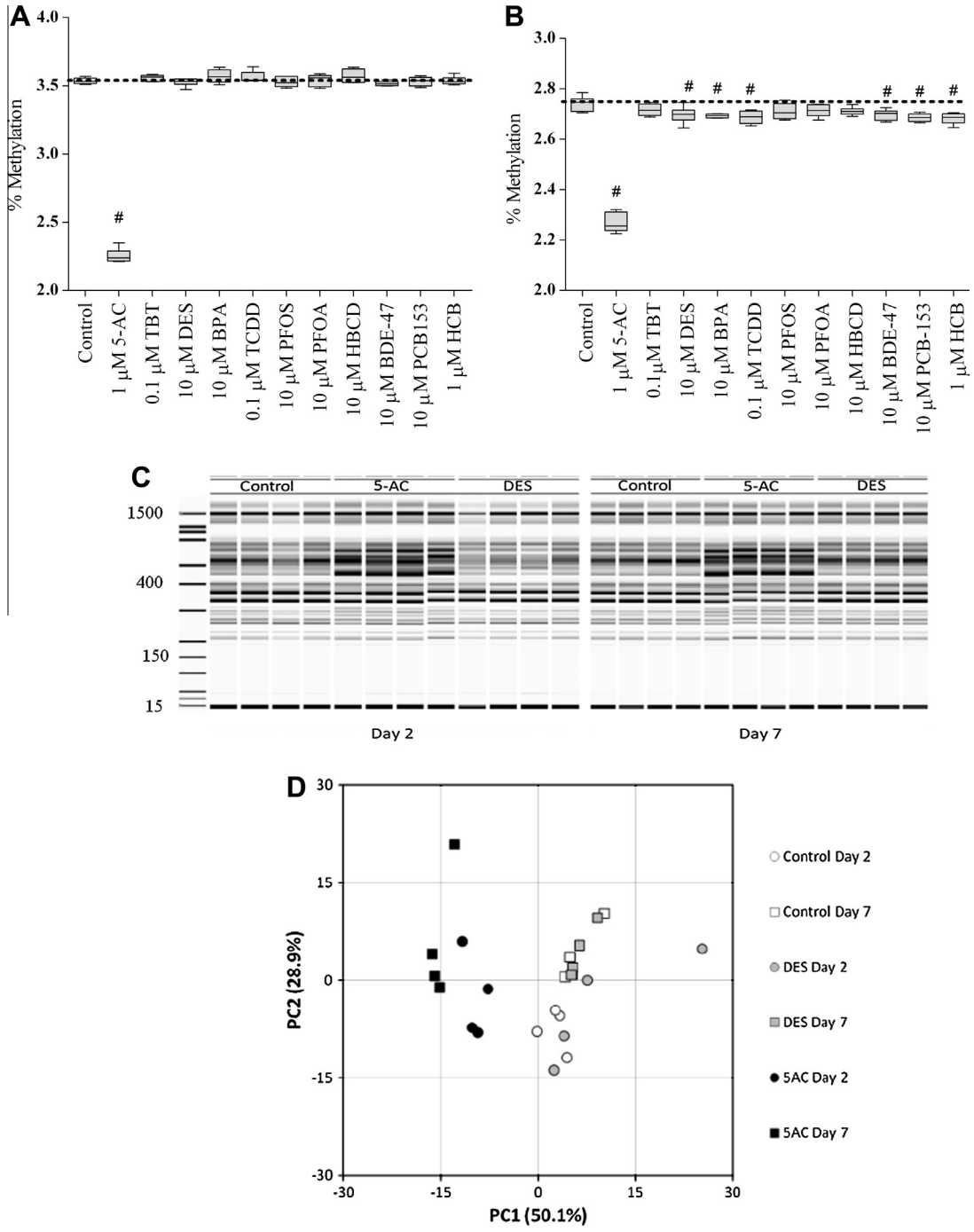

Fig. 1. Effects of chemical exposure on global DNA methylation status in neuroblastoma cells. Human SK-N-AS cells (A) and murine N2A cells, (B) were exposed to 5-azacytidine (5-AC), tributyltin (TBT), diethylstilbestrol (DES), bisphenol A (BPA), 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin (TCDD), perfluorinated octyl sulfonate (PFOS), perfluorinated octyl acid (PFOA), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47), 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153) and hexachlorobenzene (HCB) for 48 h (N2A) or 72 h (SK-N-AS) ($n = 6$). Bottom and top of the box represent 25th and 75th percentiles, line in the middle of the box represents median and the ends represent the minimum and the maximum of global DNA methylation status as measured with HPLC. #Indicates significant difference from control ($p < 0.05$). (C) Arbitrary primed PCR (AP-PCR) of N2A cells after exposure of N2A cells to 1 μM 5-AC and 10 μM DES ($n = 4$) at 2 and 7 days of exposure. Banding patterns of PCR fragments in comparison to marker are shown after peak intensities determination of the DNA sequencing obtained by electrophoresis. (D) Principal component analysis (PCA) on the quantified intensities of the different banding patterns.

