Epigenetic Assessment of Environmental Chemicals Detected in Maternal Peripheral and Cord Blood Samples

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ABSTRUCT

Epigenetic alteration is an emerging paradigm underlying the long-term effects of chemicals on gene functions. Various chemicals, including organophosphate insecticides and heavy metals, have been detected in the human fetal environment. Epigenetics by DNA methylation and histone modifications, through dynamic chromatin remodeling, is a mechanism for genome stability and gene functions. To investigate whether such environmental chemicals may cause epigenetic alterations, we studied the effects of selected chemicals on morphological changes in heterochromatin and DNA methylation status in mouse ES cells (ESCs). Twenty-five chemicals, including insecticides organophosphates insecticides, heavy metals, and their metabolites were assessed for their effect on the epigenetic status of mouse ESCs by monitoring heterochromatin stained with 4′,6-diamino-2-phenylindole (DAPI). The cells were surveyed after 48 or 96 hr of exposure to the chemicals at the serum concentrations of cord blood. The candidates for epigenetic mutagens were examined for the effect on DNA methylation at genic regions. Of the 25 chemicals, five chemicals (diethylphosphate (DEP), mercury (Hg), cotinine, selenium (Se), and octachlorodipropyl ether (S-421)) caused alterations in nuclear staining, suggesting that they affected heterochromatin conditions. Hg and Se caused aberrant DNA methylation at gene loci. Furthermore, DEP at 0.1 ppb caused irreversible heterochromatin changes in ESCs, and DEP-, Hg-, and S-421-exposed cells also exhibited impaired formation of the embryoid body (EB), which is an *in vitro* model for early embryos. We established a system for assessment of epigenetic mutagens. We identified environmental chemicals that could have effects on the human fetus epigenetic status.

Key Words: DNA methylation, embryoid body, epigenetic mutagens, ES cells, heterochromatin

INTRODUCTION

Many chemicals have been widely used in the household, agricultural, and urban environment and there are various environmental chemicals in the fetal growth environment: organophosphate insecticides (chlorpyrifos), perfluorooctane sulfonate (PFOS), di(2-ethylhexyl)phthalate (DEHP), elements of tobacco smoke (nicotine and cotinine), and heavy metals (lead (Pb), cadmium (Cd), and mercury (Hg)) [1–5]. Hg occurs in the daily intake of rice in China [6]. Prenatal exposure to environmental chemicals such as organophosphate insecticides, tobacco smoke, heavy metals, and perfluorinated compounds (PFCs) are associated with fetal growth restriction and low birth weight of infants [7–10]. The concentration of chemicals detected in the fetal environment is, however, relatively low (around 0.1–10 ppb level) and far from the pharmacological studies. Such a low concentration may accumulate in the fetus and placenta and may affect the fetal growth [11–14], and alternatively, some chemicals may have long-term effects on gene functions and stability even at low concentration without accumulation in the fetal tissues and placenta [15, 16].

Epigenetic alterations have become an emerging paradigm responsible for irreversible phenotypic change through long-term gene regulation. The epigenetic marks such as DNA methylation, histone modifications, and heterochromatin/euchromatin cause dynamic change of cellular conditions and cell types [17–19]. In mammals, heterochromatin, a highly condensed structure of chromatin, is characterized by DNA hypermethylation and histone modifications such as H3-K9 and -K27 methylation [20–22]. Pericentric regions of chromosomes involve major satellite repeats forming constitutive heterochromatin [23]. Heterochromatin can be visualized by nuclear staining with 4′,6-diamino-2-phenylindole (DAPI) in mouse cells. Chemical-induced epigenetic alterations in cell nuclei may be visualized by nuclear staining with DAPI as well as major satellite repeats.

