

RNA extraction and RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), and RT-PCR was performed using Taq DNA Polymerase (Promega) with primers as follows: 5'-CAGGAGTGTGTGAGGGAG-3', F, and 5'-GGTGTCACGTCCGACTTGC-3', R, for *Dnmt1*; 5'-ACCCATGCCAAGACTCACCTTC-3', F, and 5'-TCCACCTTCTGAGACTCTCAG-3', R, for *Dnmt3a*; 5'-TCAGACACGAAGGATGCTCC-3', F, and 5'-ACAGGGTACTCTGCACATG-3', R, for *Dnmt3b*; 5'-TTCTACAATGAGCTGCGTGTGG-3', F, and 5'-ATGGCTGGGGTGTGAAGGT-3', R, for β -actin; 5'-GGCGTTCGCTTTGAAAGGTGTTTC-3', F, and 5'-CTCGAACACATCCTTCTCT-3', R, for *Oct-4*; 5'-CCAGTCGAAGATGCTCAACA-3', F, and 5'-TGTGATGGGGTCAGAGTCAA-3', R, for *Clu*; 5'-ATCGGATATCGACCCTCAC-3', F, and 5'-ATCTGCAAAGCGTCCTTCAT-3', R, for *Dpep1* and 5'-CAACGTCTGGAAATGTGG-3', F, and 5'-CAGCCATAGTGGTGTGAA-3', R, for *Igf2r*. The PCR conditions were as follows: 95 C for 1 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 5 min.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously [20] using a Chromatin Immunoprecipitation (ChIP) Assay Kit (Cat. No. 17-295; Upstate Biotechnology, Lake Placid, NY, USA) with anti-acetylated histone H3 and H4 antibodies (Cat. No. 06-599 and 06-598; Upstate Biotechnology), anti-trimethylated H3K4 and H3K9 (Cat. No. ab8580 and ab8898; Abcam, Cambridge, UK) and anti-dimethylated H3K4, H3K9 and H3K27 (Cat. No. 07-030, 07-212 and 07-452; Upstate Biotechnology). Normal rabbit IgG (Cat. No. 12-370; Upstate Biotechnology) was used as a negative control to verify immunoprecipitation specificity. PCR was performed using primers as follows: 5'-GTGAGGTGTCGGTGACCCAAGGCAG-3', F, and 5'-CGGCTCACCTAGGGACGGTTTACC-3', R, for *Oct-4*; 5'-TGCTCTGGAGACACAGGAAA-3', F, and 5'-CTGGGAAGAAAGCCAAGAT-3', R, for *Clu* ChIP 1; 5'-ATTGCAGTGATGCCAGATGA-3', F, and 5'-ACGCACAGCAGGAGAATCTT-3', R, for *Clu* ChIP 2; 5'-CTCCTTTGTGGCTCCCTAA-3', F, and 5'-GGCTCCACAGAGTCCAAG-3', R, for *Dpep1*. PCR was performed under the following conditions: 95 C for 10 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min. The amount of each PCR product on an ethidium bromide-stained gel image was evaluated using the ImageJ software.

Results

Demethylating effect of 5azadC on repetitive sequences

We examined cell survival under different concentrations of 5azadC by WST-1 assay. After 72 h, the viable cell number was largely reduced at concentrations higher than 0.5 μ M and was severely affected at higher concentrations (Fig. 1A). A similar

effect was observed in the 96-h culture. Only minimal effects on viability were observed in cells treated with less than 0.1 μ M 5azadC.

To determine the effect of 5azadC on the methylation of repetitive sequences, cells were treated with 0.001 μ M to 5 μ M 5azadC, and the methylation status of repetitive sequences was analyzed using methylation-sensitive restriction enzymes. Southern hybridization was performed using two probes of differentially localized repetitive sequences, minor satellite repeats located in the centromeric regions and endogenous C-type viruses interspersed across the mouse genome [14, 24, 29]. Minor satellite repeats were demethylated extensively starting at the 0.1 μ M concentration (Fig. 1B), and 1 μ M was sufficient to induce the maximum demethylating effect. Similarly, endogenous viruses showed aggressive loss of methylation at the 0.1 μ M to 5 μ M treatment levels, but slight demethylation could be observed at a concentration as low as 0.001 μ M. Thus, the repetitive sequences were strongly demethylated by 5azadC. The results confirmed a previous report that 5azadC is effective in inducing demethylation dose-dependently from 0.1 μ M to 5 μ M [30].

Effect of 5azadC on genic regions

We next examined the effect of 5azadC on T-DMRs of genic regions. *Oct-4* (*Pou5f1*) has T-DMRs in the CpG-rich promoter/proximal enhancer region and distal enhancer region (Fig. 1C; 20). The DNA methylation status of the T-DMRs was analyzed using bisulfite restriction mapping focusing on *TaqI* restriction sites. At 0.001 and 0.01 μ M 5azadC, the methylation levels of the investigated regions changed little compared to the untreated ones. A significant loss of methylation in both regions was observed at 0.1 μ M, indicating that this concentration was able to induce demethylation in both genic regions and the repetitive sequences. In the 1 and 5 μ M treated samples, however, both T-DMRs had nearly the same methylation levels as in the untreated control, in contrast to the extensive demethylation observed in the repetitive sequences at these concentrations.

Sall3 has a T-DMR located at an edge of a CpG island, which is methylated only in the trophoblast cell lineage [19, 21]. The T-DMR is aberrantly methylated in the placental genome of cloned mice [21]. *Per1*, which is involved in generating circadian rhythm, has a few CpGs in the upstream promoter region. Similar to *Oct-4*, 0.001 and 0.01 μ M 5azadC had minimal effects on these loci. The methylation levels decreased significantly following 0.1 μ M 5azadC treatment, but were only slightly decreased at 1 μ M, and remained unchanged at 5 μ M.

Bisulfite sequencing was performed on several gene loci containing CpG-rich promoters, including *Oct-4*. Hypomethylation of the *Clusterin* (*Clu*) promoter is associated with high gene expression in the rat testis and epididymis [31]. *Dpep1*, a renal *Dipeptidase* gene, has been reported to be a tumor marker candidate in malignancies [32]. All investigated loci had hypermethylated promoter regions in NIH/3T3 cells (Fig. 2A). Bisulfite sequencing results for the *Oct-4* promoter region validated the restriction mapping results showing that 0.1 μ M was more effective in inducing demethylation in genic regions compared with 1 μ M. Similar dose-dependent demethylation patterns were also