2.5. 3T3-L1 differentiation and exposure

3T3-L1 cells at passage 12 were seeded in 6-wells plates (Greiner) at a density of 150,000 cells/well. Three days after seeding (day 0), differentiation was induced with culture medium supplemented with 1 μM dexamethasone (DEX), 1.67 μM insulin (INS) and 5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma–Aldrich) as previously described (Gomez et al., 2003) and refreshed after 24 h (day 1). The induction medium also contained the test chemical at the following concentrations: TCDD (0.1 nM); TBT (10 nM); HCB (1 μM); BDE-47(2.5 μM); PCB-153 (3.4 μM); or BPA, PFOA, PFOS, or HBCD (10 μM). To investigate possible dose–response effects, a minimum of 2 independent experiments were carried out, and included exposure to: TBT (10 nM, 50 nM); BPA (10 μM,

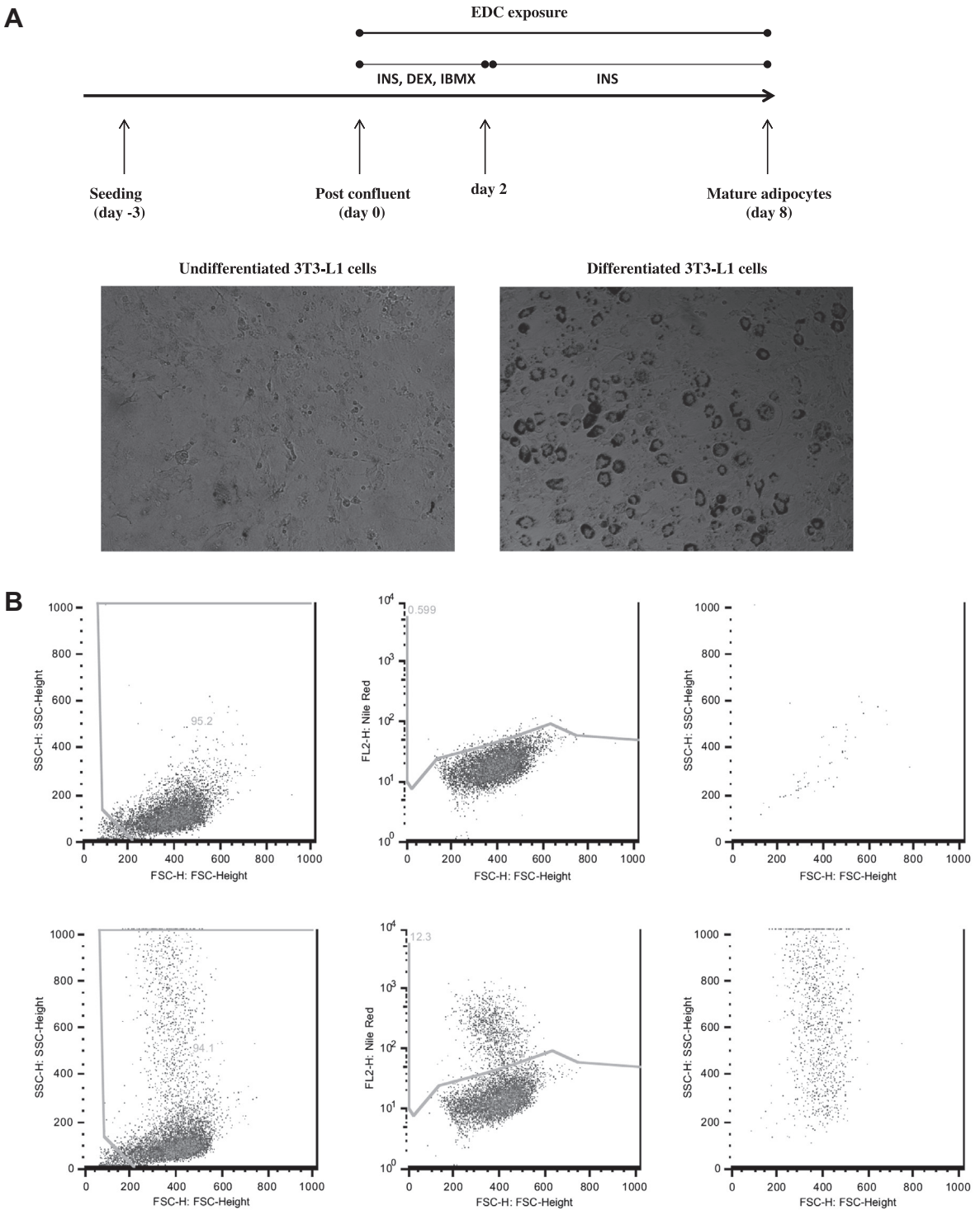

Fig. 2. Effects of chemical exposure on murine adipocyte differentiation. (A) Schematic representation of exposure regime in 3T3-L1 cells and bright field pictures of adipocyte differentiation observed in undifferentiated and differentiated 3T3-L1 cells at a 4× magnification. (B) Gating strategy on flow cytometry plots of Nile Red stained 3T3-L1 undifferentiated (upper panels) and differentiated (lower panels) cells to quantify the percentage of differentiated adipocytes in culture. Differentiated cells induced by a cocktail of DEX, INS and IBMX were identified as SSC^{hi} and Nile Red^{hi}. Left panel indicates the initial chosen gate, middle panel indicates refinement of the initial gate by selecting cells positive for Nile Red fluorescent staining and right panel shows the cells actually defined as adipocytes. (C) Effects of 8 day exposure to EDCs on differentiation of 3T3-L1 cells. 3T3-L1 cells were exposed to troglitazone (TRO), tributyltin (TBT), 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47), bisphenol A (BPA), 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin (TCDD), hexachlorobenzene (HCB), perfluorinated octyl acid (PFOA), perfluorinated octyl sulfonate (PFOS), 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153), and hexabromocyclododecane (HBCD). Three replicates were tested per concentration in two independent experiments. Undiff: undifferentiated cells exposed to medium; control: cells containing DMSO vehicle (0.1%) induced to differentiation by addition of INS, DEX and IBMX to the medium; EDC: endocrine disrupting chemicals; INS: insulin; DEX: dexamethasone; IBMX: 3-isobutyl-1-methylxanthine. Data represent mean and standard deviation of % of Nile Red and SSC positive cells. #Indicates significant difference from control ($p < 0.05$).

