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International Journal of Toxicology 2009 28: 294

DOI: 10.1177/1091581809337918

International
Journal of Toxicology
Volume 28 Number 4
July/August 2009 294-307

10.1177/1091581809337918
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Effects of Mixtures of Polychlorinated Biphenyls, Methylmercury, and Organochlorine Pesticides on Hepatic DNA Methylation in Prepubertal Female Sprague-Dawley Rats

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DNA methylation is one of the epigenetic mechanisms that regulates gene expression, chromosome structure, and stability. Our objective was to determine whether the DNA methylation system could be a target following in utero and postnatal exposure to human blood contaminants. Pregnant rats were dosed daily from gestation day 1 until postnatal day 21 with 2 dose levels of either organochlorine pesticides (OCP; 0.019 or 1.9 mg/kg/day), methylmercury chloride (MeHg; 0.02 or 2 mg/kg/day), polychlorinated biphenyls (PCBs; 0.011 or 1.1 mg/kg/day), or a mixture (Mix; 0.05, or 5 mg/kg/day) including all 3 groups of chemicals. Livers from 1 female offspring per litter were collected at postnatal day 29. Hepatic analysis revealed that the mRNA abundance for DNA methyltransferase (DNMT)-1, -3a, and -3b were significantly reduced by the high dose of PCB, that the high dose of MeHg also reduced mRNA levels for DNMT-1, and -3b, but that OCP had no significant effects compared with control. The high dose of PCB and Mix reduced the abundance of the universal methyl donor S-adenosylmethionine, and Mix also reduced global genome DNA methylation (5-methyl-deoxycytidine/5-methyl-deoxycytidine + deoxycytidine). The latter is consistent with pyrosequencing methylation analysis, revealing that the high-dose groups (except OCP) generally decreased the methylation of CpG sites (position -63 to -29) in the promoter of the tumor suppressor gene $p16^{INK4a}$. Overall, these hepatic results suggest that the DNA methylation system can be affected by exposure to high doses of blood contaminants, and that OCP is the least potent chemical group from the investigated mixtures.

Keywords: DNA methylation; rat; mixture; methylmercury; polychlorinated biphenyls; organochlorine pesticides

DNA methylation is a normal epigenetic mechanism involved in controlling DNA structure, chromosome stability, mobility of "parasitic" elements (transposons, retrotransposons, viral elements), gene imprinting, and gene expression. Abnormal DNA methylation is associated with a number of cancers, infertility, and developmental, neurological, immunological, and age-related disorders.[1] There is growing evidence that suggests that early exposure to environmental agents might lead to long-term and transgenerational effects through epigenetic mechanisms involving DNA methylation.[2-4] Global genome methylation, site-specific CpG dinucleotide methylation, and different elements of the DNA methylation system have all been shown to be affected by drugs,[5-7] radiation,[8] environmental estrogens and contaminants,[9-12] disinfection by-products,[13] and metals such as arsenic,[14,15] chromium,[16,17] nickel,[18,19] and cadmium.[20] Abnormal DNA methylation, observed in a number of diseases and cancers,[21,22] may originate from dietary insufficiency (choline, methionine, vitamin B-12, and folic acid) or disorders of the 1-carbon metabolism pathway leading to a reduced availability of the

universal methyl donor, S-adenosyl-L-methionine (SAM).[23] The DNA methylation machinery includes numerous methyl-binding proteins, DNA demethylases,[24] and families of DNA methyltransferases (DNMT-1, -2, -3) with multiple variants.[25] The DNMTs catalyze the transfer of a methyl group from SAM mostly to cytosine located next to a guanine (5′-CpG-3′).[2] During mitosis, the methylation pattern of the original DNA strand is copied mostly by the constitutively expressed DNA methyltransferase-1 (DNMT1) onto the replicating DNA. The *de novo* methyltransferases DNMT3a and 3b catalyze the transfer of a methyl group to previously unmethylated DNA during gameto- and embryogenesis. However, DNMTs often work cooperatively, and the normally low expression of DNMT3b in somatic cells is increased in cancer to cooperate with DNMT1 in silencing tumor suppressor genes.[26] An understanding of the effects of environmental contaminants on the DNA methylation machinery and its biological consequences should open avenues for better differentiating transient effects from adaptive and persistent effects, and for the development of diagnostic tools and screening methods.

Polychlorinated biphenyls (PCBs) and some organochlorine pesticides (OCPs) are legacy chemicals that were banned in the 1970s, and their levels in the environment are declining. Exposure to these chemicals is still of concern to health agencies given that they are still present in human tissues and in the human diet, particularly that of circumpolar populations who consume traditional food. Breast milk and cord blood levels of PCBs, *p,p′*-dichlorodiphenyldichloroethene (DDE), and other organochlorines are higher in the Inuit of North-Eastern Canada than many other Canadian and non-Canadian populations.[27,28] Recent papers reviewed possible associations between exposure to these chemicals and cancer,[29,30] infertility,[31] developmental anomalies,[32] endocrine disruption,[33,34] immunological function,[35] and neurological impairments.[36,37] Methylmercury (MeHg) is another contaminant of concern in Canada, given that it is substantially higher in the Canadian Arctic marine biota compared to other circumpolar countries,[38] and that fish contaminated with MeHg are components of the Inuit diet. Methylmercury accumulates in the fetus in utero, but in contrast to organochlorines, the levels of MeHg in breast milk are low.[39,40] The fetus is more sensitive than the adult to MeHg, as demonstrated by the fact that high levels of exposure result in severe neurological impairments in babies born from asymptomatic mothers,[41] whereas at low levels of exposure, subtle cognitive and language delays are observed in older children.[42,43]

There is little information about the effects on human health of chronic exposure to mixtures of contaminants during the fetal and postnatal periods. The pre- and postnatal phases are critical developmental stages because tissue differentiation, organ growth, the detoxification system, and blood barriers have not yet matured, and abnormal events during these periods can lead to long-term adverse health effects. We have shown that postnatal exposure to a high dose of a reconstituted mixture of arylhydrocarbon receptor (AhR) agonists (non-ortho PCB, polychlorinated-dibenzodioxins [PCDD], and dibenzofurans [PCDF]), prepared based on their proportion found in breast milk, decreased the mRNA expression of DNMT1 in the liver and the brain of female pups at postnatal day (PND) 21.[44] Bowers et al developed a mixture of PCBs, OCPs, and MeHg based on the chemical profiles in Canadian Arctic Inuit maternal blood, and they demonstrated that exposure of rats to this mixture during gestation and lactation produces a blood chemical profile comparable to that of humans in rodent dams and the pups.[45] When this PCB-OCP-MeHg mixture was tested at a dose that produces a serum level in the rat pups 100-fold higher than human levels, Chu et al reported a decrease in the growth rate of rat pups, clinical, biochemical, and histopathological effects.[46] Subsequent work with this mixture is currently studying adverse effects that could be attributed to the PCB, the OCP, or the MeHg component, and investigating developmental, systemic, neurotoxic, and genomic effects (only the latter has been published at this stage).[47] As part of this initiative, the current manuscript reports effects of

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this mixture and its chemical family components (PCB, OCP, MeHg) on elements of the DNA methylation system in the liver, including the abundance of SAM and DNMT mRNAs and global genome and gene-specific changes in DNA methylation. Gene-specific methylation changes will be addressed by bisulfite pyrosequencing analysis of 5 CpG sites in the promoter of the gene cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) that express the tumor suppressor protein $p16^{INK4a}$. This gene is a known DNMT methylation target for which the expression is reported to be regulated by DNA methylation during carcinogenesis and following exposure to various environmental agents.[14,17,20,48,49] Finally, the sensitivity of these end points as an indicator of exposure to xenobiotics will be compared with the changes in the abundance of cytochrome P450 1A1 (CYP1A1) mRNA. A phase I metabolism enzyme, CYP1A1 is a sensitive indicator of exposure to AhR agonists. Its expression is regulated through the classical AhR-dioxin response element pathway.[50]