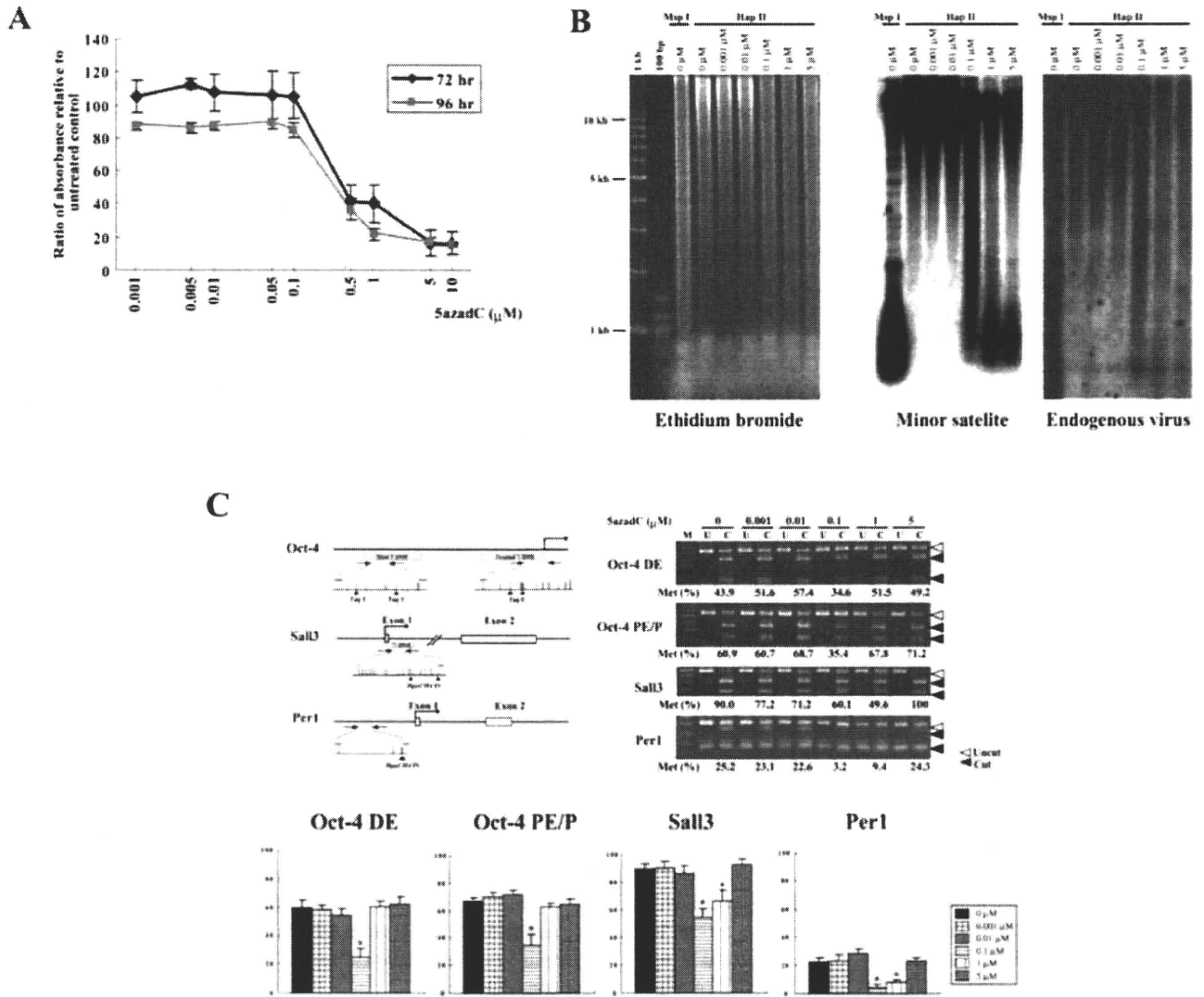


Fig. 1. Different demethylating effects of 5azadC on repetitive sequences and genic regions. **A:** A WST-1 cell proliferation assay was performed on cells treated with 5azadC at the indicated concentrations for 72 and 96 h. Cell number was estimated by the absorbance, and then represented as the ratio relative to the untreated control, the absorbance of which was arbitrarily set to 100. The values represent means \pm S.E. of 3 independent cultures. **B:** Analysis of the methylation status of repetitive sequences by Southern hybridization. Genomic DNA (5 μ g), digested with *HapII*, was hybridized to probes for minor satellite repeats and endogenous viruses. As a control for complete digestion, DNA from the untreated control was digested with *MspI*. **C:** Methylation analysis of T-DMRs by bisulfite restriction mapping. A schematic diagram of each investigated locus is shown in the top left panel. T-DMRs are represented by bars, and CG sites are represented by vertical lines. The locations of the amplified regions relative to each respective transcription start site are as follow: *Oct-4* distal enhancer (DE), -3086 to -2646; *Oct-4* proximal enhancer/promoter (PE/P), -420 to +31; *Sall3*, +1253 to +1665; and *Per1*, -1087 to -929. Each locus contains 2 *TaqI* or *HpyCH4IV* restriction sites, respectively (triangles). PCR products were digested with restriction enzymes and electrophoresed on agarose gel (top right panel). The relative DNA methylation level was calculated based on the relative intensities of cut to uncut bands, indicated below each cut lane. M, marker; U, uncut; C, cut with restriction enzyme. The bottom panel indicates the methylation level of each gene locus at each concentration; the values are presented as means \pm S.E. of 3 independent PCRs of 2 cultures. * $P < 0.05$ (Student's *t*-test).

observed in the promoter regions of *Clu* and *Dpep1*.

We also analyzed the DMR2 region of the *Igf2r* imprinted gene, which is differentially methylated depending on its parental origin [33]. Due to allele-specific methylation, half of the clones were methylated in the untreated control. As observed in other genes,

the DMR2 region appeared demethylated at 0.1 μ M, but was not affected by 1 μ M 5azadC.

These results provide evidence that 5azadC has a strong demethylating effect on repetitive sequences, but its effect on genic regions is limited to a certain effective dose. The expression levels