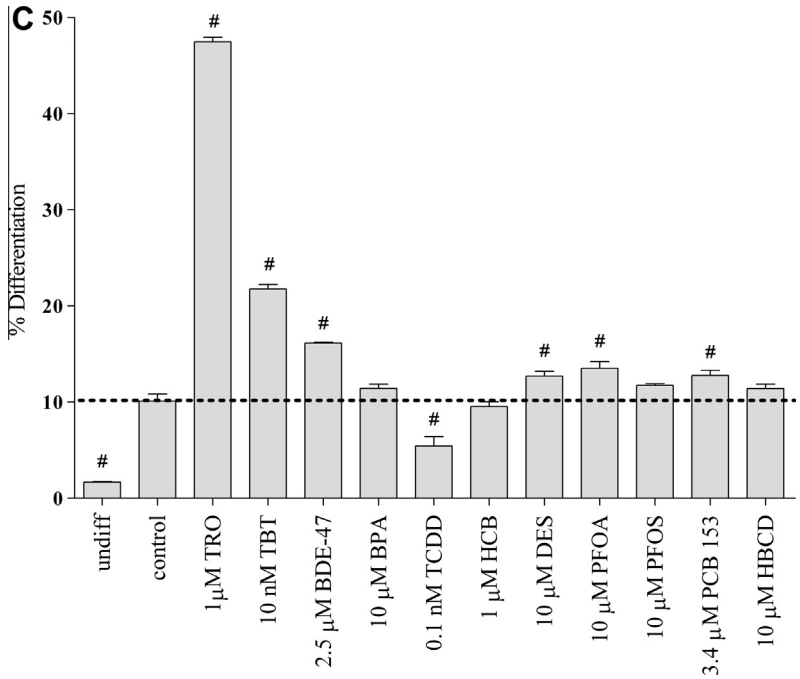

Fig. 2. (*continued*)

50 μM, 80 μM); BDE-47 (2.5 μM, 12.5 μM, 25 μM); TCDD (0.1 nM, 0.5 nM, 1 nM) and HCB (1 μM, 5 μM and 10 μM). DMSO (0.1%) was included in all experiments as a vehicle control. As a positive control for adipocyte differentiation, the PPARγ agonist troglitazone (TRO) (Grun et al., 2006) was used at a concentration of 1 μM. On day 2, the medium was refreshed with full culture medium containing the test chemicals and 1.67 μM insulin. Cells were kept in these conditions with medium refreshed every other day until day 8, when cells were rinsed with phosphate buffered saline (PBS).Three wells were tested per concentration. Undifferentiated control cells were maintained in high glucose DMEM/F12 (Gibco) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% non essential amino acids (Gibco) with medium refreshment in the same pattern as differentiated cells, however, without differentiation induction cocktail and chemicals exposure.

Differentiation of 3T3-L1 cells into adipocytes was quantified by flow cytometry as previously described by Hanlon et al. (2003) with small modifications. Briefly, cultured cells were trypsinized and resuspended in 1 ml PBS. 150 μl paraformaldehyde 4% was added to the solution to obtain a final concentration of 0.5% paraformaldehyde. Samples were kept on ice for 30 min and centrifuged for 3 min at 100*g*. After removal of the paraformaldehyde solution, 25 ng/ml Nile Red (Sigma Aldrich) dissolved in FACS buffer (0.25% BSA, 0.5 mM EDTA, 0.05% NaN_3 in 1xPBS (Sigma Aldrich) was added to the pellet and samples were analyzed. Nile Red stained cells were defined as adipocytes and were quantified by measuring 10,000 cells at a wavelength of 585 nm (fluorescent labeling) and at 488 nm (forward and side scatter, which respectively account for cell size and granularity) on a FACSCalibur Dual-Laser Benchtop Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data was acquired and analyzed with Flowjo software (Treestar, Costa Mesa, CA, USA). Differentiated adipocytes were quantified as the percentage SSC^{hi} and Nile Red^{hi} cells of total analyzed cells as described before (Gomez et al., 2003) (see Fig. 2B for gating strategy).