There are a large number of tissue-dependent and differentially methylated regions (T-DMRs) in the mammalian genome [24–26]. Embryonic stem cells (ESCs) established from the inner-cell mass have an ability for pluripotency, which is critical for mammalian development. The profile of T-DMRs—termed the 'DNA methylation profile'—is distinctive in every cell type, including ESCs and somatic cells [25, 27]. *Study of the DNA methylation profile of ESCs and somatic cells showed*

the existence of thousands of T-DMRs that are hypo- and hypermethylated in ESCs (ES hypo hyper T-DMRs) [24]. Thus, an adequate DNA methylation profile during self-renewal and differentiation is critical for pluripotency. Recently, enrichment of short interspersed elements has been observed as a genomic property of regions harboring hypomethylated T-DMRs in ESCs [28]. Therefore, DNA methylation and heterochromatin signals will be useful for epigenetic assessment.

Chemicals that cause alterations in epigenetic systems are called epimutagens [29]. A DNA demethylating agent, 5-azacytidine, which is a cytidine analog, is a well-established epimutagen that causes reactivation of silenced genes [30]. It is known that Trichostatin A (TSA), an inhibitor of histone deacetylases, causes alteration in epigenetic regulation and nuclear structure of the cells [31]. Common chemicals in the environment also cause epigenetic alteration. We previously demonstrated that dimethyl sulfoxide (DMSO), which has been used as a cryopreservant for various cell lines, altered DNA methylation status in both gene areas and condensed repetitive sequences during the differentiation of ESCs into the embryoid body (EB) [32]. Carcinogenic nickel compounds have also been reported as an epigenetic modifier that induces histone methylation and changes in gene expression and chromatin structure [33]. Thus, environmental chemicals may have the potential to affect epigenetic states in the embryonic tissue or cells.

We here report the epigenetic effects of 25 environmental chemicals at low concentration, which were detected in maternal peripheral blood and cord blood, on heterochromatin signals and DNA methylation status of T-DMRs in mouse ESCs.

MATERIALS AND METHODS

Maternal peripheral blood and cord blood samples.

Maternal peripheral blood and cord blood samples at term were collected at Yamaguchi University during 2008–2010 for analysis of 3-phenoxybenzoic acid (3-PBA), 3,5,6-trichloro-2-pyridinol (TCP), dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), perfluorooctanoate (PFOA), and PFOS (Table 1). The blood samples were collected at Tokai University during 2005–2007 for other chemicals. This study was approved by the Ethics Committee of Yamaguchi Grand Medical Center, the Yamaguchi University School of Medicine, and the Human Subjects Committee of Tokai University Hospital. All samples were taken with written informed consent.

Sample preparation and chromatography conditions.

Determination of the serum concentrations of chemicals except for organophosphate and pyrethroid insecticides (Group A) and heavy metals (Group E) and volatile organic compounds (VOCs) (Group F) was as previously described [3, 4, 34, 35]. For octachlorodipropyl ether (S-421) (Group A), polybrominated diphenyl ethers (PBDEs) (Group D) and tin (Sn) (Group E), the exposure level to cells was determined by concentrations of nonserum sample, breast milk (S-421), and adult urine (penta BDE [36] and Sn). Concentration of dimethyldithiophosphate (DMDTP), diethyldithiophosphate (DEDTP), and deca BDE was based on the data from previous reports [37, 38].

The determination of 3-PBA and TCP (Group A) was by a modified previous method [39, 40] using liquid chromatography–tandem mass spectrometry (LC/MS/MS) (Alliance2695 and Quattro micro; Waters, Milford, MA) equipped with an electrospray ionization probe. After addition of beta-glucuronidase for derivatization, samples were purified with an Oasis HLB extraction column (Waters), and then fractionated by reverse-phase chromatography on a symmetric C18 column (50 mm × 2.1 mm i.d., 3.0 μm film thickness; Waters) with 35% acetonitrile and 0.1% acetic acid. Following sample load, MS/MS was performed in the negative-ion mode.