Methods

Animal Treatment

Animal treatment and housing conditions were approved by the Animal Care Committee of Health Canada and conformed to the Guidelines of the Canadian Council on Animal Care. Housing, handling and breeding conditions, origin of test chemicals (analytical grade, 99% pure), and methods for the preparation and validation of the mixture were all as previously described.[46] Female Sprague Dawley rats (200-230 g) were mated with males (320-350 g) purchased from Charles River Laboratories (St. Constant, Québec). The day of detection of a vaginal plug was denoted as gestation day 0 (GD 0). All pregnant dams were dosed daily from GD 1 to postnatal day (PND) 21 by providing them with a Teddy Graham cookie (Nabisco Ltd., Toronto, ON) laced with a measured dose (adjusted for body weight every day) of the mixture dissolved in corn oil. The mixtures are described in Table 1. Pregnant dams were exposed to either corn oil as control (CTRL), a low dose (L) or high dose (H) of OCP (0.019 or 1.9 mg/kg/day), methylmercury chloride (MeHg, 0.02 or 2 mg/kg/day), PCB (0.011 or 1.1 mg/kg/day), or a complete mixture including all these chemicals (Mix: 0.05, or 5 mg/kg/day). The dams were monitored for parturition beginning on GD 18, and litters were culled to 4 males and 4 females on PND4. One female pup from each litter was sacrificed by decapitation at PND29, providing the following number of investigated pups per treatment group: 11 CTRL, 10 OCP-L, 10 OCP-H, 10 PCB-L, 11 PCB-H, 9 MeHg-L, 10 MeHg-H, 11 Mix-L, and 9 Mix-H. At PND99, liver samples were also collected from older siblings treated with the high doses (11 CTRL, 10 OCP-H, 10 PCB-H, 10 MeHg-H, and 9 Mix-H). Male pups were not investigated, given that PCBs and AhR-agonists are classical hepatocarcinogens mostly in females.[51]

Table 1. Chemical Composition of the High-dose Contaminant Mixture[a]

Contaminants	Dose (mg/kg)	% of Mixture
1. Organochlorine pesticides		
Aldrin	0.0049	0.098
β-Hexachlorocyclohexane	0.0746	1.49
Dieldrin	0.0223	0.45
Hexachlorobenzene	0.2961	5.92
Heptachlor epoxide	0.0232	0.46
Mirex	0.0291	0.58
Toxaphene	0.0699	1.40
Oxychlordane	0.1359	2.72
trans-Nonachlor	0.2203	4.40
cis-Nonachlor	0.0525	1.05
p,p′-DDE	0.9187	18.37
p,p′-DDT	0.0569	1.38
Total	*1.9*	*38.1*
2. Di-*ortho* PCBs[b]		
52 2,2′,5,5′-TetraCB	0.0154	0.31
99 2,2′,4,4′,5-PeCB	0.0973	1.95
101 2,2′,4,5,5′-PeCB	0.0145	0.29
128 2,2′,3,3′,4,4′-HxCB	0.0071	0.14
138 2,2′,3,4,4′,5-HxCB	0.2146	4.29
153 2,2′,4,4′,5,5′-HxCB	0.3177	6.35
170 2,2′,3,3′4,4′,5-HpCB	0.0562	1.12
180 2,2′,3,4,4′,5,5′-HpCB	0.1522	3.04
183 2,2′,3,4,4′,5,6-HpCB	0.0193	0.39
187 2,2′,3,4′,5,5′,6-HpCB	0.0795	1.59
Total	*0.97*	*19.5*
3. Mono-*ortho* PCBs		
28 2,4,4′-TriCB	0.0072	0.14
105 2,3,3′4,4′-PeCB	0.0165	0.33
118 2,3,4,4′,5-PeCB	0.0727	1.45
156 2,3,3′4,4′,5-HxCB	0.029	0.58
Total	*0.13*	*2.5*
4. Methyl mercury chloride	1.997	39.9
Total Mix	5.0001	100

[a] The polychlorinated biphenyl treatment included the di-ortho and mono-ortho substituted chemicals, for a total of 14 congeners and a combined dose of 1.1 mg/kg.
[b] Polychlorinated biphenyl numbering system according to Ballschmiter and Zell.[82]

Measurement of SAM

S-Adenosyl methionine is a universal methyl donor involved in various methylation reactions, including DNA methylation. The concentration of SAM was quantified from liver samples using a commercially available fluorescence assay kit (Mediomics LLC, St. Louis, MO, USA). Samples (0.5 to 1 g) were homogenized in 2 volumes (v/g) of 50 mM NH_4HCO_3 and clarified by centrifugation (11 750 $\times$ *g*, 30 min, 4°C). The supernatant was diluted with cold absolute (100%) ethanol to a final concentration of 70% and maintained in an ice bath for 30 minutes prior to being clarified by centrifugation (11 750 $\times$ *g*, 30 min, 4°C). The supernatant was lyophilized and resuspended in cold buffer S (20 mM Hepes, pH 8.0, 100 mM NaCl and 0.5 mM 2-mercaptoethanol) prior to a final clarification by centrifugation (11 750 $\times$ *g*, 15 min, 4°C). S-Adenosyl methionine concentrations were measured in this supernatant at 3 dilution levels using a fluorescence microplate reader (excitation 485 nm, emission 665 nm) and a 9-point standard curve (0-50 µM SAM), as described by the manufacturer's protocol (Mediomics LLC, St. Louis, MO, USA). All samples were measured in a single assay. The intra-assay coefficient of variation calculated over the range of the standards was 10.4%.

Measurement of 5-Methyl-deoxycytidine as an Indicator of Global Genome DNA Methylation

5-Methyl-deoxycytidine (5mdC) is measured as an indicator of the level of DNA methylation in the genome. It is expressed as a percentage ratio of 5mdC/(5mdC + dC), in which the measurement of dC also provides a correction for the amount of DNA in the sample.

Total DNA was prepared from liver samples using the Qiagen DNAeasy kit (Qiagen, Mississauga, Ontario, Canada). The concentration of purified DNA was measured for each sample using a spectrophotometer at 260 nm. For each sample, 10 µg DNA (usually in 50 µL) was first denatured for 3 minutes in a 100°C water bath and then chilled immediately in ice water. After adding a 1/10 volume of ammonium acetate buffer (0.1 M, pH 5.3) containing 20 units of nuclease P1 (Sigma Chemical Co., Canada), the mixture was incubated at 45°C for 2 hours in an autoshaker (Thermomixer-R, Eppendorf AG). A 1/5 volume of ammonium bicarbonate buffer (0.5 M, pH 8.0) including 0.02 unit of snake venom phosphodiesterase I (Sigma Chemical Co.) was added and incubated at 37°C for 2 hours. Then, 5 units of shrimp alkaline phosphatase (1.23 units/µL glycerol solution from Sigma Chemical Co.) was added, and the mixture was incubated at 37°C for 1 hour. Finally, the mixture was incubated at 65°C to inactivate the shrimp alkaline phosphatase, then the samples were spun at 16 000 $\times$ *g* for 10 minutes, and the supernatant was stored at -20°C until analysis by liquid chromatography–ultraviolet electrospray ionization–mass spectrometry (LC-UV/ESI-MS). To control for DNA hydrolysis and analytical variability, aliquots of pooled rat genomic DNA were prepared and included in each batch of samples.

Ten nucleotides (cytosine [C], deoxycytidine [dC], 5-methyl-cytosine [5mC], 5-methyl-deoxycytidine [5mdC], uracil [U], adenine [A], deoxyadenosine [dA], guanine [G], deoxyguanosine [dG], and thymine [T]) were quantified using a LC-UV/ESI-MS Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA), with a Waters Atlantis dC_{18} column (3 µm, 150 $\times$ 2.1 mm; Waters, Milford, MA, USA) at 30°C. Liquid chromatography separation was performed at a flow rate of 220 µL/min using a solution of 0.1% (v/v) formic acid and a linear gradient of methanol increasing from 2% to 29% at a rate of 1.5% per minute. Methanol was further increased to 80% in 2 minutes and maintained for 11 minutes. The injection volume was 10 µL. Optimal ESI was conducted using nitrogen as the nebulizer (45 psi, 8 L/min) and drying gas (350°C). The capillary voltage of MS was maintained at + 3000 V, and the sampling orifice was set for the positive mode. The MS was operating in both scan and SIM mode, and spectra were acquired over the range of 100 to 650 Da. The diode array detector wavelength was set at 280 nm. Quantification was accomplished by analyzing the UV readings and confirmed by MS scan using single ion monitoring for pairs of ions: m/z 244.1/112.2, 228.2/112.2, 258.3/126.1, 242.1/126.3, 245.1/112.9, 268.1/136.1, 252.3/135.9, 284.1/152.3, 268.1/152.3, and 243.3/127.2 for C, dC, 5-mC, 5-mdC, U, A, dA, G, dG, and T, respectively.