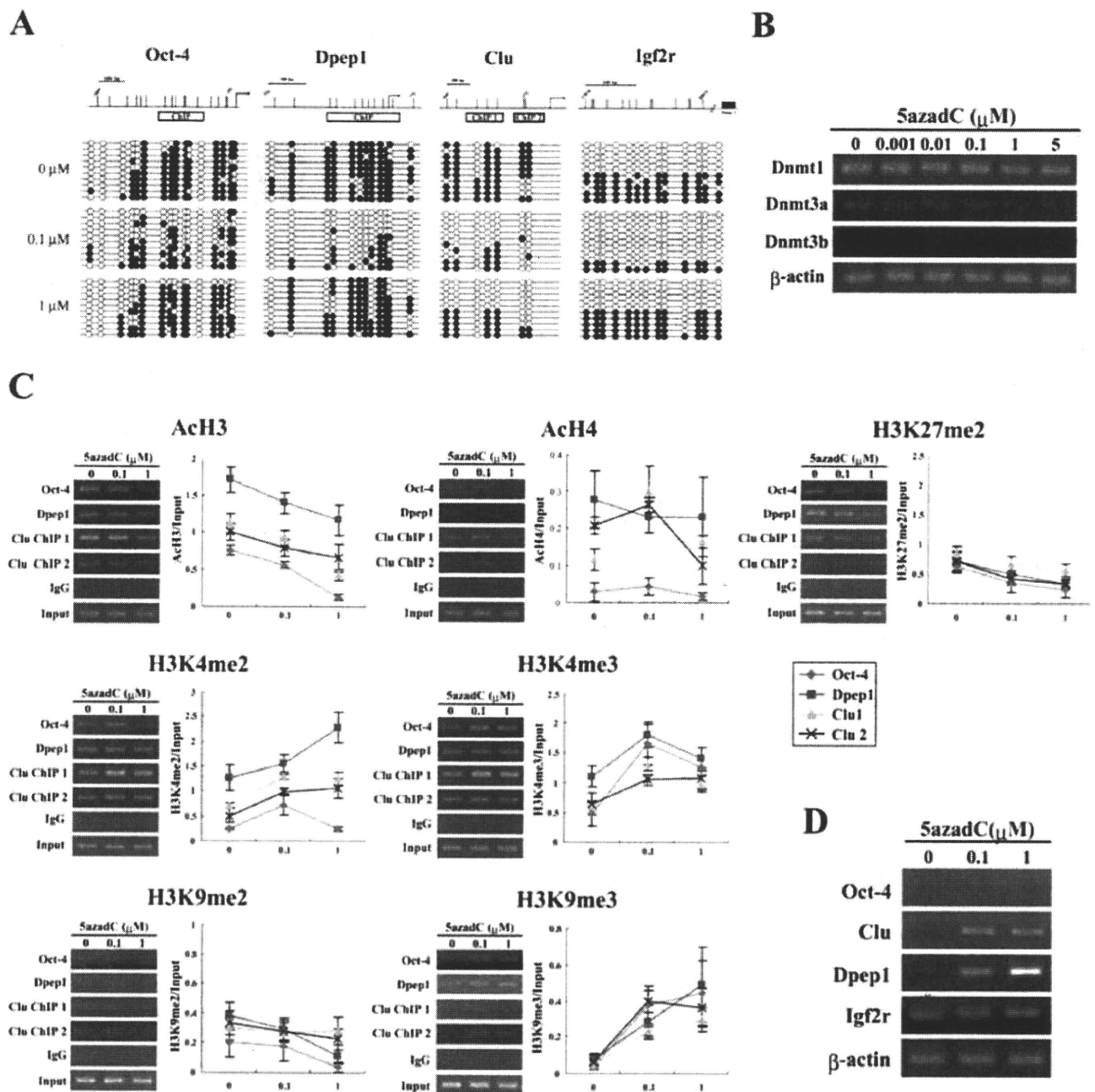


Fig. 2. Effect of 5azadC on DNA methylation, histone modification and gene expression. **A:** DNA methylation analysis of 4 gene loci, *Oct-4*, *Clu*, *Dpep1* and *Igf2r*, using bisulfite sequencing. The top panel shows the location of individual CpG sites (vertical lines) in genic regions amplified by PCR. The 0, 0.1 and 1 μ M 5azadC-treated DNA was bisulfite converted and sequenced. The methylation status of each CpG site is represented by open (unmethylated) or closed (methylated) circles. **B:** Expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* at 0–5 μ M 5azadC analyzed by RT-PCR. The expression level of β -actin was used as the internal control. **C:** Histone modification analysis of 0, 0.1 and 1 μ M 5azadC-treated samples by ChIP. Analyzed regions are indicated by boxed “ChIP” in the schematic diagram in A. DNA immunoprecipitated with respective antibodies was amplified by PCR and electrophoresed (left panels). The relative intensities of PCR bands to those of the input DNA, presented as the means \pm S.E. of 3 independent PCRs of 3 cultures, are shown in the right panels. **D:** RT-PCR results for the expression of each gene following 5azadC treatment.

of *Dnmt1*, *Dnmt3a* and *Dnmt3b* were not affected by 5azadC at concentrations from 0.001 to 5 μ M (Fig. 2B), indicating that the partial retention of methylation observed at high concentrations of 5azadC was not due to increased Dnmt expression.

5azadC induced a unique combination of histone tail modifications

Since 0.1 and 1 μ M 5azadC showed rather unexpected, different demethylation effects on genic regions, we investigated changes in histone modifications by performing a ChIP assay with antibodies against euchromatic and heterochromatic marks. The AcH3 level in genic regions continuously declined as the concentration of 5azadC increased (Fig. 2C), which is in contrast to the condition in decondensed chromatin [26]. Such changes were not observed in AcH4, which was less enriched in genic regions.

Both the H3K4me2 and H3K4me3 levels increased with 5azadC treatment, in almost all investigated regions, and high levels were correlated with increased gene expression (Fig. 2D). Elevation of H3K4me2 in *Dpep1* at 1 μ M 5azadC corresponded to a remarkable transcriptional increase, and the relatively low abundance of H3K4me2 in the *Oct-4* promoter region at any level of 5azadC did not induce *Oct-4* expression. H3K4me3 was more enriched by 0.1 μ M than by 1 μ M 5azadC treatment.

Heterochromatin-associated H3K9me2 and H3K27me2 marks continuously decreased with 5azadC treatment. In contrast, elevation of H3K9me3 was observed, with higher enrichment at 1 μ M compared with 0.1 μ M 5azadC in most regions. Altogether, 5azadC treatment at different concentrations was accompanied by a distinct combination of changes in euchromatic and heterochromatic histone marks in genic regions.

Increase of H3K9me3 correlates with partially methylated regions

Previous studies show that H3K9 methylation directs DNA methylation [34, 35]. To date, our data have shown that 5azadC induces partial demethylation, not complete demethylation, in genic regions. To determine whether increased H3K9me3 is associated with DNA methylation, we investigated enrichment of H3K9 methylation in the *Dpep1* and *Clu* promoter regions in Dnmt-deficient cells. The promoter regions of *Dpep1* and *Clu* were heavily methylated in wild type ES cells and were demethylated in *Dnmt1*^{-/-} and *Dnmt3a*^{-/-}*3b*^{-/-} (Fig. 3A). The increase of H3K9me3 in the genic regions of Dnmt knockout cells was not as obvious as those in the 5azadC-treated NIH/3T3 cells (Fig. 3B). Therefore, increased H3K9me3 correlated with partially methylated regions, such as in 5azadC-treated cells. Similar to 5azadC-treated cells, H3K9me2 decreased in *Dnmt1*^{-/-} cells, but was increased in *Dnmt3a*^{-/-}*3b*^{-/-} cells.

Discussion

The current results demonstrate that 5azadC has differential effects on non-genic repetitive sequence and genic regions (Fig. 4). Importantly, non-genic repetitive sequences are susceptible to 5azadC, whereas genic regions are only demethylated by effective low doses.

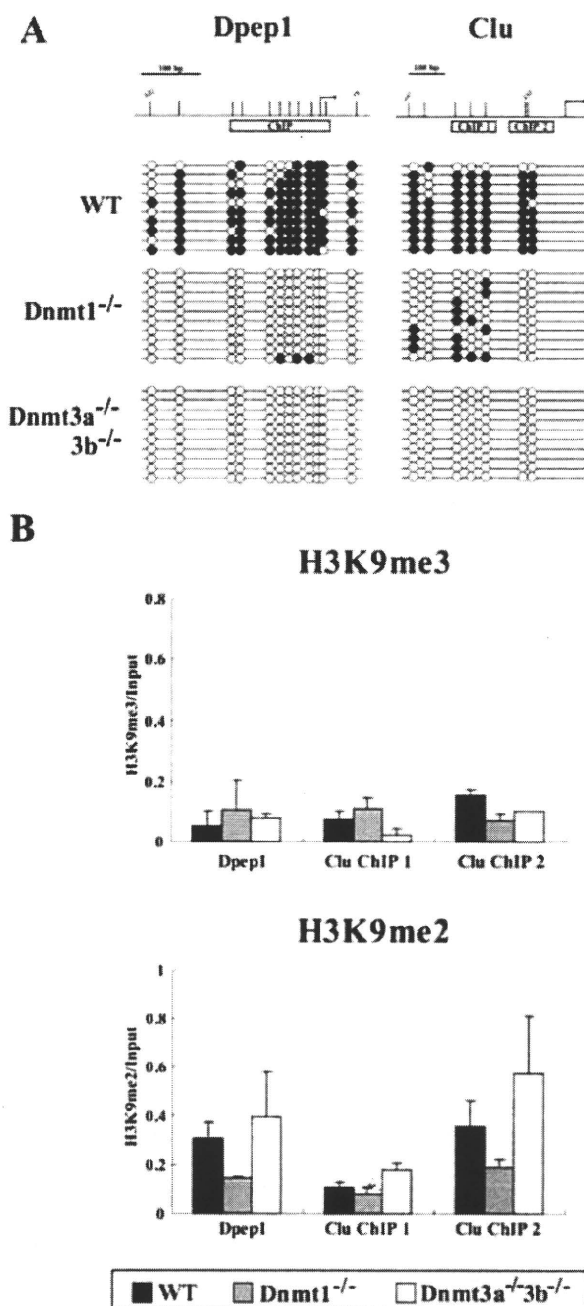


Fig. 3. DNA methylation and H3K9 methylation status of *Dpep1* and *Clu* in Dnmt knockout ES cells. A: DNA methylation analysis of *Dpep1* and *Clu* by bisulfite sequencing. B: H3K9 di- and tri-methylation levels in the regions shown in panel A assessed by ChIP. The bar charts show the relative intensities of PCR bands to those of the input; the values are presented as means \pm S.E. of 3 independent PCRs of 2 cultures.