For S-421 (Group A), sample preparation from human milk was performed according to the

method described in Kakimoto et al. [41] with slight modifications, and the concentration of S-421 was determined by gas chromatography–mass spectrometry (GC/MS).

DMP, DEP, DMTP, and DETP (Group A) were determined using the method described in Ueyama et al. [42] with modifications. Samples were purified on a C18 cartridge column (Aisti Science, Wakayama, Japan), and then deproteinized with acetonitrile. After derivatization with pentafluorobenzylbromide, samples were purified using a three-layer column of Florisil, PSA, and anhydrous sodium sulfate. The concentrations of DMP, DEP, DMTP, and DETP were determined using gas chromatography with flame-photometric detector (GC/FPD) (Agilent Technologies, Tokyo, Japan) equipped with an LVI-S200 injection port (Aisti Science) on a DB-1701 or DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies).

The determination of heavy metals (Group E) were by a modified previous method [43]. Serum and urine samples were mixed with HNO_3 in a Nano-Band MV (GL Science, Tokyo, Japan) overnight. After microwave digestion, samples were mixed with internal standard (scandium (Sc), yttrium (Y), iridium (Ir)) and subjected to analysis by inductively coupled plasma–mass spectrometry (ICP/MS) (Agilent Technologies 7500i).

VOCs (Group F) in serum samples were determined using the previously described procedure [44]. Samples were diluted with saturated saline solution and spiked with internal standard (toluene-d_8) in a CV-22 vial (Chromacol, Trumbull, CT). VOCs were determined by AUTO MASS SYSTEM II (JEOL, Tokyo, Japan) coupled with Tekmar 7000 headspace sampler (Tekmar Co., Cincinnati, OH). Separation was accomplished with a Vocol column (60 mm × 0.25 mm i.d., 1.5 μm film thickness; Supelco, Bellefonte, PA). Analysis was performed in a selected ion monitoring mode and quantification masses for each of the analytes.

ESC culture and exposure of chemicals.

ESCs (J1) derived from 129 strain mice were cultured on 0.1% gelatin-coated dishes with ES medium, containing 15% fetal bovine serum (FBS; Biowest, Nuaille, France), 2 mM of L-glutamine (Invitrogen, Tokyo, Japan), 100 mM of nonessential amino acid (Invitrogen), 100 mM of

2-mercaptomethanol (Invitrogen), 1 mM of Na-pyruvate (Invitrogen), 50 U/mL of penicillin, 50 μg/mL of streptomycin (Invitrogen), and 2000 U/mL of leukemia inhibitory factor (LIF, ESGRO; Chemicon, Temecula, CA) in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals, Osaka, Japan). To induce formation of EBs, ESCs were transferred to bacterial Petri dishes after removal of feeder cells. EBs were cultured in DMEM containing 10% FBS (JRH, Lenexa, KS), 50 U/mL penicillin, and 50 μg/mL streptomycin.

ESCs or EBs from J1 ESCs were cultured with chemicals at the serum level concentrations or tenfold higher level (Table 1). Selenium (Se), Cd, Hg, and Pb were dissolved in 0.0025% nitric acid (HNO_3). Sn was diluted with 0.007% hydrochloric acid (HCl). The other chemicals including TSA (Sigma-Aldrich, Tokyo, Japan) were dissolved in 0.1% ethanol (EtOH).

Slide preparation for heterochromatin analysis.

In the heterochromatin and DNA methylation analyses, all reagents, unless otherwise stated, were purchased from Wako Pure Chemicals. ESCs exposed to chemicals or their solvents were fixed with fix solution, consisting of EtOH (A) and acetic acid (B) (A/B:3/1, v/v). After centrifuging and removal of the supernatant liquid, the cells were suspended with 20–50 μL of fix solution, and then the interphase nuclei were spread on the glass slide. Prepared glass slides were stored at –20 °C until DAPI staining or DNA fluorescence *in situ* hybridization (DNA-FISH) analysis. For heterochromatin analysis with DAPI staining, the nuclei were stained with 0.5 μg/mL of DAPI (Dojindo, Kumamoto, Japan) for 5 min at room temperature, followed by washing in phosphate-buffered saline (PBS). The samples were mounted with antifade medium, PermaFluor Aqueous Mounting Medium (Thermo Scientific, Inc., Wilmington, DE).