Real-time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated using RNeasy mini-kits and the RNase-free DNase set (Qiagen). The Qiagen

Omniscript Reverse Transcriptase kit was used for reverse transcription (RT). For negative control samples to test for residual DNA contamination, RT enzyme was omitted. The resulting cDNA was amplified by quantitative real-time polymerase chain reaction (PCR) analysis using QuantiTect SYBR Green PCR Master Mix (BioRad, Mississauga, ON, Canada), RT products, and primers (forward and reverse). The temperature cycles were 15 minutes at 95°C, then 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C. The PCR primers (forward and reverse), designed using Beacon Designer 2.0 (BioRad) were: 5′-CAA TGA GCT GCG TGT GGC-3′ and 5′-GGG TCA TCT TTT CAC GGT TGG-3′ for β-*actin* (NM031144), 5′-AAC GGA ACA CTC TCT CTC ACT CA-3′ and 5′-TCA CTG TCC GAC TTG CTC CTC-3′ for *dnmt-1* (AF116344), 5′-ATA GAA GTC AAG CGG GAA GTG-3′ and 5′-GGA GGC TGA GGT ATT GAT TGG-3′ for *dnmt-3a* (XM220319), 5′-CGT GGT AGG AGA TGG AGA TGG-3′ and 5′-TGG AGA TAC TGT TGC TGT TTC G-3′ for *dnmt-3b* (BN000397), 5′-CTT CAC ACT TAT CGC TAA TGG-3′ and 5′-TTG GGT CTG AGG CTA TGG-3′ for *Cyp1A1* (NM 012540), and 5′-CTT CAC CAA ACG CCC CGA ACA C-3′ and 5′-CGG GAG AGG GTG GTG GGG TC-3′ for $p16^{ink4a}$ (NM031550). The results were normalized for the abundance of β-*actin* mRNA.

CpG Methylation Analyses of the Gene *Cdkn2a* by Pyrosequencing

Total DNA was extracted from liver samples using the DNAeasy kit (Qiagen, Valencia, CA), and the samples were shipped to an independent laboratory for blind analysis and further processing of the samples (EpigenDx, Inc., Worcester, MA, USA). The nucleotide sequence 1369-1427 (accession number AB081658) of the rat gene cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) was investigated. This sequence included 7 CpG dinucleotides located upstream of the start codon (A in ATG is position 0). The pyrogram was disrupted at position -16 likely because of a point mutation A→G, which prevented the quantification of the percentage methylation of the 2 CpG dinucleotides closest to the start codon (positions -14 and -9). Therefore, the methylation analysis focused on the CpG dinucleotides located at positions -63, -55, -40, -29, and -23.

Sodium bisulfite conversion was performed using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). The bisulfite-treated DNA (1 μL, approximately 100 ng) was amplified by PCR reaction (95°C 15 min; 45 × [95°C, 30 s; 59°C, 30s; 72°C, 30 s]; 72°C, 5 min, 4°C) in 30 μL buffer including 3 mM $MgCl_2$, 200 μM of each dNTP, 6 pM of forward (5′-GGAAGGAGGGTTTTATTGGTT-3′) and reverse (5′-biotin-CTACAAAAAACTCCATACTACTCC-3′) primers, and 0.75 U of HotStar Taq Polymerase. The PCR product, with 1 strand 5′ end biotin labeled, was captured on streptavidin-coated beads (GE Health Care Life Sciences, Baie d'Urfé, Québec, Canada). The biotinylated strand of DNA was separated and purified by alkali treatment and subjected to pyrosequencing using the sequencing primer, 5′-GGGTTTTATTGGTTATATTA -3′. The pyrosequencing analysis was performed using the PyroMarkMD system (Biotage, Uppsala, Sweden), and the degree of methylation was analyzed as an artificial "C/T SNP" using the AQ mode. The percentage methylated C was calculated as: C peak height × 100 / (C peak height + T peak height). As quality control, the completeness of the sodium bisulfite reaction was confirmed by the analysis of the conversion of Cs→Ts at sites that were not part of CpG dinucleotides. The absence of PCR bias (ie, disproportional amplification of 1 allele over another) and the capability for an absolute quantification of methylation was verified by testing the linearity of stepwise increments in methylation from samples prepared by mixing unmethylated and artificially methylated DNA. The 0% methylated DNA was derived from normal DNA extracted from rat blood. The 100% methylated DNA was artificially fully methylated in vitro using the SssI enzyme system according to the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA). These 2 DNA controls were mixed at an increasing proportion of methylated DNA (0%, 2.5%, 5%, 10%, 20%) before bisulfite modification. The pyrosequencing analysis revealed a strong linear correlation (r^2 = 0.999) between the expected and measured methylation levels.

Statistical Analysis

The normality of the data was verified by goodness-of-fit Shapiro test. Data failing normality were log transformed. If normality was reached, the data were analyzed by analysis of variance (ANOVA) followed

by control to treatment means comparison using the Tukey-Kramer HSD test for comparison of all pairs. If the log-transformed data failed the normality test, the data were analyzed using the nonparametric Wilcoxon test when 2 groups were compared, and the Kruskal-Wallis rank sum test when differences among more than 2 groups were tested.

Three assays were performed to measure the mRNA abundance for DNMT1, 3a, 3b, and CYP1A1 at PND29 (Figure 3). The analysis of all control rats was repeated in each assay, explaining the presence of 3 control bars in Figure 3. Assay 1 included all samples from the control and the PCB-H groups, Assay 2 included controls and the other high-dose groups (OCP-H, MeHg-H, Mix-H), and Assay 3 included controls with all low-dose groups. Levels of p16 mRNA were analyzed in 2 assays, 1 for the low doses and a second assay for the high-dose groups. Levels of CYP1A1 mRNA at PND99 were analyzed in a single assay. In the text, comparisons to the control group refer to the control of the respective assay. The data for DNMT3a and $p16^{INK4a}$ were normally distributed, and comparisons among treatment groups (excluding the control) were analyzed using a 2-way ANOVA, testing for effects of treatment (OCP, PCB, MeHg, Mix), dose (low, high), and their interactions. This step was possible given that all the data are expressed as a percentage relative to the mean of the respective control group, and the variance of the control groups obtained in the different assays were not significantly different (O'Brien, Brown-Forsyth, and Levene tests). Further mean comparisons for these 2 end points were performed as described in the previous paragraph for the other data sets.

Regressions between 5mdC and dC were analyzed using the simultaneous linear regression models of SAS, version 9.1 TS Level 1M3 XP_PRO Platform (SAS Institute, Inc., Cary, NC, 2002), whereas other analyses were performed using the software JMP, version 3 (SAS Institute Inc., Cary, NC, 1998), all considering $P \leq .05$ as the statistically significant level.