Demethylation of repetitive sequences was concentration-dependent, whereas genic regions were only demethylated at effective concentrations, but not at higher concentrations. Thus,

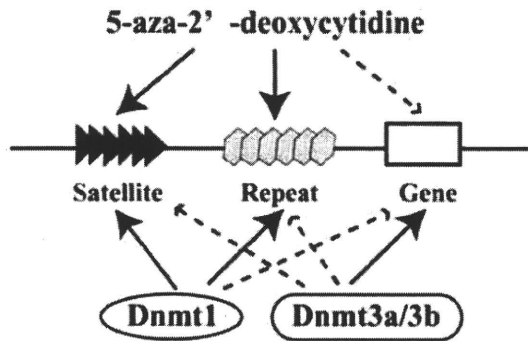


Fig. 4. Summary of the effect of 5azadC on repetitive sequences and genic regions. Repetitive sequences (black arrows) are strongly demethylated by 5azadC, but genic regions are only demethylated at low doses (dotted arrow). The present study suggests the preference of 5azadC for Dnmt1, as Dnmt1 has a functional preference for repeats, whereas Dnmt3a and Dnmt3b functions on genic regions [24].

increasing the treatment dosage might not effectively induce demethylation in targeted genic regions, but will lead to genome-wide hypomethylation in non-cancer cells. Loss of methylation promotes additional genomic changes, including increased mutation rate and pericentromeric rearrangement [17, 36]. Thus, before using 5azadC, precise doses should be determined to achieve localized hypomethylation, but avoid concurrent global hypomethylation.

Some reports have shown that 5azadC treatment is followed by decreased H3K9 methylation [37–39]. Our results also showed decreased H3K9me2, but increased H3K9me3 in all genic regions after 5azadC treatment. H3K9me3, but not H3K9me2, is a mark for cytosine methylation in chromatin regions in *Neurospora crassa* [35]. Thus, increased H3K9me3 might be associated with partial methylation in 5azadC-treated cells, indicating a functional difference between H3K9me2 and H3K9me3 in mammalian cells.

Unusual histone tail modifications were observed following 5azadC treatment, in contrast to their authentic roles in DNA methylation and gene silencing [25, 26]. Such changes might reflect mechanisms to prevent full demethylation in genic regions. As DNA methyltransferases are associated with H3K9 methyltransferases [40, 41], accumulation of histone methyltransferases, which confers increase in H3K9me3, may also attracts Dnmts. Recruitment of MeCPs to the methylated DNA region is associated with a corepressor complex containing mSin3 and histone deacetylases (HDACs), causing reduction of AcH3 [42, 43]. Given that Dnmt1 has preference for repetitive sequence, whereas Dnmt3a and 3b favor gene regions [24], the current study may reflect the preference of 5azadC for Dnmt1 (Fig. 4). Thus, Dnmt3 might be associated with H3K9 methyltransferases for maintaining the methylation level in genic regions due to the functional cooperation of Dnmt1 and Dnmt3 [22–24, 44, 45].

Gene activation by 5azadC seems to involve a complex mechanism of action, in addition to DNA demethylation [37, 39]. We showed that genes were upregulated at 0.1 μM and that expressions

were maintained or increased at 1 μM , despite higher methylation levels observed at 1 μM . Although transcriptional activation could be explained by an increase of H3K4 methylation marks [46], AcH3 concurrently decreased drastically. Interestingly, H3K4me3 abundance was lower in the 1 μM treatment than in the 0.1 μM treatment and was accompanied by DNA hypermethylation. A similar study on cancer genes suggested that histone modifications of demethylated genes do not fully recover to euchromatic states [47]. Interestingly, recent findings have shown that H3K9me3 is associated with active genes [48–49] and is dynamically present in active transcribed regions together with heterochromatin protein, HP1 γ [50], suggesting the role of H3K9me3 in 5azadC-induced gene activation. Thus, gene activation by 5azadC seems to be more complex than the authentic model of epigenetic gene activation and silencing.

Although studies have shown that the toxicity of the 5azadC treatment is not due to a demethylating effect [51], our results raise questions concerning the 5azadC toxicity mechanism. Repetitive sequences were partially demethylated at 0.1 μM and were extensively demethylated at 1 and 5 μM . Concurrent with demethylation, cell viability was minimally affected at 0.1 μM , but was retarded at 1 and 5 μM , reflecting that the induced cytotoxicity might be an effect of global demethylation in the repetitive sequences. In contrast, genic regions were hypomethylated at 0.1 μM , and were methylated at 1 and 5 μM 5azadC, probably as a resistance mechanism against cell death.

This study provides an explanation of the mechanisms behind the dual modes of action of the demethylating drug 5azadC. At low concentrations, the demethylating effect is potent in both repetitive and genic regions. At high concentrations, repetitive sequences are highly demethylated, which potentially leads to toxicity. DNA methylation in genic regions is less affected, which might be due to changes in histone modification attempting to maintain the DNA methylation level. Thus, an appropriate dose of 5azadC should be wisely chosen for therapeutic use.

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Determination of Phthalates in Diet and Bedding for Experimental Animals Using Gas Chromatography-Mass Spectrometry

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Abstract We have developed a gas chromatography-mass spectrometry method to measure five phthalates (dibutyl phthalate, butylbenzyl phthalate, di-2-ethylhexyl phthalate, diisooctyl phthalate, and diisononyl phthalate) in diets and beddings for experimental animals. The recoveries from diets and beddings spiked with five phthalates were 98.8%–148% with coefficients of variation of 0.4%–7.8% for diets and 94.7%–146% with coefficients of variation of 1.0%–5.0% for beddings. We analyzed commercial animal diets and beddings, and found that the levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Keywords Phthalates · Animal diets · Beddings · Gas chromatography-mass spectrometry

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Phthalates are used as additives, as solvents, and as plasticizers in many consumer products. The widespread manufacture, use, and disposal of phthalates have caused ubiquitous environmental pollution and humans are regularly exposed to phthalates (Blount et al. 2000; Silva et al. 2004; Kato et al. 2005). Certain phthalates, such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), have been shown to disrupt development of the reproductive tract in male rodents in an antiandrogenic manner (Parks et al. 2000). Concern has been raised about phthalates in relation to effects on the reproductive tract in adult males and the development of male offspring in humans (Duty et al. 2003; Swan et al. 2005).

When animal toxicology of phthalates is studied, it is necessary to take into account the exposure from the diet and the experimental environment. Since the complete exclusion of phthalate may well be impossible, the precise concentration of phthalates in the diet, bedding and water used for feeding, the air in the experimental animal room, etc. could be important information needed to attain reliability of animal experiments. To our knowledge, no study of phthalate contamination of diets or experimental environments has been reported.