DNA-FISH.

DNA-FISH using the major satellite repeat sequence, according to the previous reports [45, 46]. Probes specific to major satellite sequences in pericentric regions were labeled with biotin using Biotin-Nick Translation Mix (Roche Diagnostics, Tokyo, Japan). The interphase nuclei on a glass

slide were immersed in 2x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) for 30 min at 37 °C, and then dehydrated with 70%, 80%, and 95% EtOH each for 2 min at room temperature. After denaturation with 70% formamide in 2x SSC (pH 7.0) for 2 min at 70 °C, the samples were dehydrated again with cold 70%, 80%, and 95% EtOH each for 2 min at –20 °C. The probes were diluted to 10 ng/mL with hybridization solution (50% formamide, 10% dextran sulfate, 50 mM phosphate buffer in 2x SSC, pH 7.0), followed by denaturation for 5 min at 95 °C. Denatured probe was added to the nuclear samples and incubated for 16 hr at 37 °C. The samples were then immersed in wash buffer (100 mM Tris-Cl, 150 mM NaCl, 0.05% Tween-20, pH 7.0) for 5 min at room temperature. Following incubation in blocking solution (100 mM Tris-Cl, 150 mM NaCl, 0.5% blocking regent (Roche Diagnostics), pH 7.5) for 30 min at 37 °C, the samples were incubated with 10 μg/mL of biotinylated antistreptavidin (Vector Laboratories, Burlingame, CA) for 60 min at 37 °C. After three sets of washing in wash buffer, the samples were incubated with 5 μg/mL of Texas red-conjugated streptavidin (Vector Laboratories) for 30 min at 37 °C, followed by three sets of washing. Finally, 10 μg/mL of biotinylated antistreptavidin was added to the samples again for 30 min at 37 °C, and then the nuclei were counterstained with DAPI at room temperature.

Immunofluorescence.

ESCs were fixed in 4% paraformaldehyde for 10 min. After permeabilization with 0.2% Triton X-100 for 5 min, samples were treated with blocking buffer (5% BSA, 0.1% Tween-20 in PBS) for 30 min. The samples were incubated with anti-mouse heterochromatin protein 1a (HP1a, Chemicon, Temecula, CA) dissolved in blocking buffer (1:500) for 45 min, followed by three sets of washing in PBS containing 0.05% Tween-20. After incubation with fluorescent secondary antibody (anti-mouse IgG-FITC conjugated, KPL, Gaithersburg, MD) dissolved in blocking buffer (1:20) for 60 min, the samples were washed again. Finally, the samples were mounted with PermaFluor Aqueous Mounting Medium, containing 0.2 μg/mL of DAPI on the glass slide. All reactions were performed at room temperature.

2-mercaptomethanol (Invitrogen), 1 mM of Na-pyruvate (Invitrogen), 50 U/mL of penicillin, 50 μg/mL of streptomycin (Invitrogen), and 2000 U/mL of leukemia inhibitory factor (LIF, ESGRO; Chemicon, Temecula, CA) in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals, Osaka, Japan). To induce formation of EBs, ESCs were transferred to bacterial Petri dishes after removal of feeder cells. EBs were cultured in DMEM containing 10% FBS (JRH, Lenexa, KS), 50 U/mL penicillin, and 50 μg/mL streptomycin.

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Image acquisition and counting heterochromatin foci.