Results

The hepatic concentrations of the universal methyl donor SAM were significantly reduced in the high-dose PCB and Mix groups compared with control (Table 2). To assess a possible effect of a reduced availability of SAM on global genome DNA methylation, an LC-UV/ESI-MS method was developed to quantify nucleosides and to calculate the percentage ratio of 5mdC ($100 \times 5mdC/5mdC + dC$) as an indicator of global genome DNA methylation. This LC-UV/ESI-MS method separates and quantifies 10 nucleosides (Figure 1) with confirmed identity using distinct molecular ions. The limit of detection was less than 0.02 μg. Table 3 shows that $3.49\% \pm 0.06\%$ of the deoxycytidine is methylated in the DNA of the control group. This percentage of methylation is lower in the group treated with a low dose of PCB ($P = .02$) than in the control, whereas the high dose of mixture induced a tendency for reducing global genome DNA methylation ($P = .053$). It is only in the high-dose Mix group that a statistically significant linear relationship could be obtained between the availability of SAM and global genome DNA methylation ($r^2 = 0.5$, $P = .03$). As expected, given the large number of deoxycytidine in the genome, the differences in global genome DNA methylation (Table 3) are small among treatment groups, therefore the

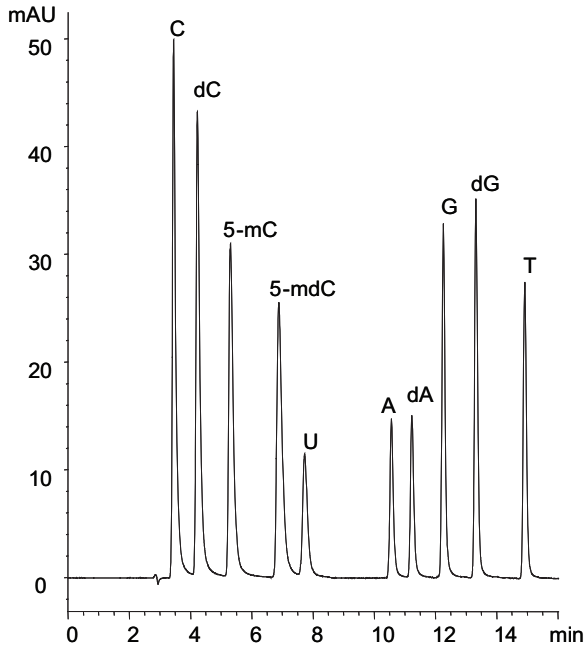

Figure 1. Chromatogram of the DNA nucleosides separated by LC-UV, showing different elution time for cytosine (C), 2'-deoxyribo-cytosine (dC), 5-methyl-cytosine (5-mC), 5-mdC; uracyl (U), adenosine (A), 2'-deoxyribo-adenosine (dA), guanosine (G), 2'-deoxyribo-G (dG), and thymidine (T). Electrospray ionisation-mass spectrometry electropherogram was used to confirm the peak identity using pairs of specific molecular ions.

Table 2. Abundance of Hepatic SAM (nM/g, mean ± SE) Following In Utero and Lactational Exposure to 2 Dose Levels of Either the OCP, PCBs, MeHg, or the Mix Including All These Chemicals

Control	Dose[a]	OCP	PCB	MeHg	Mix
7.37 ± 0.36	Low	7.55 ± 0.44	7.22 ± 0.33	7.24 ± 0.39	7.08 ± 0.22
	High	7.42 ± 0.21	5.79 ± 0.43*	6.84 ± 0.53	6.19 ± 0.23*

Abbreviations: MeHg, methylmercury chloride; Mix, all chemicals; OCP, organochlorine pesticides; PCBs, polychlorinated biphenyls; SAM, S-adenosylmethionine.

* Significantly different from control ($P < .05$). One rat of the high-dose PCB group (10.23 nM/g) was removed from the data set as an outlier that was more than 3 standard deviations ($3 \times SD = 4.05$) away from the mean. A 2-way analysis of variance, excluding the control group, indicated effects of treatment ($P = .03$) and dose ($P = .006$). Further comparisons revealed that SAM was less abundant in the PCB and Mix groups than in the OCP group.

[a] OCP: 0.019 or 1.9 mg/kg/day; PCBs: 0.011 or 1.1 mg/kg/day; MeHg: 0.02 or 2 mg/kg/day; Mix: 0.05, or 5 mg/kg/day (the addition of all chemicals).

Table 3. Treatment Effects on Global DNA Methylation Indicated by Differences in the Percentage Ratio of 5mdC/(5mdC + dC) Measured by Liquid Chromatography–Ultraviolet Electrospray Ionization–Mass Spectrometry (mean ± SE)

Control	Dose[a]	OCP	PCB	MeHg	Mix
3.49 ± 0.06	Low	3.29 ± 0.07	3.25 ± 0.06[b]	3.32 ± 0.05	3.37 ± 0.08
	High	3.56 ± 0.57	3.35 ± 0.09	3.52 ± 0.13	3.26 ± 0.09[c]

Abbreviations: MeHg, methylmercury chloride; Mix, all chemicals; OCP, organochlorine pesticides; PCBs, polychlorinated biphenyls.

[a] OCP: 0.019 or 1.9 mg/kg/day; PCBs: 0.011 or 1.1 mg/kg/day; MeHg: 0.02 or 2 mg/kg/day; Mix: 0.05, or 5 mg/kg/day (the addition of all chemicals).

[b] Significantly different from control ($P = .02$). This low-dose effect may not be robust given that it is not supported by any other observations.

[c] Tendency for a difference with control ($P = .053$).

relationship between dC and 5mdC was further investigated by a simultaneous linear regression analysis (Figure 2). Briefly, simultaneous linear regression models were fitted ($5mdC = a + b \times dC$) to the full data set for each treatment by dose combination. The overall F test was significant ($F = 2.85$, $P < .008$), indicating that the slope for at least 1 of the treatment dose combinations was different. When the Mix-H group was removed from the data and the simultaneous regression model re-run, there was no statistical difference between the slopes of the remaining treatment by dose combination groups ($F = 0.58$, $P = .77$). Therefore, only the group Mix-H had a statistically significantly different slope compared to the other groups (Figure 2), which supports abnormal DNA methylation in that group. The data for the Mix-H group are located more to the upper right area of the graph only because more genomic DNA was analyzed in some samples of this group. The quantity of total nucleotides ($5mdC + dC + dA + dT + dG$) measured in the 4 rats to the right of the graph range from 11.47 to 11.60 μg, whereas the average for all samples was 8.57 ± 1.63 μg (mean ± SD). The amount of genomic DNA analyzed has no impact on the slope.

Figure 3 summarizes the treatment effects on DNMTs, CYP1A1, and $p16^{INK4a}$ mRNA abundance. Only the high-dose PCB and MeHg treatments significantly reduced the DNMT1 mRNA abundance below the control level (statistically significant comparisons to the control group are indicated by asterisks). Additional analysis revealed that DNMT1 mRNA abundance was significantly less in the Mix than in the OCP groups, and a significant difference between the low-dose and high-dose PCB treatments. Only the high-dose PCB treatment significantly reduced the DNMT3a mRNA abundance below the control level. In addition, DNMT3a mRNA was significantly less abundant in the Mix than in the OCP group (treatment group effects are indicated by capital letters). Abundance of DNMT3b mRNA was significantly reduced by all high-dose treatments compared with low-dose treatments (brackets), but only the high-dose PCB and high-

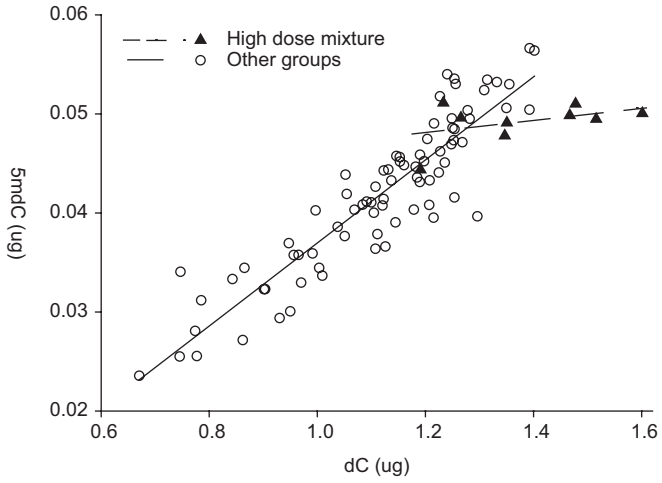

Figure 2. Regression lines between the amount of 5-methyldeoxycytidine (5mdC) and deoxycytidine (dC) measured in each liver DNA sample. Simultaneous linear regression models fitted ($5mdC = a + b \times dC$) to the full data set indicated that the slope for at least 1 of the treatment dose combinations was different ($P < .008$). When the Mix-H group was removed from the data and the simultaneous regression model re-run, there was no statistical difference between the slopes of the remaining treatment-by-dose combination groups, which demonstrates that the high-dose mixture group had a statistically significantly different slope compared to the other groups.