2.6. Global methylation – HPLC

After exposure, cells were lysed and DNA was extracted with the nucleospin tissue DNA purification kit (Macherey Nagel, Düren, Germany) according to the manufacturers' instructions and stored at −20 °C until further analysis. An additional RNA clean up step was added prior to DNA extraction by treatment of lysates with 1 mg/ml RNAse A (Sigma–Aldrich) for 5 min at room temperature. Global methylation was analyzed according to Rozhon et al. (2008). In brief, DNA samples (1 μg) were digested to single nucleotides with a combination of DNAse I (Sigma) and Nuclease P1 (Sigma). Samples were further digested with alkaline phosphatase (New England Biolabs, Hitchin, UK). Deoxycytidine (dC) and 5-methyl deoxycytidine (5MdC) were quantified with an HPLC-UV system (Shimadzu) equipped with a 125 × 4 mm nucleosil 100–10 SA column (Macherey–Nagel) and a mobile phase consisting of 40 mM acetic acid in 15% acetonitrile (pH 4.8) at a flow rate of 0.6 ml/min. Global methylation status was determined by expressing the level of methylated deoxycytidines (5MdC) as a percentage of the total deoxycytidines (dC + 5MdC). Five (3T3-L1 cells) and six (N2A and SK-N-AS cells) replicates were tested per concentration.

2.7. Global methylation – arbitrary primed-PCR

For measuring more subtle changes in DNA methylation, DNA samples ($n = 4$) from one experiment with N2A cells described above were analyzed with an AP-PCR method (Bachman et al., 2006) with modifications. In brief, 0.5 μg of DNA was digested with 5 U of the RSAI restriction enzyme (New England Biolabs) for 1 h in 30 μl reactions. Samples were further digested overnight with 5 U of the methylation sensitive restriction enzyme HPAII (New England Biolabs). A volume of 5 μl of the digestion product was used to perform AP-PCR using the following primer: 5′-AACCCTCACCCTAACCCCGG3′ in a 25 μl reaction. Next, 1 μl of the PCR product was analyzed on the Experion Bio Analyzer (Bio-Rad, Veenendaal, The Netherlands) using a 1K DNA analysis chip

(Bio-Rad). From the Experion software (Bio-Rad) a compare run was made with all the different exposures. The raw data was exported to excel and saved as a tab separated text file. This file was imported in spec align (Cartwright group) for peak intensities calculations of the electropherograms. A base line was determined with a window size of 1. Subsequently, the baseline was subtracted. Peaks were identified with the pick peak function, with variables: Base cutoff: 0.1 Window: 1 Height 1.5. An average spectrum was made from all the spectra. Using this average spectrum as a reference, spectra were aligned with a combination of fast fourier transform and peak matching with the variables: Scale: 2, Max shift: 20, Look ahead: 1.

2.8. Statistical analysis

Univariate treatment effects were determined by one-way ANOVA analysis ($p < 0.05$) on data that were normally distributed by themselves or after ln-transformation (Shapiro–Wilk test). Significant differences from the vehicle control treatment were determined by Dunnet's post hoc multiple comparison test for data with equal variances (Levene's test) or by Dunnett's T3 test for data with unequal variances. Simple linear regression analysis was used to investigate the relationship between adipocyte differentiation and global DNA methylation. All univariate and regression analyses were performed using the SPSS Software Package (Version 16.0, 2007). Multivariate principal component analysis (PCA) on the Pareto variance weighted peak intensities of the electropherograms obtained in the AP-PCR experiments was performed in Sirius 7.1 software (Pattern Recognition Systems as, 2006).

3. Results

3.1. Effects of EDCs on global DNA methylation in neuroblastoma cells

To examine direct effects of EDCs on global methylation levels, we first exposed two neuronal cell lines to selected chemicals, representing major classes of environmental contaminants. Exposure of the human neuroblastoma cell line SK-N-AS to the known DNA methyltransferase-1 (DNMT1) inhibitor 5-AC, resulted in a significant decrease in global DNA methylation status (2.25% ±0.05) compared to control (3.54% ±0.02) (Fig. 1A). Exposure of the murine neuroblastoma cell line N2A to 5-AC led to a similar reduction in global DNA methylation (2.26% ±0.04, compared to 2.76% ±0.03 in the control; Fig. 1B). Exposure to the selected endocrine disrupting compounds, however, did not result in any effects on global DNA methylation in the human cell line while DES (2.70% ±0.03), BPA (2.69% ±0.01), TCDD (2.69% ±0.02), PCB-153 (2.68% ±0.02), BDE-47 (2.70% ±0.02) and HCB (2.68% ±0.02) caused modest yet significant reduction of global DNA methylation in the murine cell line.