Image acquisition of DAPI staining and DNA-FISH was performed using a fluorescent microscope (BZ-8000; KEYENCE, Osaka, Japan), and 5–10 visual fields were analyzed for individual samples. The exposure time of fluorescence by DAPI staining and major satellite DNA-FISH was fixed to 1/1.5 sec and 1.0 sec, respectively. After deconvolution of the fluorescent image by BZ-8000 software, the number of DAPI and major satellite signals in the nuclei was determined using ImageJ software provided by the National Institute of Health (http://rsb.info.nif.gov/ij/). Briefly, RGB images were first converted to 8-bit grayscale by linearly scaling min–max to 0–255. Next, the thresholds of intensity in DAPI and DNA-FISH images were decided within 79–84 and 15–20, respectively. Finally, the number of signals per nucleus (n = 30–80) was determined by ImageJ. Statistical analysis between solvent- and chemical-exposed cells was performed using the Wilcoxon test.

Combined Bisulfite Restriction Analysis (COBRA) assay.

Genomic DNA extraction and bisulfite conversion were carried out as previously described [26]. DNA methylation analysis was performed based on the previous finding [24] focusing on 48 T-DMRs by COBRA assay [47].

Genomic DNA was extracted from ESCs in lysis buffer (100 mM Tris-Cl (pH 8.0), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 200 μg/mL Proteinase K) at 55 °C for 30 min. After treatment with PCI, consisting of phenol (A), chloroform (B), and isoamyl alcohol (C) (A/B/C:50/49/1, v/v) for removal of proteins, RNaseA (Roche Diagnostics) was added and genomic DNA was purified by EtOH precipitation. Genomic DNA was digested with a restriction enzyme, *Hind* III (TaKaRa, Kyoto, Japan), and purified by EtOH precipitation. After denaturation of digested genomic DNA with 0.3 M NaOH, sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 mM, respectively. The bisulfite reaction was performed on a PCR machine as follows: 20 cycles of 95 °C for 30 sec and 55 °C for 15 min, followed by 55 °C for 10 hr. Bisulfite-treated genomic DNA was purified with QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), and desulfonated with 0.3 M NaOH at 37 °C for 15 min, followed by EtOH precipitation. Purified

bisulfite-treated DNA was amplified with BioTaq (BIOLINE, London, UK) using specific primers for gene loci (Supplemental Table 1). Amplified PCR products were digested with *Hpy* CH4 IV (New England BioLabs, Inc., Beverly, MA) at 37 °C for 3 hr, and then analyzed by microchip electrophoresis using MCE-202 (MultiNA; Shimadzu, Kyoto, Japan). The DNA methylation level by COBRA assay was calculated using the formula: estimated methylation degree (%) = $100 \times I^C/(I^C + I^{CU})$, where I^C and I^{CU} represent the sum of the intensities of digested bands and undigested bands, respectively.

Statistical Analysis.

Statistical comparisons of the number of DAPI signals were performed using the Wilcoxon test, and those of DNA methylation and area of EBs were performed using the Student's *t* test.

RESULTS

Chemical screening by heterochromatin marks.

Outline of the chemical exposure experiments was illustrated in Fig. 1. We first examined the effect of the 25 chemicals detected in maternal serum of pregnant mothers and cord blood (Table 1, Groups A–G) by counting the number of DAPI signals colocalized with signals of HP1a and major satellite DNA (Fig 2A). Reduction of numbers was observed in ESCs treated with TSA (1.0 nM) for 48 hr, but not with TSA at a lower concentration (0.2 nM) or control solvent (Fig. 2B). The other solvents, EtOH (0.1%), HCl (0.007%), or HNO_3 (0.0025%) for other chemicals, also had no effect on the DAPI signals in the treated ESCs (Fig. 2C).