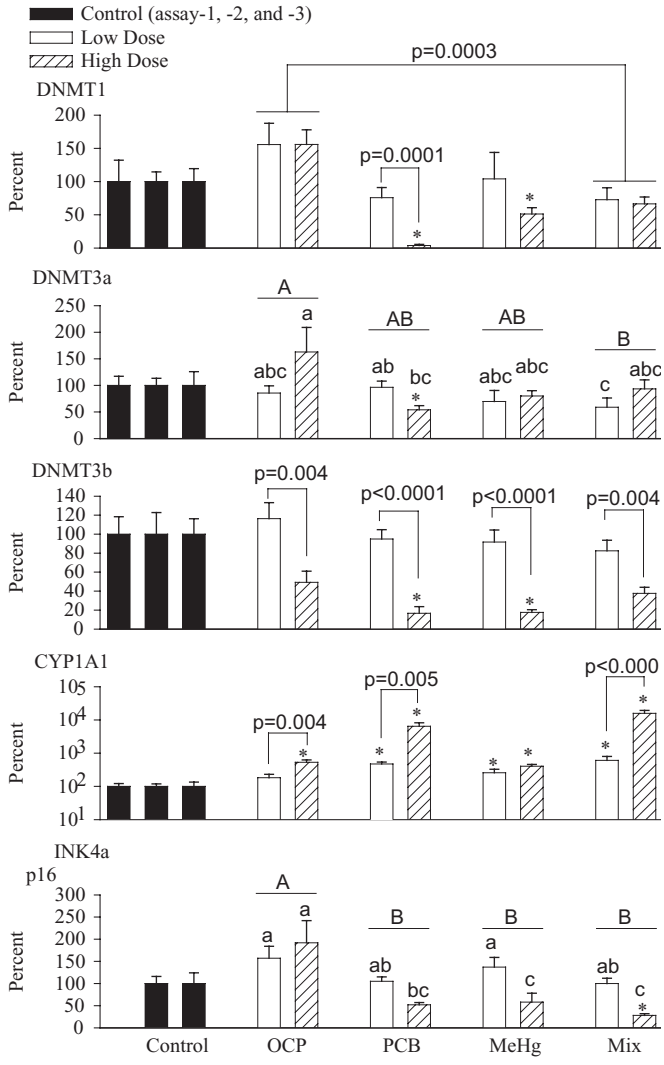

Figure 3. Changes in the abundance of DNMT-1, -3a, -3b, CYP1A1, and $p16^{INK4a}$ mRNAs following *in utero* and lactational exposure to either the complex mixture (Mix), or to its components including organochlorine pesticides (OCP), polychlorinated biphenyls (PCB), and methylmercury chloride (MeHg), at two dose levels. Mean ± SE. *significantly different from control ($P < 0.05$). Hooked-lines describe specific comparisons with the associated p value. Normally distributed data are identified with letters. Capital letters are describing the treatment effects from two way-ANOVAs, whereas small letters identify mean differences revealed by the all pair comparisons Tukey-Kramer HSD test. The letters "a" or "A" are associated with the largest values, and means not sharing a letter are significantly different ($P < 0.05$; for example A is different from B but is not different from AB). Note the log scale for CYP1A1.

dose MeHg treatments reduced mRNA abundance below the control level (asterisks). Over all the DNMT data, only the high-dose PCB and MeHg treatments changed the mRNA abundance relative with controls (asterisks): PCB affected all 3 DNMTs, decreasing hepatic mRNA abundance for DNMT1, 3A, and 3B to 4%, 54%, and 17% of the control values, respectively, whereas MeHg reduced DNMT1 and DNMT3B mRNA levels to 51%, and 18%, of the control value, respectively. Levels of mRNA in the OCP and the Mix groups were not significantly different from control.

Except for the low-dose OCP treatment group, CYP1A1 mRNA was significantly more abundant in all treatment groups compared with control (Figure 3, see asterisks). In all high-dose groups, mRNA was significantly more abundant than in the low-dose groups, except for the MeHg groups, where the abundance in the low-dose and high-dose groups was not significantly different. A synergic effect is apparent in the Mix high-dose group (5-, 64-, 4-, and 158-fold increase above control in the OCP, PCB, MeHg, and Mix group, respectively; note the log scale), but not among the low-dose groups (1.8-, 4.7-, 2.6-, and 6-fold increase above control in the OCP, PCB, MeHg, and Mix group, respectively).

For the cyclin-dependent kinase inhibitor $p16^{INK4a}$, a known methylation target for DNMT3b,

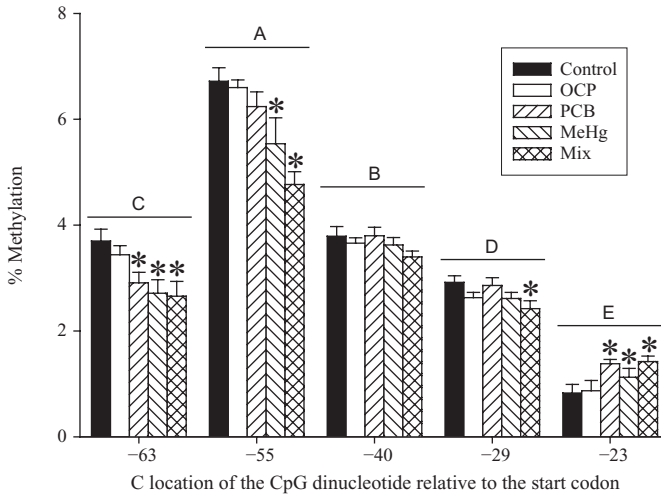

Figure 4. Pyrosequencing methylation analysis revealing the effects of high-dose treatments on the percentage of methylated C from 5 CpG dinucleotides located 63, 55, 40, 29, and 23 nucleotides upstream of the start codon (A in ATG is position 0) of the rat gene cyclin-dependent kinase inhibitor 2A (*Cdkn2a*-p16^{INK4a}, accession number AB081658, studied sequence 1369-1427). Mean ± SE. *Significantly different from control group ($P < .05$) within CpG location. Different capital letters indicate CpG sites with significantly different ($P < .05$) percentage methylation, from the highest (A) to the lowest percentage methylation (E).

mRNA abundance was significantly reduced below the control level only by the high-dose Mix group (28% of control, asterisk). In the OCP group, mRNA was significantly more abundant than in the other groups (capital letters). Abundance of mRNA was significantly reduced in the high dose compared with the low dose only in the MeHg- or Mix-treated groups.

The mRNA expression of these 5 genes was also measured at PND99 in liver samples from sibling females of control and high-dose groups. Significant effects ($P < .05$) could be detected only for CYP1A1 mRNA, which was more abundant in the PCB and the Mix groups compared with other groups (Control, 100 ± 33; OCP, 258 ± 76; PCB, 3570 ± 1075; MeHg, 91 ± 30; Mix, 1950 ± 445).

The reduction in mRNA expression of p16^{INK4a}, the decrease in availability of SAM, and the reduction in global genome DNA methylation, all in the high-dose Mix group, led us to investigate at PND 29 the methylation level of the CpG dinucleotides close to the initiation sites in the promoter region of the gene for p16^{INK4a}. Figure 4 demonstrates a consistent pattern of methylation with significantly different levels of methylation over all CpG sites (capital letters) and significant treatment effects (asterisks). Although OCP treatment had no significant effects at any of the CpG dinucleotides, other treatments altered methylation of CpG at all sites except position -40. Generally, methylation levels were decreased by PCB, MeHg, and Mix treatments. The CpG dinucleotide located at position -23 appears to be different from the others since methylation was increased by most treatments (except OCP). In all cases, Mix appears to produce the greatest change in methylation levels, whereas OCP treatment never induced significant effects. Accurate measurements of the methylation level for the 2 CpG dinucleotides closest to the start codon (location -14 and -9) could not be obtained due to a disruption of the pyrogram, which suggests single-nucleotide polymorphism (SNP) attributable to a point mutation A→G (location -16) that occurred at a frequency that followed a mendelian inheritance pattern (+/+ 31%, +/- 48%, and -/- 21% of rats).