As measurement of global DNA methylation takes into account the methylation of all cytosines present in the isolated DNA, we next performed an analysis to measure the effects of EDCs on methylation of sites in genomic regions that contain a high frequency of cytosine–guanine nucleotides (CpG islands), which are more important for gene regulation than CpG methylation in random regions of DNA. A modified AP-PCR approach was used, based on the digestion of genomic DNA with the methylation sensitive restriction enzyme HPAII followed by PCR amplification with an arbitrary primer that specifically binds CpG rich regions combined with capillary gel electrophoresis (Bachman et al., 2006). This experiment was limited to examination of the effects of 5-AC and DES, an EDC shown previously to cause changes in methylation *in vivo* (reviewed in Harris and Waring, 2012). Treatment of N2A cells with 5-AC resulted in a clear difference in banding patterns when genomic DNA was digested with HPAII (Fig. 1C). Principal component analysis (PCA) on the quantified intensities of the different bands (Fig. 1D) resulted in a separate cluster of 5-AC treated samples (black symbols) when compared to control samples (white symbols), confirming that there were indeed differences in DNA methylation in CpG rich regions. No obvious differences in banding patterns were found during exposure of N2A cells to DES for 2 days and 7 days, however (Fig. 1D, grey symbols). Taken together, the subtle reduction of global DNA methylation observed in the N2A cell line under exposure to DES could not be correlated with changes in the methylation pattern of CpG rich regions.

3.2. Effects of EDCs on adipocyte differentiation

Given the modest effects of EDCs on global methylation in neuroblastoma cell line, we consequently examined the effects of EDCs on global methylation in a differentiation model, as DNA methylation during differentiation is dynamic (Sakamoto et al., 2008) and potentially more sensitive to chemical insult. Therefore, we further studied the effects of EDCs on the differentiation of 3T3-L1 preadipocytes, a well established *in vitro* model. After reaching confluency, 3T3-L1 preadipocytes can be induced to differentiate using a cocktail of dexamethasone, insulin and 3-isobutyl-1-methylxanthine for 2 days and subsequently maintained in the presence of insulin for another 6 days (Fig. 2A). At the start of differentiation, cells were exposed to selected EDCs and differentiation was quantified by measuring the percentage of adipocytes by flow cytometry.

Differentiated adipocytes were identified based on two characteristics of lipid accumulation, i.e. Nile Red fluorescent lipid staining (Fig. 2B, middle panel) and surface granularity due to lipid droplets in the cytoplasm (Fig. 2B, right and left panel). As expected, the cocktail induced differentiation of 10.1% (±1.3) of all cells, compared to 1.7% (±0.1) in the undifferentiated state (Fig. 2C). As expected, exposure to the PPARγ agonist troglitazone (TRO) and the positive control TBT, led to an increase in adipocyte differentiation with average values of 47.5% (±0.9) for TRO and 21.8% (±0.8) for TBT (Fig. 2C). Interestingly, exposure to 2.5 μM BDE-47 (16.1% ±0.2), 10 μM PFOA (13.5% ±1.2) and 3.4 μM PCB-153 (12.8% ±0.9) led to a significant increase in differentiation whereas exposure to 0.1 nM TCDD (5.4% ±1.7) led to a significant decrease in differentiation. For 10 μM BPA (11.4% ±0.8), 1 μM HCB (9.5% ±0.9), 10 μM PFOS (11.7% ±0.3) and 10 μM HBCD (11.4% ±0.8) no significant changes in differentiation were found (Fig. 2C). When tested at a five times higher concentration in subsequent experiments, 50 μM BPA (15.3% ±1.3) significantly increased differentiation, while 5 μM HCB (8.1% ±.1%), 50 μM PFOA (12% ±2%) and 17 μM PCB-153 (11.2% ±0.3) did not result in increased differentiation (data not shown).

3.3. Effects of EDCs on global DNA methylation in adipocytes

In order to examine the effects of EDCs on global DNA methylation in adipose cells in more details, we performed subsequent experiments using two chemicals that showed enhanced adipocyte differentiation at five times higher concentrations (BPA, BDE-47), as well as one chemical with inhibitory effects on adipogenesis (TCDD) and one "negative" chemical (HCB). We exposed 3T3-L1 cells to three increasing concentrations of the test chemical. A dose-related increase in adipocyte differentiation was observed for BDE-47 and BPA (Fig. 3A). TCDD showed a decrease in adipocyte differentiation in all concentrations tested whereas HCB did not show significant changes in differentiation regardless the test concentration (Fig. 3A). When analyzing global DNA methylation with our HPLC approach, a modest but significant decrease in global DNA methylation