We have developed a gas chromatography-mass spectrometry (GC-MS) method to determine five commonly used phthalates (DBP, DEHP, BBzP, DINP and diisooctyl phthalate (DIOP)) in animal diets and beddings. The method was used for the analysis of commercial diets and beddings for experimental animals. Additionally, in order to investigate the other causes of rodent exposure to phthalates in feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room.

Materials and Methods

DBP, DEHP, BBzP, DIOP, DINP, DBP-3,4,5,6-d₄, DEHP-3,4,5,6-d₄, BBzP-3,4,5,6-d₄, and DOP-3,4,5,6-d₄ were purchased from Kanto Chemical (Tokyo, Japan). DNP-3,4,5,6-d₄ was purchased from Hayashi Pure Chemicals (Osaka, Japan). Phthalic acid esters and analytical grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical. Pesticide analysis grade sodium sulfate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 µm pore size) was purchased from Varian (CA, USA). Florisil® PR was purchased from Wako Pure Chemical Industries. The water used for extraction was prepared by washing distilled water with hexane.

Rodent diets were obtained from CLEA Japan Inc. (Tokyo, Japan), Oriental Yeast Co. Ltd (Tokyo, Japan), and Nihon Nosan Kogyo Co. Ltd (Tokyo, Japan). Animal beddings were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan), Japan SLC, Inc. (Hamamatsu, Japan), CLEA Japan Inc., and Oriental Yeast Co. Ltd.

Since some of phthalates analyzed in this study are very abundant and contamination is practically unavoidable (Takatori et al. 2004), the contamination preventive measures were given as follows. No plastic apparatus was used in these experiments. The reagents and solvents were used just after opening or were not left for a long time after opening. All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil® PR were heated at 200°C for 2 h. Before starting the experiment, we took various precautionary measures, such as shortening fingernails, washing hands well with soap, covering the laboratory bench with aluminum foil, etc. A blank analysis was carried out before sample analysis in each batch. The evaporator was cleaned before use by evaporating 10 mL of acetone.

A Florisil and Bondesil-PSA column was prepared by packing Florisil (1 g), Bondesil-PSA (0.5 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm × 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Stock solutions of native standards (DBP, DEHP, BBzP, DIOP, DINP) and isotope-labeled internal standards (DBP-3,4,5,6-d₄, DEHP-3,4,5,6-d₄, BBzP-3,4,5,6-d₄, DOP-3,4,5,6-d₄, DNP-3,4,5,6-d₄) were prepared in acetonitrile and stored at -20°C in Teflon-capped glass bottles until use. They were mixed at the desired ratio and serially diluted for calibration curves. The peak area ratio of analyte to isotope-labeled internal standard was used for quantification.

Reproducible calibration curves for five phthalates were obtained with correlation coefficients greater than 0.999 (known concentration vs analyte/internal standard). They were linear over the range of 10–1,000 ng/mL for DBP, BBzP and DEHP and 50–1,000 ng/mL for DIOP and DINP.

The present method for analysis of phthalates in diets is a modification of that described by Tsumura et al. (2001). A sample of the diet (5 g) was weighed into a centrifuge tube, followed by water (5 mL) and acetonitrile (20 mL), and spiked with isotopically labeled internal standards (4 µg/mL, 25 µL). The sample was homogenized for 1 min using a homogenizer (Phycotron, Microtec Co. Ltd), and then centrifuged at 3,000 rpm for 5 min. The acetonitrile layer was collected, and the residual homogenate was extracted again with 75% acetonitrile in water (20 mL). The acetonitrile layers were combined, sodium chloride (1.5 g) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was collected, hexane saturated with acetonitrile (4 mL) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in water (2 mL) and hexane (5 mL) and the mixture was shaken vigorously for 30 s. The solution was centrifuged at 3,000 rpm for 5 min, and the hexane layer was removed and saved. Hexane (3 mL) was added to the water layer, and extracted as described above. The hexane layers were combined and loaded onto a Florisil and Bondesil-PSA dual layer column, which was preconditioned with acetone (10 mL) and hexane (10 mL). After washing the column with hexane (3 mL), phthalates were eluted with 5% acetone in hexane (10 mL). The eluate was evaporated to dryness under reduced pressure at 35°C, and then dissolved in hexane (1 mL). An aliquot of each sample (2 µL) was injected into a GC-MS system.

The present method for analysis of phthalates in bedding is a modification of that described by Tsumura et al. (2001). A sample of bedding for experimental animals (5 g) was weighed into a centrifuge tube followed by acetone (40 mL), and spiked with isotopically labeled internal standards (4 µg/mL, 25 µL). The sample was left for 1 h and then shaken vigorously for 10 min. The solution was evaporated to dryness under reduced pressure at 35°C. The residue was treated as described for diets.

Analysis of phthalates in water was carried out as described (Glick 1998). Water (30 mL) from a tap in the experimental animal room was weighed into a centrifuge tube followed by hexane (10 mL), spiked with isotopically labeled internal standards (4 µg/mL, 25 µL), then shaken vigorously for 10 min. The hexane layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in hexane (1 mL) and an aliquot of each sample (2 µL) was injected into a GC-MS system.

Analysis of phthalates in air was carried out according to the method reported by the Ministry of Health, Labour and Welfare of Japan (2000). Sampling was done using an SP208-10L (GL Science, Tokyo, Japan) pump attached to an AERO cartridge SDB 400 (GL Science) with a sampling rate of 5 L/min for 24 h. After the addition of isotopically labeled internal standards (4 µg/mL, 25 µL), the collected phthalates were extracted with acetone (2 mL) by ultrasonication for 10 min. After centrifugation at 3,000 rpm for 5 min, the supernatant was collected. An aliquot of each sample (2 µL) was injected into a GC-MS system.

GC-MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30 m HP-5MS SV column (J & W Scientific, CA, USA) with 0.25 mm i.d. and 0.5 µm film thickness was used. The initial oven temperature was 80°C. After holding at the initial temperature for 2 min, the temperature was increased to 240°C at a rate of 40°C/min, and then to 300°C at a rate of 10°C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min). The ion source temperature was 230°C and electron ionization was used as the ionization mode. The

injection port was kept at 250°C. The ions used for selected ion monitoring are summarized in Table 1. DIOP was determined as the two highest peaks, and DINP as the five main peaks in the chromatogram as described by Tsumura et al. (2001).

Results and Discussion

The recoveries from diets and beddings spiked with 100 ng/g of DBP, BBzP and DEHP and 500 ng/g of DIOP and DINP were examined. Overall recoveries of the three repeated measurements are summarized in Table 2; these values were 98.8%–148% with coefficients of variation (CV) of 0.4%–7.8% for diets and 94.7%–146% with CV of 1.0%–5.0% for beddings. Recoveries of BBzP, DIOP and DINP were satisfactory (98.8%–113% for diets; 94.7%–119% for beddings); however, those of DBP and DEHP were relatively high (123%–148%).

The elimination of phthalate contamination is key for precise measurement. Blanks originating from sample analysis were therefore examined using phthalates-free water instead of diet and bedding. The blanks from diet sample analysis were 5.0 (±1.9) ng/g for DEHP, <3.0 ng/g for DBP and BBzP, and <20 ng/g for DIOP and DINP (n = 5), and those from bedding sample analysis were 6.6 (±2.5) ng/g for DEHP, 3.1 (±0.3) ng/g for DBP, <3.0 ng/g for BBzP, and <20 ng/g for DIOP and DINP (n = 5). The limit of quantification (LOQ) for each of five phthalates was calculated as 10S₀, where S₀ is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as 10S₁, where S₁ is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of standard sample. The LOQ for each of five phthalates is summarized in Table 2. With our careful control of contamination, sample analysis was achieved with a low-level background, which allowed us to evaluate the amount of phthalates precisely. Typical chromatograms of a standard mixture and of diet and bedding samples are shown in Fig. 1.