Discussion

The study of epigenetic effects of environmental contaminants, including mechanisms of DNA methylation, is a relatively new area of investigation in toxicology. By investigating the rat liver, this project demonstrates that in utero and lactational exposure to a mixture of persistent organochlorine pollutants and MeHg decreases the availability of SAM, DNMT mRNA abundance, and DNA methylation as supported by small decreases in global genome DNA methylation and in CpG methylation in a segment of the promoter of the p16^{INK4a} gene. These changes occur concomitantly with hepatic inflammation, vacuolation, and hypertrophy, but no hyperplasia, necrosis, or changes in liver weight, as reported in other rats treated with the same mixture.[46] The current investigation suggests that the OCP component of the mixture is the least potent chemical group in affecting the DNA methylation system, as it had no obvious effects on DNMT mRNA abundance, SAM concentration, and DNA methylation. Interestingly, consistent with the current reduction in DNA methylation in the rat, a negative correlation was recently observed between serum level of persistent organic pollutants and global genome DNA methylation in Greenlandic Inuit.[52] Similar associations have also been reported in other human populations exposed to chemicals.[53,54]

The changes in the abundance of the different DNMT mRNAs induced by the chemical groups suggest chemical- and mixture-specific effects. Compared with controls, PCB treatment reduced mRNA expression of the 3 DNMTs and MeHg treatment reduced the expression of 2 DNMTs, whereas OCP treatment did not affect any. The observation that the DNMT mRNA levels in the Mix group are statistically similar to the controls suggests antagonism among chemical components, an effect particularly evident for DNMT1. Explaining the origin of these chemical-specific imbalances in DNMT mRNA expression would require the investigation of end points known to be relevant to the DNMT promoters, including occupation of dioxin response elements, the methylation of CpG dinucleotides, and the role of Sp regulatory sites,[55,56] as well as microRNAs known to interfere with DNMT expression.[57,58] The general pattern of reduction in DNMT mRNA expression is consistent with the reduction in DNA methylation observed by both techniques (LC-UV/ESI-MS and sodium bisulfite pyrosequencing), but at this stage no specific relationship can be established between mRNA expression and DNA methylation. The literature reports that in some instances DNMTs work independently, but in others they work cooperatively.[26,59] The expression of many genes is often regulated beyond the mRNA level by translational or post-translational mechanisms, and others found no correlation between DNMT mRNA expression and DNA methylation in cancerous or noncancerous tissues.[60,61]

In contrast to DNMT mRNA abundance, mRNA levels of CYP1A1 appear to exhibit a synergistic increase. However, using other rats treated with the same mixtures, ethoxyresorufin-*o*-deethylase (the classical enzyme reaction for assessing CYP1A1 activity) measurements did not reveal a synergistic increase; the PCB and Mix treatments induced enzyme activities that were similar and approximately 136-fold above control level.[62] This increase in enzyme activity is close to the 158-fold increase in CYP1A1 mRNA abundance reported here. These changes in CYP1A1 mRNA abundance and activity suggest significant and complex detoxification response, which was likely associated with increases in the amount of proteins that are known to sequester contaminants, including CYP proteins, metallothionein,[63,64] and glutathione involved in oxidative stress modulation.[65] As an indicator of exposure and effects, changes in the expression of DNMT mRNAs are not as sensitive as changes in CYP1A1 mRNA expression. However, whereas increases in CYP1A1 expression are normal detoxification responses, the biological consequences of the treatment-specific reductions in DNMT mRNA levels require further investigation.

The high dose of Mix and PCBs reduced the availability of SAM, the universal methyl donor involved in methylation reactions. It is well known that changes in the availability of SAM are associated with DNA hypomethylation and hepatocarcinogenesis.[23,66] Pyrosequencing provides robust quantitative methylation analysis for sequential series of CpG dinucleotides,[67] and our results (Figure 4) appear to follow an additive pattern among chemical treatments leading to a consistent reduction in methylation in the Mix group, which is in line with the reduction in availability of SAM (Table 2) and global genome methylation (Table 3). Global genome hypomethylation and site-specific hypermethylation are usually landmarks in the progression of carcinogenic events. Hypomethylation is involved with the activation of prometastatic genes and other tumor-promoting genes,[68-70] and it precedes genomic damage and cancer.[71,72] Perhaps exposure to these contaminants decreases global genome methylation and induces a predisposition to hepatocarcinogenesis. This hypothesis would be in line with the observations that PCBs and AhR agonists are classical hepatocarcinogens, mostly in females.[51]

The high-dose Mix treatment decreased the abundance of $p16^{INK4a}$ mRNA, and this might be another observation supporting a predisposition to hepatocarcinogenesis. This protein originates from the INK4b-ARF-INK4a locus; its elevated expression causes a G1-phase cell-cycle arrest that is dependent on functional retinoblastoma protein.[73] $p16^{INK4a}$ has an important role in oncogene-induced senescence, a tumorigenesis barrier that slows or inhibits the progression of preneoplastic lesions to neoplasia.[74] A decrease in $p16^{INK4a}$ expression is common in various cancers and suggests increased mitotic activity with less time for DNA repair. Reduction in $p16^{INK4a}$ mRNA expression can be due to mechanisms unrelated to DNA methylation (eg, polycomb repressor proteins and mRNA stability, reviewed in Jacobs and de Lange[75]), but it is often associated with DNA methylation of its promoter. Our investigation could not demonstrate a link between mRNA expression and DNA methylation. Others were able to associate hypermethylation of the $p16^{INK4a}$ promoter region

with a reduced mRNA expression in chromium-,[17] arsenic-,[14,76] radiation-,[49] or 2-acetylaminofluorene (2-AAF)-treated rats.[77] Comparisons with the current investigation are hazardous, given the differences in chemical treatments, in the various techniques used to assess the methylation levels, in sex differences in chemical sensitivity and effects on DNA methylation,[77] and most importantly, in the DNA segment investigated. Our results are consistent with other pyrosequencing analysis of this promoter region, showing low methylation level of the CpG sites upstream of the transcriptional start site,[78] as well as with bisulfite genomic sequencing results demonstrating very little methylation of the CpG sites in this promoter region in normal lung tissue and in preneoplasia-containing tissues in the rat.[79] In fact, Blanco et al showed increases in methylation of CpG sites occurring only downstream of the transcriptional start site in lung tumors (a DNA segment not investigated here).[79] Perhaps the small increase in methylation at position 23 (the position closest to the transcriptional start site in Figure 4) is part of a progressive process, given that silencing of $p16^{INK4a}$ precedes the DNA methylation changes that were suggested to maintain rather than initiate gene silencing.[80] Investigating human livers and hepatocarcinomas using pyrosequencing, and reporting only the average methylation level over multiple CpG sites of the $p16^{INK4a}$ promoter, Kondo et al obtained, in normal tissues, a mean DNA methylation level of 3%, which is very close to our observations in the rat (Figure 4).[61] This percentage rises early during carcinogenesis to reach 37% in cancer.[61] These comparisons with human tissues may suggest that the changes observed here have not reached the carcinogenic process involving hypermethylation of the $p16^{INK4a}$ promoter.

Finally, the current pyrosequencing analysis supports the existence of an SNP in the *p16*^{INK4a} gene (A→G, location -16) that followed a mendelian inheritance pattern. Single-nucleotide polymorphisms are more frequent in outbred strains like the Sprague Dawley rat than in inbred strains and complicate the interpretation of methylation analysis. This SNP, however, may not have a biological impact on protein activity given that it is not part of an exon.

In summary, this project assessed the impact on components of the DNA methylation system of in utero and lactational exposure to a mixture of environmental contaminants based on the chemicals measured in Inuit blood, and that generates in the high-dose rat pups a serum level of organochlorines 100 times the human level.[46] The decreases in abundance of DNMT1, 3a, and 3b mRNA induced by some treatment groups and the decrease in the availability of SAM and in DNA methylation as observed by 2 independent techniques (pyrosequencing of some CpG sites in the promoter of the gene $p16^{INK4a}$ and global genome DNA hypomethylation by LC-UV/ESI-MS in the Mix group) support that the DNA methylation system could be affected following exposure to environmental contaminants. Compared with the PCB and MeHg component of the complex mixture, the OCP group was the least potent in affecting the current series of end points. Methylation of DNA is of fundamental importance in cellular and molecular biology, and the identification of the specific DNA loci where these changes have biological impact is slowly emerging.[81] Given the biological importance of changes in DNA methylation, it is plausible that exposure to contaminants induces site-specific changes in methylation that could be predictive of adverse health effects, and this possibility warrants further investigation.