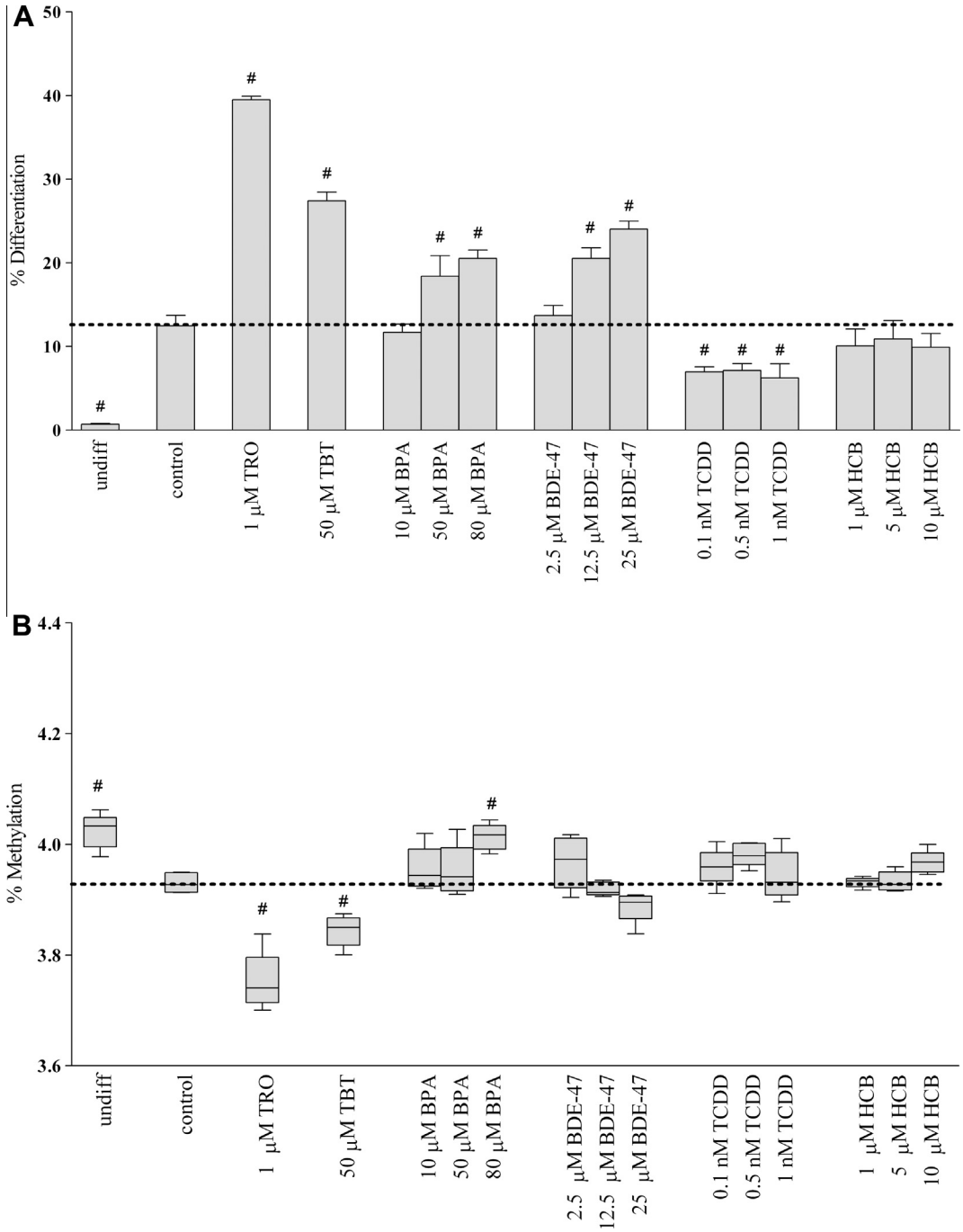

Fig. 3. Dose–response effects of EDC exposure on murine adipocyte differentiation and global methylation *in vitro*. 3T3-L1 cells were exposed to BPA, BDE-47, TCDD or HCB for 8 days of differentiation. (A) Ten thousand cells were stained and analyzed for differentiation with flow cytometry ($n = 3$). Data represent mean and standard deviation of %of Nile Red and SSC positive cells. Three replicates were tested per concentration. Data is representative of two independent experiments. (B) Global DNA methylation status at day 8 analyzed with HPLC. All concentrations were tested in five replicates. Bottom and top of the box represent 25th and 75th percentiles, line in the middle of the box represents median and the ends represent the minimum and the maximum of all the data. (C) Negative correlation ($r = 0.821$; $p < 0.05$) between adipocyte differentiation and global DNA methylation by combination of all exposure conditions. Each point represents the average status of DNA methylation and adipocyte differentiation for each concentration tested per chemical. Undiff: undifferentiated cells exposed to medium; control: cells containing DMSO vehicle (0.1%) induced to differentiation by addition of INS, DEX and IBMX to the medium; TBT: tributyltin; TRO: troglitazone;; BPA: bisphenol A; TCDD: 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin; HCB: hexachlorobenzene; BDE-47: 2,2′,4,4′-tetrabrominated diphenyl ether; #indicates significant difference from control ($p < 0.05$).

status was found in differentiated control cells (3.93% ±0.02) compared to non-induced undifferentiated cells (4.02% ±0.03) (Fig. 3B). A further decrease in global methylation status was found for cells treated with TRO (3.75% ±0.05) and TBT (3.84% ±0.03). Additional AP-PCR experiments with TRO and TBT, however, did not reveal any changes in methylation of CpG rich regions (data not shown). At the highest concentration tested, BPA led to a significant increase in global DNA methylation. BDE-47 showed a dose–response trend of reduced methylation. TCDD showed no significant effects on global DNA methylation status (Fig 3B). When all exposure conditions were compared, a significant negative relationship ($r = 0.821$; $p < 0.05$) was observed between global methylation status and adipocyte differentiation (Fig. 3C).