Table 1 Retention times, quantification and confirmation ions for the measured phthalates: dibutyl phthalate, DBP; butyl benzyl phthalate, BBzP; di-2-ethylhexyl phthalate, DEHP; diisooctyl phthalate, DIOP; diisononyl

Analyte	Retention time (min)	Quantification ion	Confirmation ion
DBP	8.0	149	205, 223
DBP-d ₄	8.0	153	209, 227
BBzP	10.2	149	91, 206
BBzP-d ₄	10.2	153	91, 210
DEHP	11.3	149	167, 279
DEHP-d ₄	11.3	153	171, 283
DIOP	11.5–12.1	149	279
DOP-d ₄	12.5	153	283
DINP	12.8–13.9	149	293
DNP-d ₄	14.2	153	297

Table 2 Recoveries and LOQ of phthalates

Analyte	Diet			Bedding		
	Recovery (%)	CV ^a (%)	LOQ ^b (ng/g)	Recovery (%)	CV (%)	LOQ (ng/g)
DBP	123	7.8	10	132	5.0	5
BBzP	98.8	0.4	10	102	1.0	10
DEHP	148	1.8	20	146	4.2	25
DIOP	109	0.5	50	119	4.3	50
DINP	113	1.3	50	94.7	4.3	50

Results are means of three replicate determinations

^a CV coefficients of variation

^b LOQ limit of quantification

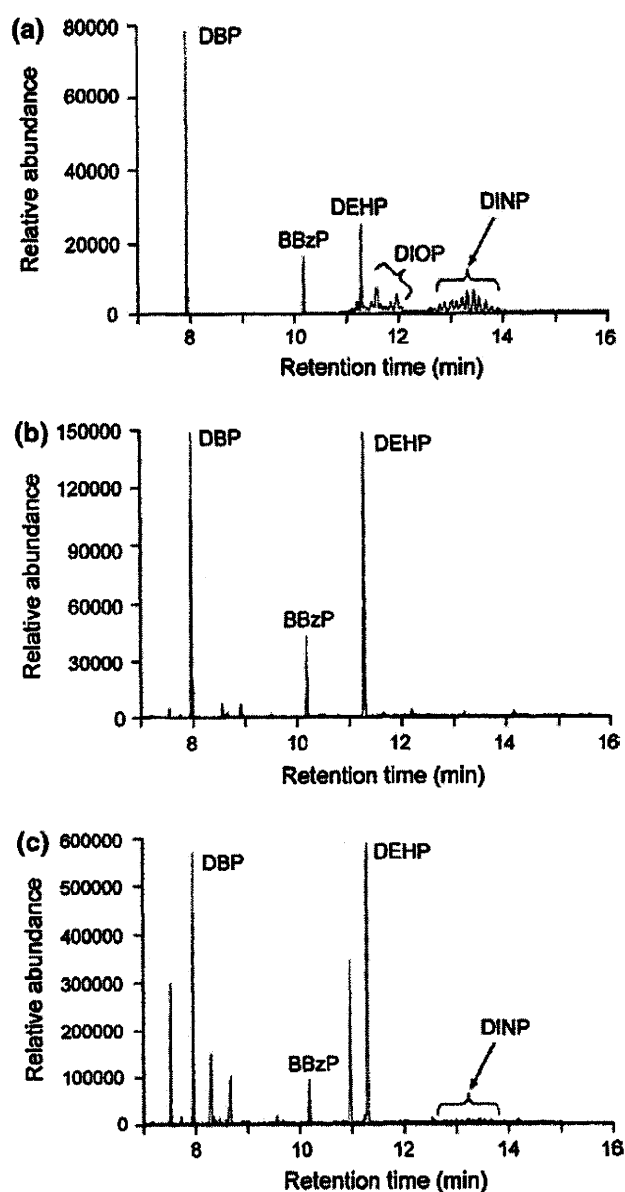


Fig. 1 Typical selected ion monitoring chromatograms of a a standard mixture of five phthalates, b diet and c bedding

We evaluated the suitability of the method for detecting the levels of phthalate in 12 commercial animal diets and 13 bedding materials (Table 3). All samples were analyzed just after opening the package. Two of the most frequently detected phthalates were DBP, which was found in all diets and beddings, and DEHP, which was found in all diets and in 77% of the beddings. BBzP was not detected in most samples (15%–33% detectable), and DINP was detected in only one sample of bedding. DIOP was not detected in any diet or bedding. The levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Table 3 Concentrations of phthalates in commercial animal diets and beddings (ng/g)

Sample no.	DEHP	DBP	BBzP	DIOP	DINP	Sum
<i>Diet</i>						
D1	143	179	<LOQ	<LOQ	<LOQ	322
D2	160	46.3	<LOQ	<LOQ	<LOQ	206
D3	116	25.1	<LOQ	<LOQ	<LOQ	141
D4	511	146	157	<LOQ	<LOQ	814
D5	156	41.4	<LOQ	<LOQ	<LOQ	197
D6	146	134	<LOQ	<LOQ	<LOQ	280
D7	118	36.1	<LOQ	<LOQ	<LOQ	154
D8	205	80.4	<LOQ	<LOQ	<LOQ	285
D9	281	205	22.2	<LOQ	<LOQ	508
D10	422	403	18.4	<LOQ	<LOQ	843
D11	257	344	<LOQ	<LOQ	<LOQ	601
D12	431	944	33.4	<LOQ	<LOQ	1,410
<i>Bedding</i>						
B1	449	1,380	<LOQ	<LOQ	<LOQ	1,830
B2	<LOQ	757	<LOQ	<LOQ	<LOQ	781
B3	280	19.6	<LOQ	<LOQ	<LOQ	300
B4	498	765	<LOQ	<LOQ	<LOQ	1,260
B5	<LOQ	6.0	<LOQ	<LOQ	<LOQ	20.5
B6	187	128	<LOQ	<LOQ	<LOQ	315
B7	262	130	440	<LOQ	<LOQ	832
B8	420	66.4	<LOQ	<LOQ	<LOQ	486
B9	132	538	<LOQ	<LOQ	<LOQ	670
B10	5,070	1,390	900	<LOQ	198	7,560
B11	547	381	<LOQ	<LOQ	<LOQ	928
B12	443	55.5	<LOQ	<LOQ	<LOQ	499
B13	<LOQ	30.5	<LOQ	<LOQ	<LOQ	46.5

Results are means of duplicate determinations

The results of our study demonstrated that the levels of phthalates varied from sample to sample. The highest value for the concentration of five phthalates in diets (1,410 ng/g) was 10 times higher than the lowest value. If a rat weighing 150 g eats 12 g of this diet daily (Poon et al. 1997), 16,920 ng of phthalates would be ingested, which corresponds to an oral administration of 0.113 mg/kg per day. The levels of phthalates in the diets analyzed in this study are about 20–2,000 times lower than those fed in reported developmental and reproductive toxicity experiments (Poon et al. 1997; Tyl et al. 1988; Arcadi et al. 1998). However, it is necessary to take into account the progress of the contamination during storage after opening the packaging of diets.