Based on the levels of the 25 chemicals in the specimens (Table 1, Groups A–G), we assessed the effects of the chemicals on ESCs for 48 hr culture with each chemical at low (serum level, ×1, Table 1) and high (10 times, ×10, Table 1) concentrations (Exp. 1 in Fig. 1). DEP at serum concentration (0.1 ppb) significantly decreased the number of DAPI signals (Figure 2D). Cotinine (Group B) and Hg (Group E) also caused a decrease in DAPI signals at the serum concentration. In contrast, S-421 (Group A) and Se (Group E) increased the number of signals. Chemicals such as TCP, DMP, DETP, and DMDTP in Group A, deca BDE in Group D and mono(2-ethylhexyl)phthalate (MEHP) in group G exhibited effects on the number of DAPI signals only at high concentrations ($p < 0.01$) (Fig. 2D). It is noteworthy that five chemicals (DEP, Hg, cotinine, Se, and S-421) could affect the epigenetic status of ESCs at the concentration in the fetal environment.

Effects of DEP, Hg, cotinine, Se, and S-421 on the size of heterochromatin signals.

We further evaluated the effects of the five chemicals (DEP, Hg, cotinine, Se, and S-421) by examining the size of signals with both DAPI staining and DNA-FISH with major satellite repeats, in which signals were merged closely together in all preparations (Fig. 2E).

The size of the heterochromatin signals by DAPI and DNA-FISH were decreased by treatments with DEP and Se, but not in the Hg, cotinine, and S-421 treatments (Fig. 2F), although DEP, Hg, and cotinine decreased the number of signals and Se and S-421 increased the number as observed in the

screening using DAPI staining. These data indicate that the effects of DEP are, at least, distinct from those of Hg and cotinine, and the effect of S-421 is distinguished from that of Se. These distinct reactions by chemicals suggested that the size of heterochromatin signals should provide another dimension of heterochromatin status in addition to the number of DAPI signals, and that DEP, Hg, cotinine, Se, and S-421 have distinct effects on the epigenetic status of ESCs.

Effects of DEP, Hg, cotinine, Se, and S-421 on DNA methylation status at T-DMRs in ESCs.

To explore the effects of five chemicals (DEP, Hg, cotinine, Se, and S-421) on the epigenetic status of the genome at serum level concentrations, we investigated the DNA methylation status of 48 T-DMRs of genic regions, in which the methylation degree (%) varied from 0 to 80 in controls by COBRA assay using *Hpy* CH4 IV (Figure 3A, Exp. 2 in Fig. 1, and Supplemental Table 2).

DNA methylation levels in the control and Hg-exposed cells were 62.2% ± 0.79% and 72.3% ± 2.38% at *Rnd2* T-DMR, respectively, indicating that Hg caused hypermethylation (Fig. 3A and 3B). DNA methylation status at the *Aebp2* and *Prickle2* T-DMRs was also changed, resulting in hypomethylation in Se-exposed cells. DNA methylation levels in the control and Se-exposed cells were 28.0% ± 1.04% and 18.9% ± 0.64% at the *Aebp2* T-DMR, and 21.5% ± 0.70% and 16.0% ± 0.73% at the *Prickle2* T-DMR, respectively (Fig. 3B). These results indicate that Hg and Se affect the epigenetic status of DNA methylation as well as higher-ordered levels (heterochromatin), and that simple DAPI staining of ESCs enables us to screen for chemicals that affect epigenetic status.

Studies on prolonged effects of DEP, Hg, cotinine, Se, and S-421.

Changes in DNA methylation, which has been regarded as a stable epigenetic mark, prompted us to assess the prolonged effects of chemicals. We examined heterochromatin in ESCs exposed to the chemicals DEP, Hg, cotinine, Se, and S-421 at the serum level concentrations for 48 hr followed by 48 hr culture after their removal (Exp. 3 in Fig. 1). In the pretreatment of ESCs with Hg, cotinine, Se, and S-421, the number of DAPI signals in ESCs was reversed to almost the same levels as their controls (Fig. 4A, upper panel), suggesting that altered heterochromatin was reversible in the cells exposed to

these four chemicals. In contrast, the reduced number of DAPI signals in DEP-exposed cells did not recover to the control level after removal of DEP (Fig. 4A, upper panel). In addition, the area occupied by major satellite signals also remained at affected levels even after removal of DEP (Fig. 4A, lower panel). Thus, DEP-induced heterochromatin change was irreversible, even after 48 hr.