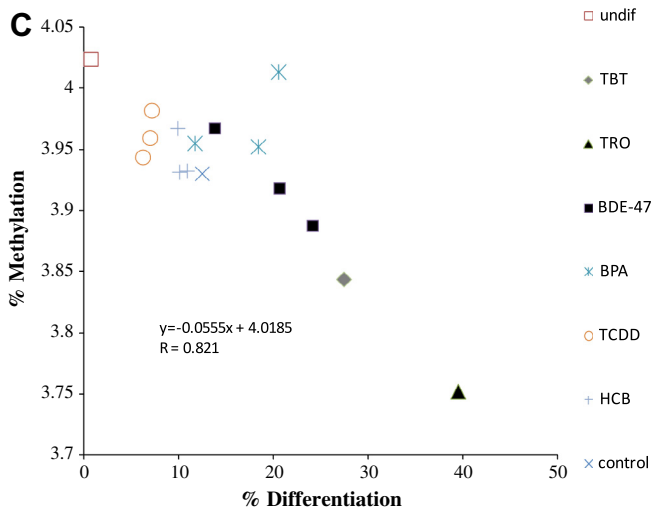

Fig. 3. (*continued*)

4. Discussion

Evidence is accumulating that early life exposure to EDCs may contribute to the development of obesity in later life. Altered epigenetic gene regulation has been proposed as a mechanism by which EDCs influence developmental programming, leading to latent onset of disease. The goal of this study was to examine the effects of selected EDCs on DNA methylation and adipocyte differentiation using *in vitro* models. The regulation of DNA methylation is vital for embryonic development and is deregulated in diseases such as cancer (Dahl and Guldberg, 2003). Exposure to chemicals during critical periods of development can induce heritable and persistent changes in DNA methylation and, therefore, in gene regulation, which may ultimately lead to adverse biological effects (Bombail et al., 2004).

Our first approach was to study effects of EDCs on DNA methylation in human and murine neuroblastoma cell lines. These cell lines were selected as models for the neural system, as the hypothalamic regulation of hunger and satiety are important functions that may be affected in obesity. In both cell lines, treatment with the DNMT-1 inhibiting agent 5-AC led to global DNA hypomethylation. Modest global hypomethylation was observed in the murine cell line following exposure to DES, BPA, TCDD, PCB-153, BDE-47 or HCB but not in the human cell line. We are the first to report an altered global methylation status in neuroblastoma cells as a consequence of EDCs exposure; however the importance of this finding remains to be elucidated. Subsequent studies with DES using AP-PCR did not reveal changes in global methylation of CpG islands, indicating that further examination of other gene loci undergoing methylation is needed. Our results also suggest that the effects of EDCs on global methylation status might be cell type specific. The lack of effects of EDCs observed in SK-N-AS cells may also be due to the hypermethylated nature of this cell line (Buckley et al., 2011).

To link the effects of EDCs on global methylation status to functional changes during development and differentiation, the 3T3-L1 adipocyte differentiation model was used. Our results demonstrate that EDCs can alter murine adipocyte differentiation *in vitro*, and that increased adipocyte differentiation correlates with global DNA demethylation. The process of differentiation of 3T3-L1 cells from preadipocytes to adipocytes has been reported earlier to be accompanied with genome-wide changes by temporal methylation/demethylation, depending on the differentiation stage (Sakamoto et al., 2008). Accordingly, genome-wide analysis of DNA methylation of human preadipocytes and mature adipocytes found hypomethylation occurring in 2701 genes and hypermethylation in 1070 genes after differentiation (Zhu et al., 2012), suggesting an overall reduction in methylation which supports our findings. It is important to investigate whether demethylation associated to exposure to chemicals during differentiation of 3T3-L1 cells occurs through a passive mechanism (e.g. impairment of remethylation by DNMTs) or an active mechanism (e.g. through influencing enzymatic modification of the methylated cytosines) (Bhutani et al., 2011).

We demonstrate here, for the first time, that a ubiquitous polybrominated diphenyl ether in the environment, BDE-47, increases adipocyte differentiation in a dose-related manner in 3T3-L1 cells. A recent *in vivo* study has demonstrated hypomethylation of DNA isolated from brain tissue in adult female mice prenatally exposed to BDE-47 (Woods et al., 2012). Importantly, animal studies have also indicated that *in vivo* exposure to BDE-47 results in effects on adipogenesis and adipocyte metabolism. A study by Hoppe and Carey, 2007 demonstrated *in vivo* features of metabolic obesity in 6-week old Sprague–Dawley male rats exposed to penta-BDE, a technical mixture of BDEs containing high BDE-47 content. Daily exposure of juvenile rats to 14 mg/kg BW penta-BDE for 4 weeks showed no effect on animal or adipocyte size but insulin metabolism of isolated adipocytes was clearly affected. Perinatal exposure of Wistar rats to low concentrations of BDE-47 (0.002 and 0.2 mg/kg body weight/day) resulted in increased body weight in offspring, as well as effects on glucose and insulin metabolism in males (Suvorov et al., 2009). In a subsequent study of global gene expression in liver DNA isolated from BDE-47 exposed offspring, the same authors demonstrated altered gene expression particularly in metabolic pathways (Suvorov and Takser, 2010). Interestingly, these results indicated that direct interaction with retinoic X receptor (RXR) and its upstream cascade may be involved in the metabolic effects of BDE-47. RXR and PPARγ are key regulatory transcription factors in the adipogenic pathway, and EDCs such as TBT have been shown to induce adipocyte differentiation by activating these receptors (Grun et al., 2006). Currently, a study is underway in our laboratory to elucidate the role of RXR and PPARγ and related genes as targets of BDE-47 (Kamstra et al., in preparation).