In terms of the contamination level in beddings, one sample (no. B10), made of recycled paper, showed a remarkable level of contamination at a concentration of

Table 4 Concentrations of five phthalates in water supplied to the animals, and samples of air taken from the animal room

Sample	DEHP	DBP	BBzP	DIOP	DINP	Sum
<i>Water (ng/mL)</i>						
Water1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
<i>Air (ng/m³)</i>						
Air1	31.4	357	<LOQ	<LOQ	<LOQ	388
Air2	27.6	320	<LOQ	<LOQ	<LOQ	348
Air3	31.9	261	<LOQ	<LOQ	<LOQ	293

Results are means of duplicate determinations

five phthalates of 7,560 ng/g. Although we cannot estimate the exposure to phthalates from beddings through oral intake by licking and dermal absorption, it would be better not to use heavily contaminated bedding to avoid the potential exposure to high levels of phthalates.

In order to investigate the other causes of rodent exposure to phthalates under various feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room (Table 4). None of the five phthalates was detected in the water (lower limits of quantification: DBP, BBzP and DEHP, 0.5 ng/mL; DIOP and DINP, 2 ng/mL). DBP and DEHP were detected in all samples of air with concentration ranges of 261–357 ng/m³ and 27.6–31.9 ng/m³, respectively. Other three phthalates were not detected (lower limits of quantification: BBzP, 5 ng/m³; DIOP and DINP, 20 ng/m³). The contamination level in air taken from the animal room was 293–388 ng/m³ for five phthalates. The average exposure from air amounts to 101 ng/day when we assume an average daily inhalation of 0.29 m³ air per rat (The ICH Steering Committee 1997). This level of exposure by inhalation would be about 125 times lower than the intake from diets. These results indicate that the major source of exposure to phthalates may be the diet, although the possibility cannot be denied completely that direct incorporation of these phthalates from the lung without hydrolysis occurs.

In summary, we have developed a GC-MS method to determine five phthalates in diets and beddings used for experimental animals. We analyzed commercial animal diets and beddings, and found that both of them were polluted by phthalates, especially DBP and DEHP, suggesting that dietary exposure to phthalates routinely occurs. The total exclusion of phthalates from the experimental environment is probably impossible. Therefore, the contamination levels in the diet and bedding should be measured. Additionally, it would be wise to monitor the

contamination levels of water and the air in the animal room, although the exposure from water and air was low.

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Determination of Five Phthalate Monoesters in Human Urine Using Gas Chromatography-Mass Spectrometry

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Abstract We have developed a gas chromatography-mass spectrometry (GC-MS) method to determine five phthalate monoesters (monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-(2-ethylhexyl) phthalate (MEHP), monoisononyl phthalate (MINP) and monobenzyl phthalate (MBz)) in human urine. Human urine samples were subjected to enzymatic deconjugation of the glucuronides followed by extraction with hexane. The extracted phthalate monoesters were methylated with diazomethane, purified on a Florisil column and then subjected to GC-MS analysis. The recoveries from urine spiked with five phthalate monoesters were 86.3%–119% with coefficients of variation of 0.6%–6.1%. We measured phthalate monoester levels in human urine by analyzing 36 samples from volunteers. MBP and MEP were detected in all

samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINPs were not detected in most samples (6% detectable). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP than men. The estimated daily exposure levels for the four parent phthalates excluding diisononyl phthalate ranged from 0.27 to 5.69 $\mu\text{g}/\text{kg}/\text{day}$ (median).

Keywords Phthalate monoesters · Exposure · Urine · Gas chromatography-mass spectrometry

Phthalates, diesters of phthalic acid, are widely used in many consumer products and as additives, solvents and plasticizers. Humans are ubiquitously exposed to phthalates (Blount et al. 2000a; Silva et al. 2004) and patients undergoing medical procedures such as transfusions, dialysis, cardiopulmonary bypass or aphaeresis are potentially more heavily exposed (Calafat et al. 2004; Green et al. 2005; Inoue et al. 2005). Certain phthalates such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP) lead to testicular toxicity in rodents in an antiandrogenic manner (Parks et al. 2000). DBP, DEHP, BBzP, and their metabolites, mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and mono-(2-ethylhexyl) phthalate (MEHP) are teratogenic in animals (Gray and Gangolli 1986; Mylchreest et al. 1998; Parks et al. 2000; Ema and Miyawaki 2001a, b). It has been reported that urinary concentrations of four phthalate metabolites, monoethyl phthalate (MEP), MBP, MBzP, monoisobutyl phthalate, were significantly associated with reduced anogenital distance in human male infants which was the first

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demonstration of the subtle developmental effects in human (Swan et al. 2005).

More recent studies concerning general human exposure to phthalates have been carried out by measuring phthalate monoester metabolites mainly in urine (Koch et al. 2003; Hauser 2008), because phthalate diesters metabolize rapidly to their monoesters and are excreted in urine either as hydrolytic monoesters or the oxidative products (Silva et al. 2003). Furthermore, potential problems of diester contamination during sample collection, storage and analysis procedures may make it difficult to measure low baseline levels of phthalate diesters in serum and/or urine (Blount et al. 2000b). Several reports have described the assessment of phthalate exposure in the general population in the USA (Kohn et al. 2000) and Europe (Koch et al. 2003). However, reports relating to the Japanese are limited; Itoh et al. (2005) measured MBP and MEHP in urine samples from the general population.

For the quantitative detection of phthalate monoester metabolites in urine, a high-performance liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been developed (Silva et al. 2003). All the above-mentioned reports used LC-MS/MS method. To our knowledge, there are no reports of studies on phthalate monoester determination in urine using gas chromatography-mass spectrometry (GC-MS). In the present study we have developed a new method for the quantitative determination of five phthalate monoesters (MEP, MBP, MEHP, monoisononyl phthalate (MINP) and MBzP) in human urine using GC-MS. The method was used for analysis of human urine samples.

Materials and Methods

The study participants were 36 volunteers who were recruited from the staff of Aichi Prefectural Institute of Public Health, Nagoya, Japan. All of the volunteers reside in the Aichi Prefecture, Japan. Of the participants, 64% (23/36) were men and 36% (13/36) were women. 31% (11/36) were under 39 years of age, 25% (9/36) were between 40 and 49 years of age, and 44% (16/36) were over 50 years of age.

MEP, MBP, mono-2-ethylhexyl phthalate (MEHP), monoisononyl phthalate (MINP), MBzP, and their $^{13}\text{C}_4$ -labeled internal standards were purchased from Cambridge Isotope Laboratories (MA, USA). Florisil[®] PR, β -glucuronidase solution from *Escherichia coli* and pesticide analysis grade sodium sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 μm pore size) was purchased from Varian (CA, USA). Phthalic acid esters, analysis grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical (Tokyo, Japan). The water

used for the extraction was prepared by washing distilled water with hexane.

All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil[®] PR were heated at 200°C for 2 h. A reagent blank was analyzed before sample analysis in each batch.

A Florisil column was prepared by packing Florisil (1 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm \times 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Diazomethane was prepared by adding 0.5 g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to 3 mL of 20% sodium hydride solution; the diazomethane gas thus generated was dissolved in 10 mL of ice-cold methyl *t*-butyl ether.