Acknowledgments

This work was supported by the Northern Contaminants Program, Department of Indian Affairs and Northern Development (DIAND) and by Health Canada.

The authors are grateful to Dr. Nanqin Li for the preparation and analysis of the dosing solutions; to Leonora Marro for statistical advices; to Karen Leingartner, Bruce Martin, Susan Kelly, and Kelly Brennan for their technical assistance; to Liying Yan (EpigenDx Inc, Worcester, MA) for the pyrosequencing analysis; and to Dr. Tim Schrader and Dr. Craig Parfett for carefully reviewing this manuscript.

References

1. Allis CD, Jenuwein T, Reinberg D, Caparros M-L. *Epigenetics*. New York, NY: Cold Spring Harbor; 2007.
2. Szyf M. The dynamic epigenome and its implications in toxicology. *Toxicol Sci*. 2007;100:7-23.
3. Kovalchuk O, Baulch JE. Epigenetic changes and nontargeted radiation effects—is there a link? *Environ Mol Mutagen*. 2008;49:16-25.

4. Skinner MK. What is an epigenetic transgenerational phenotype? F3 or F2. *Reprod Toxicol*. 2008;25:2-6.
5. Brueckner B, Kuck D, Lyko F. DNA methyltransferase inhibitors for cancer therapy. *Cancer J*. 2007;13:17-22.
6. Kovalenko VM, Bagnyukova TV, Sergienko OV, et al. Epigenetic changes in the rat livers induced by pyrazinamide treatment. *Toxicol Appl Pharmacol*. 2007;225:293-299.
7. Tryndyak VP, Kovalchuk O, Muskhelishvili L, et al. Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. *Mol Carcinog*. 2007;46:187-197.
8. Koturbash I, Rugo RE, Hendricks CA, et al. Irradiation induces DNA damage and modulates epigenetic effectors in distant bystander tissue in vivo. *Oncogene*. 2006;25:4267-4275.
9. Bachman AN, Kamendulis LM, Goodman JI. Diethanolamine and phenobarbital produce an altered pattern of methylation in GC-rich regions of DNA in B6C3F1 mouse hepatocytes similar to that resulting from choline deficiency. *Toxicol Sci*. 2006;90:317-325.
10. Alworth LC, Howdeshell KL, Ruhlen RL, et al. Uterine responsiveness to estradiol and DNA methylation are altered by fetal exposure to diethylstilbestrol and methoxychlor in CD-1 mice: effects of low versus high doses. *Toxicol Appl Pharmacol*. 2002;183:10-22.
11. Dolinoy DC, Jirtle RL. Environmental epigenomics in human health and disease. *Environ Mol Mutagen*. 2008;49:4-8.
12. Sadikovic B, Andrews J, Rodenhiser DI. DNA methylation analysis using CpG microarrays is impaired in benzopyrene exposed cells. *Toxicol Appl Pharmacol*. 2007; 225:300-309.
13. Tao L, Wang W, Li L, Kramer PK, Pereira MA. DNA hypomethylation induced by drinking water disinfection by-products in mouse and rat kidney. *Toxicol Sci*. 2005;87:344-352.
14. Chanda S, Dasgupta UB, Guhamazumder D, et al. DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol Sci*. 2006;89:431-437.
15. Xie Y, Liu J, Benbrahim-Tallaa L, et al. Aberrant DNA methylation and gene expression in livers of newborn mice transplacentally exposed to a hepatocarcinogenic dose of inorganic arsenic. *Toxicology*. 2007;236:7-15.
16. Shiao YH, Crawford EB, Anderson LM, Patel P, Ko K. Allele-specific germ cell epimutation in the spacer promoter of the 45S ribosomal RNA gene after Cr(III) exposure. *Toxicol Appl Pharmacol*. 2005;205:290-296.
17. Kondo K, Takahashi Y, Hirose Y, et al. The reduced expression and aberrant methylation of p16(INK4a) in chromate workers with lung cancer. *Lung Cancer*. 2006;53:295-302.
18. Salnikow K, Zhitkovich A. Genetic and Epigenetic Mechanisms in Metal Carcinogenesis and Cocarcinogenesis: Nickel, Arsenic, and Chromium. *Chem Res Toxicol*. 2007;21:28-44.
19. Chen H, Ke Q, Kluz T, Yan Y, Costa M. Nickel ions increase histone h3 lysine 9 dimethylation and induce transgene silencing. *Mol Cell Biol*. 2006;26: 3728-3737.
20. Benbrahim-Tallaa L, Waterland RA, Dill AL, Webber MM, Waalkes MP. Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of de novo DNA methyltransferase. *Environ Health Perspect*. 2007;115:1454-1459.
21. Poirier LA. The effects of diet, genetics and chemicals on toxicity and aberrant DNA methylation: an introduction. *J Nutr*. 2002;132(8 Suppl):2336S-2339S.
22. James SJ, Melnyk S, Pogribna M, Pogribny IP, Caudill MA. Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology. *J Nutr*. 2002;132(8 Suppl):2361S-2366S.
23. Pascale RM, Simile MM, De Miglio MR, Feo F. Chemoprevention of hepatocarcinogenesis: S-adenosyl-L-methionine. *Alcohol*. 2002;27:193-198.
24. Kress C, Thomassin H, Grange T. Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. *Proc Natl Acad Sci U S A*. 2006;103:11112-11117.
25. Wang J, Bhutani M, Pathak AK, et al. Delta DNMT3B variants regulate DNA methylation in a promoter-specific manner. *Cancer Res*. 2007;67:10647-10652.
26. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*. 2002;416:552-556.
27. Butler WJ, Seddon L, McMullen E, et al. Organochlorine levels in maternal and umbilical cord blood plasma in Arctic Canada. *Sci Total Environ*. 2003; 302:27-52.
28. Dewailly E, Ayotte P, Bruneau S, et al. Inuit exposure to organochlorines through the aquatic food chain in Arctic Quebec. *Environ Health Perspect*. 1993;101:618-620.
29. Knerr S, Schrenk D. Carcinogenicity of "non-dioxinlike" polychlorinated biphenyls. *Crit Rev Toxicol*. 2006;36:663-694.
30. Brody JG, Moysich KB, Humblet O, et al. Environmental pollutants and breast cancer: epidemiologic studies. *Cancer*. 2007;109(12 Suppl):2667-2711.
31. Younglai EV, Wu YJ, Foster WG. Reproductive toxicology of environmental toxicants: emerging issues and concerns. *Curr Pharm Des*. 2007;13:3005-3019.
32. Rogan WJ, Ragan NB. Some evidence of effects of environmental chemicals on the endocrine system in children. *Int J Hyg Environ Health*. 2007;210:659-667.
33. Zoeller RT. Environmental chemicals impacting the thyroid: targets and consequences. *Thyroid*. 2007;17: 811-817.
34. Schoeters G, Den Hond E, Dhooge W, van Larebeke N, Leijs M. Endocrine disruptors and abnormalities of