In addition to BDE-47, our study confirmed the adipogenic effects of BPA and TBT in 3T3-L1 cells, but also showed novel effects of these chemicals on global DNA methylation. Recently, BPA has been shown to induce adipogenesis in 3T3-L1 cells at concentrations as low as 100 nM (Chamorro-Garcia et al., 2012). BPA has been shown previously to increase adipogenesis *in vitro* by a mechanism involving the phosphatidylinositol 3-kinase, the insulin signaling pathway that plays a role in terminal adipocyte differentiation (Masuno et al., 2005), though a role for estrogen receptor activation has also been suggested (Casals-Casas and Desvergne, 2011). Interestingly, we found global DNA hypermethylation at the highest concentration tested. This is an intriguing finding as we found that in most cases, increased adipocyte differentiation was accompanied by hypomethylation. DNA hypermethylation by BPA may be related to increased DNMT activity, as perinatal exposure to BPA has been shown to result in hypermethylation of estrogen receptors promoter regions in testis from male rats concurrent with a 2-fold increase in de novo DNMT3a and DNMT3b expression at transcript and protein level (Doshi et al., 2011). In addition to BPA, the adipogenic effect of TBT has been reported earlier (Inadera and Shimomura, 2005; Grun et al., 2006). Interestingly, we showed in this study that global DNA hypomethylation was induced by TBT to a level similar to that of the pharmacological PPARγ agonist troglitazone, despite differences in adipocyte differentiation. TBT has been shown previously in adipose-derived stem cells to cause hypomethylation of the promoter region of the fatty acid binding protein 4, an important gene

in adipogenesis (Kirchner et al., 2010). In fish, TBT has been shown to induce hypomethylation in liver DNA which was associated with changes in levels of the substrate and products of methyltransferases (Wang et al., 2009).

Exposure to TCDD led to a decrease in 3T3-L1 differentiation, a finding in agreement with Phillips et al. (1995), who reported a decrease in number of fat cells after addition of 10 nM TCDD to the 3T3-L1 culture. Hanlon et al. (2003) also reported inhibited adipogenesis in 3T3-L1 cells during 10 nM TCDD exposure, suggesting a mechanism of suppression of PPARγ1 through an aryl hydrocarbon receptor (AhR) dependent process. Interestingly, we found no changes in global methylation status despite the pronounced inhibition of adipocyte differentiation. This is a rather surprising finding, given that a recent study demonstrates that TCDD exposure causes stable and heritable changes in sperm DNA methylation in male rats (Manikkam et al., 2012). It is possible that the methylation changes induced by TCDD are tissue specific, or limited to a selected number of genes that cannot be detected by the global HPLC method.

A number of chemicals tested in this study showed either non dose-dependent (PFOA, PCB-153) effects or no effects on 3T3-L1 differentiation (HCB, HBCD, PFOS). These results are not consistent with the reports in the literature of the potential obesogenic effects of some of these chemicals. For example, epidemiological studies have shown that prenatal exposure to HCB and PCB-153 is associated with higher BMI in children (Smink et al., 2008; Verhulst et al., 2009). Both PFOA and PFOS are partial agonists of PPARγ when tested at a concentration range of 1–200 μM (van den Heuvel et al., 2006), and *in utero* exposure to PFOA has shown increased body weight in adult CD-1 mice (Hines et al., 2009). Recent studies in 3T3-L1 cells, however, show no effects of PFOA on adipocyte differentiation to concentrations up to 75 μM (Taxvig et al., 2012). To our knowledge, nothing has been reported on the obesogenic effects of HBCD. We cannot exclude that the concentrations used in this study were too high or the inappropriate exposure scenario was used to induce adipogenic effects. Therefore, testing the chemicals at a range of concentrations which also includes the ones relevant for human exposure levels is recommended. It is also possible that stimulation of adipocyte differentiation is not the mechanism underlying potential obesogenic effects of these chemicals.

In conclusion, a number of EDCs, including BDE-47, TBT and BPA, were found to enhance adipogenesis and TBT and BPA to alter global DNA methylation in 3T3-L1, an *in vitro* murine preadipocyte model, shedding light on a mechanism by which EDCs may contribute to the developmental programming of obesity. A trend indicating that global hypomethylation accompanies murine adipocyte differentiation was observed. This study demonstrates that the 3T3-L1 cells may be a suitable model to screen EDCs for effects on adipogenesis and global DNA methylation, though effects on a human *in vitro* model should also be studied. For a better understanding of the role of chemical alterations in DNA methylation in the development of obesity and related disorders, further studies should focus on specific loci methylation. In addition, knowledge on epigenetics is accumulating in a fast pace, and the complexity and dynamics of this field is becoming increasingly relevant. Potential effects of chemicals on epigenetic gene regulation may go far beyond interference with DNA methylation, indicating that future studies should consider alternative epigenetic mechanisms such as histone modifications, chromatin remodeling and non-coding RNAs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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