Effects of DEP, Hg, cotinine, Se, and S-421 on differentiation potentials in formation of EBs.

To test the effects of the chemicals on the developmental potential, we investigated the effects of chemicals on EB formation (Exp. 4 in Fig. 1). Although exposure to cotinine and Se had no effect on EB formation judged by the sizes of EBs between chemical-exposed and control cells, in the presence of DEP, Hg, and S-421 at low concentration, the size of EBs was significantly smaller than that of the controls (Fig. 4B).

DISCUSSION

The assessment of environmental chemicals has usually been performed with high doses of chemicals resulting in growth arrest in the previous studies. However, many chemicals have been detected in human samples with much lower concentrations at which severe growth arrest and cell death are not observed. In the present study, using mouse ESCs as an *in vitro* model for early embryos, we demonstrated that at least five chemicals (DEP, cotinine, Hg, Se, and S-421) have epimutagenic effects at the serum concentration of maternal peripheral blood and cord blood. The majority of the chemicals (20/25) examined had no effect on heterochromatin formation at the serum level, whereas the 10 times or more higher pharmacological dose may have some effects. In addition, exposure to Hg and Se caused alteration of DNA methylation status at unique gene loci harboring T-DMRs, DEP shows a long-term effect, and DEP, Hg, and S421 affect the developmental potentials. Thus, certain chemicals that are detected in human samples affect the epigenetic levels. A summary of the results is shown in Fig. 4C.

Of the five epimutagens, DEP-exposed ESCs did not recover from the altered heterochromatin status even after removal of the chemical, whereas other chemicals (Hg, cotinine, Se, and S-421) exhibited reversal of the number of DAPI signals after removal of the chemicals. This implies that DEP can cause long-lasting heterochromatin changes even at concentrations that have been detected in the serum of cord blood. DEP is a metabolite of chlorpyrifos, which is a component of pesticides. Prenatal exposure to pesticides caused lasting developmental disorders such as neural dysfunction in children even after birth [48, 49]. The EB is an *in vitro* model for differentiating early embryos [50], and EB formation was impaired in the presence of serum levels of DEP, Hg, and S-421, which caused altered heterochromatin formation in ESCs. In particular, DEP-induced epigenetic changes should be long lasting even at serum level concentrations. Collectively, certain chemicals such as DEP have the potential to cause epigenetic dysfunction in developing early embryos.

The genes showing disturbed DNA methylation status upon Hg or Se exposure have important roles in fetal development. *Aebp2* is a component of the epigenetic regulator, Polycomb Repression Complex 2 [51]. *Prickle2* and *Rnd2* are related to neural differentiation [52, 53]. This implies that the

genes with various functions regulated by DNA methylation are affected in ESCs that are thought of as an *in vitro* model of early embryonic cells.

The cytotoxic effect of chemicals is more severe in early stages of development than in adulthood [54, 55]. Heavy metals such as Se and Cd have been reported to cause epigenetic alteration in human cancer cell lines or fibroblasts [56, 57], although the cells were treated with high doses, more than 10 times those of serum level. On the other hand, human prostate cancer cell line LNCsP, exposed to Se for four days, did not exhibit altered DNA methylation [58]. In the present study, Hg and Se at serum levels caused altered heterochromatin marks and DNA methylation changes in ESCs.

In conclusion, the method using mouse ESCs to assess the epimutagens was simple and sensitive. We have demonstrated that DEP, Hg, cotinine, Se, and S-421 altered epigenetic systems at serum level concentrations detected in human maternal peripheral blood and cord blood.

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