pubertal development. *Basic Clin Pharmacol Toxicol*. 2008;102:168-175.
35. Hertz-Picciotto I, Park HY, Dostal M, et al. Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development. *Basic Clin Pharmacol Toxicol*. 2008;102:146-154.
36. Jacobson JL, Jacobson SW. Prenatal exposure to polychlorinated biphenyls and attention at school age. *J Pediatr*. 2003;143:780-788.
37. Korrick SA, Sagiv SK. Polychlorinated biphenyls, organochlorine pesticides and neurodevelopment. *Curr Opin Pediatr*. 2008;20:198-204.
38. Braune BM, Outridge PM, Fisk AT, et al. Persistent organic pollutants and mercury in marine biota of the Canadian Arctic: an overview of spatial and temporal trends. *Sci Total Environ*. 2005;351-352:4-56.
39. Sundberg J, Oskarsson A, Albanus L. Methylmercury exposure during lactation: milk concentration and tissue uptake of mercury in the neonatal rat. *Bull Environ Contam Toxicol*. 1991;46:255-262.
40. Sakamoto M, Kubota M, Matsumoto S, Nakano A, Akagi H. Declining risk of methylmercury exposure to infants during lactation. *Environ Res*. 2002;90:185-189.
41. Harada M. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Crit Rev Toxicol*. 1995;25:1-24.
42. Grandjean P, Weihe P, White RF, et al. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicol Teratol*. 1997;19:417-428.
43. Saint-Amour D, Roy MS, Bastien C, et al. Alterations of visual evoked potentials in preschool Inuit children exposed to methylmercury and polychlorinated biphenyls from a marine diet. *Neurotoxicology*. 2006;27:567-578.
44. Desaulniers D, Xiao GH, Leingartner K, et al. Comparisons of brain, uterus and liver mRNA expressions of *cytochrome P450s, Dnmt-1, and catechol-o-methyltransferase*, in prepubertal female Sprague Dawley rats exposed to aryl hydrocarbon receptor agonists. *Toxicol Sci*. 2005;86:175-184.
45. Bowers WJ, Nakai JS, Yagminas A, Chu I. Disentangling the developmental and neurobehavioural effects of perinatal exposure to a chemical mixture found in blood of Arctic populations: differential toxicity of mixture components. *Organohalogen Compounds*. 2004;66:3112-3119.
46. Chu I, Bowers WJ, Caldwell D, et al. Toxicological effects of in utero and lactational exposure of rats to a mixture of environmental contaminants detected in Canadian Arctic human populations. *J Toxicol Environ Health A*. 2008;71:93-108.
47. Padhi BK, Pelletier G, Williams A, et al. Gene expression profiling in rat cerebellum following in utero and lactational exposure to mixtures of methylmercury, polychlorinated biphenyls and organochlorine pesticides. *Toxicol Lett*. 2008;176:93-103.
48. Cui X, Wakai T, Shirai Y, et al. Arsenic trioxide inhibits DNA methyltransferase and restores methylation-silenced genes in human liver cancer cells. *Hum Pathol*. 2006;37:298-311.
49. Kovalchuk O, Burke P, Besplug J, et al. Methylation changes in muscle and liver tissues of male and female mice exposed to acute and chronic low-dose X-ray-irradiation. *Mutat Res*. 2004;548:75-84.
50. Perdew GH. Ah receptor binding to its cognate response element is required for dioxin-mediated toxicity. *Toxicol Sci*. 2008;106:301-303.
51. Persky VW. Health effects of occupational exposure to PCBs. In: Robertson LW, Hansen LG, eds. *PCBs: Recent Advances in Environmental Toxicology and Health Effects*. Lexington: The University Press of Kentucky; 2001:99-102.
52. Rusiecki JA, Baccarelli A, Bollati V, et al. Global DNA hypomethylation is associated with high serum persistent organic pollutants in Greenlandic Inuit. *Environ Health Perspect*. 2008;116:1547-1552.
53. Bollati V, Baccarelli A, Hou L, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res*. 2007;67:876-880.
54. Baccarelli A, Wright RO, Bollati V, et al. Rapid DNA Methylation Changes after Exposure to Traffic Particles. *Am J Respir Crit Care Med*. 2009;179:572-578.
55. Jinawath A, Miyake S, Yanagisawa Y, Akiyama Y, Yuasa Y. Transcriptional regulation of the human DNA methyltransferase 3A and 3B genes by Sp3 and Sp1 zinc finger proteins. *Biochem J*. 2005;385(Pt 2):557-564.
56. Nayyar T, Zawia NH, Hood DB. Transplacental effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the temporal modulation of Sp1 DNA binding in the developing cerebral cortex and cerebellum. *Exp Toxicol Pathol*. 2002;53:461-468.
57. Fabbri M, Garzon R, Cimmino A, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A*. 2007;104:15805-15810.
58. Benetti R, Gonzalo S, Jaco I, et al. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat Struct Mol Biol*. 2008;15:268-279.
59. Suetake I, Shinozaki F, Miyagawa J, Takeshima H, Tajima S. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J Biol Chem*. 2004;279:27816-27823.
60. Park HJ, Yu E, Shim YH. DNA methyltransferase expression and DNA hypermethylation in human hepatocellular carcinoma. *Cancer Lett*. 2006;233:271-278.
61. Kondo Y, Shen L, Suzuki S, et al. Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. *Hepatol Res*. 2007;37:974-983.

62. Leingartner K, Nakai J, Bowers WJ. Perinatal Exposure to a Northern Contaminant Mixture: Effects of Component Interactions on Hepatic Microsomal Enzyme Induction, Thyroid Hormones, and Hematological Parameters in Sprague-Dawley Dams and Postnatal Day 21 Pups. 15th North American Regional Meeting of the International Society for the Study of Xenobiotics, Poster no. 405; 2008.
63. Yoshida M, Satoh M, Shimada A, et al. Maternal-to-fetus transfer of mercury in metallothionein-null pregnant mice after exposure to mercury vapor. *Toxicology*. 2002; 175:215-222.
64. Nishimura N, Miyabara Y, Suzuki JS, et al. Induction of metallothionein in the livers of female Sprague-Dawley rats treated with 2,3,7, 8-tetrachlorodibenzo-p-dioxin. *Life Sci*. 2001;69:1291-1303.
65. Ballatori N. Transport of toxic metals by molecular mimicry. *Environ Health Perspect*. 2002;110(Suppl 5):689-694.
66. Ghoshal K, Li X, Datta J, et al. A folate- and methyl-deficient diet alters the expression of DNA methyltransferases and methyl CpG binding proteins involved in epigenetic gene silencing in livers of F344 rats. *J Nutr*. 2006;136:1522-1527.
67. Mikeska T, Bock C, El Maarri O, et al. Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J Mol Diagn*. 2007;9:368-381.
68. Pakneshan P, Szyf M, Rabbani SA. Methylation and inhibition of expression of uPA by the RAS oncogene: divergence of growth control and invasion in breast cancer cells. *Carcinogenesis*. 2005;26:557-564.
69. Phillips JM, Goodman JI. Identification of genes that may play critical roles in phenobarbital (PB)-induced liver tumorigenesis due to altered DNA methylation. *Toxicol Sci*. 2008;104:86-99.
70. Shukeir N, Pakneshan P, Chen G, Szyf M, Rabbani SA. Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis in vitro and in vivo. *Cancer Res*. 2006; 66:9202-9210.
71. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell*. 2006; 9:199-207.
72. Licchesi JD, Westra WH, Hooker CM, et al. Epigenetic alteration of Wnt pathway antagonists in progressive glandular neoplasia of the lung. *Carcinogenesis*. 2008; 29:895-904.
73. Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol*. 2006;7:667-677.
74. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*. 2006; 444:633-637.
75. Jacobs JJ, de Lange T. p16INK4a as a second effector of the telomere damage pathway. *Cell Cycle*. 2005;4:1364-1368.
76. Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S. Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4a and RASSF1A and induces lung cancer in A/J mice. *Toxicol Sci*. 2006;91: 372-381.
77. Bagnyukova TV, Tryndyak VP, Montgomery B, et al. Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. *Carcinogenesis*. 2008;29:638-646.
78. Bastide K, Guilly MN, Bernaudin JF, et al. Molecular analysis of the Ink4a/Rb1-Arf/Tp53 pathways in radon-induced rat lung tumors. *Lung Cancer*. 2009;63:348-353.
79. Blanco D, Vicent S, Fraga MF, et al. Molecular analysis of a multistep lung cancer model induced by chronic inflammation reveals epigenetic regulation of p16 and activation of the DNA damage response pathway. *Neoplasia*. 2007;9:840-852.
80. Bachman KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell*. 2003;3:89-95.
81. Irizarry RA, Ladd-Acosta C, Wen B, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet*. 2009;41:178-186.
82. Ballschmiter K, Zell M. Analysis of polychlorinated biphenyls by capillary gas chromatography. *Anal Chem*. 1980;302:20-31.