Urine (2 mL) was placed into a centrifuge tube to which ammonium acetate (0.5 mL) was added and the sample was spiked with isotopically labeled internal standards (50 μL , 1 $\mu\text{g}/\text{mL}$). After β -glucuronidase (30 μL , 50 units/mL) was added, the sample was incubated at 37°C for 60 min. The sample solution was then acidified to pH 2 with 10% sulfuric acid. After adding hexane (5 mL) and sodium chloride (1 g), the sample solution was mixed for 3 min by vigorous shaking and then centrifuged at 3,000 rpm for 5 min. The hexane layer was collected and dried under a stream of nitrogen gas at 35°C. Diazomethane (0.5 mL) was added to the residue and the mixture was allowed to stand for 30 min at room temperature. The solution was dried under a stream of nitrogen gas at 35°C. The residue was dissolved in hexane (5 mL) and then loaded onto a Florisil column, which had been preconditioned with acetone (10 mL) and hexane (10 mL). After washing with hexane (3 mL), the phthalate monoester fraction was eluted with 5% acetone-hexane (10 mL). The eluate was concentrated to 1.0 mL under a stream of nitrogen gas at 35°C. An aliquot of each sample (2 μL) was injected into a GC-MS system.

GC-MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30-m HP-5MS SV column (J & W Scientific, CA, USA) of i.d. 0.25 mm and a film thickness of 0.5 μm was used. The initial oven temperature was 80°C. After holding at the initial temperature for 3 min, the temperature was increased to 240°C at a rate of 20°C/min, and then to 300°C at a rate of 10 °C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min. The ion source temperature was 230°C and electron ionization was used as the ionization mode. The injection port was kept at 250°C. The ions used for selected ion monitoring (SIM) are summarized in Table 1; the ions observed as the base peak were used for quantification and

Table 1 Monitored ions for quantification and confirmation, and retention times for the measured methyl derivatives of phthalate monoesters

Analyte	Monitored ions (<i>m/z</i>)		Retention time (min)
	Quantification	Confirmation	
MEP	163	149, 176	9.04
MEP- ¹³ C ₄	167	153, 180	9.04
MBP	163	149, 181	10.18
MBP- ¹³ C ₄	167	153, 185	10.18
MEHP	163	149, 181	12.09
MEHP- ¹³ C ₄	167	153, 185	12.09
MINP	163	149, 181	12.42
MINP- ¹³ C ₄	167	153, 185	12.42
MBzP	163	91, 164	12.71
MBzP- ¹³ C ₄	167	91, 168	12.71

Abbreviations: MEP monoethyl phthalate, MBP mono-*n*-butyl phthalate, MEHP mono-2-ethylhexyl phthalate, MINP monoisononyl phthalate, MBzP monobenzyl phthalate

the second and third most abundant ions were used for confirmation. The concentrations of five phthalate monoesters in urine were corrected with ¹³C₄-labeled internal standards. Reproducible calibration curves for the methyl derivatives of the five phthalate monoesters were obtained with correlation coefficients greater than 0.999 (known concentration versus analyte/internal standard) and they were linear over the range of 2–100 ng/mL.

We calculated the estimated daily intake of phthalates by using the following equation (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005): intake (μg/kg/day) = ME × CE / (f × 1,000) × (MW_d/MW_m). ME is the creatinine-adjusted concentration of the phthalate monoester (μg/g creatinine), CE is the personal daily creatinine excretion (mg/kg/day), which was calculated using the following equation (Kawasaki et al. 1991): CE (male) = -12.63 × age + 15.12 × body weight (kg) + 7.39 × height (cm) - 79.90, or CE (female) = -4.72 × age + 8.58 × body weight (kg) + 5.09 × height (cm) - 74.95. The values of the ratios of urinary excretion to total elimination (f) were 0.024 for DEHP, 0.69 for DEP and DBP, and 0.73 for BBzP (Koch et al. 2003). MW_d and MW_m are the molecular weights of the parent diesters and their monoesters, respectively.

The Mann–Whitney *U*-test was used for statistical analyses. Differences were considered to be significant at *p* < 0.05.

Results and Discussion

For extraction of phthalate monoesters in biological samples, different solid-phase extraction (SPE) and solvent extraction methods are used. Among a variety of

commercially available solid-phase extraction cartridges, we examined three types of cartridge; Absolut NEXUS (VARIAN), GL-Pak Glass SPE PLS-3 (GL Science, Tokyo, Japan) and Oasis[®] HLB Glass (Waters, MA, USA). However, the background levels of MEHP and MBP for all cartridges were high at about 60 and 8 ng/mL, respectively, even though the cartridges were prewashed with acetone and hexane repeatedly. We then examined hexane and acetonitrile for extraction of phthalate monoesters. Although both solvents extracted phthalate monoesters in urine effectively, acetonitrile also extracted co-existing substances in urine. Hexane was consequently selected as the extraction solvent.

For purification of methyl derivatives of phthalate monoesters methylated with diazomethane, we examined the Florisil single layer column and the Florisil and Bondesil-PSA dual layer column. A Florisil single layer column was selected because both columns showed the same clean-up effect. For elution from a Florisil column, we examined a mixture of solvents; acetonitrile/hexane and acetone/hexane. Acetone/hexane was selected because this solvent mixture gave the most satisfactory recoveries, while acetonitrile/hexane gave poor recoveries of methylated MEP (<10%). The optimized procedures including the extraction followed by Florisil column purification are described in the Materials and Methods section. Typical SIM chromatograms of a mixture of methyl derivatives of five standard phthalate monoesters and a urine sample are shown in Fig. 1.

The recoveries from urine spiked with 250 ng/mL of each of the five phthalate monoesters were examined by calculating the ratio of the amount of analytes recovered

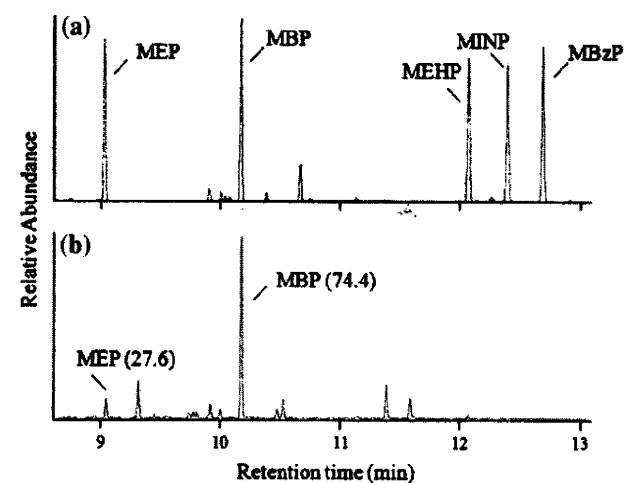


Fig. 1 Typical SIM chromatograms of **a** a mixture of methyl derivatives of five standard phthalate monoesters and **b** a urine sample. Numbers in parentheses indicate the found concentrations (ng/mL)

Table 2 Recoveries and LOQ of phthalate monoesters

Analyte (ng/mL)	Recovery (%)	CV ^a (%)	LOQ ^b
MEP	86.3	2.2	1
MBP	119	6.1	1
MEHP	100	0.6	5
MINP	98.0	0.8	1
MBzP	89.1	2.1	5

Results are means of three replicate determinations

^a CV coefficients of variation

^b LOQ limit of quantification

after Florisil column purification to the amounts originally added. Overall recoveries and coefficients of variation were found to be satisfactory; these values were 86.3%–119% and 0.6%–6.1%, respectively (Table 2). To determine the background levels of the five phthalate monoesters originating from sample preparation, a blank test was carried out using hexane-washed water instead of urine. The blanks ($n = 3$) contained < 1.0 ng/mL for MBP, $1.6 (\pm 0.09)$ ng/mL for MEP, $3.0 (\pm 0.5)$ ng/mL for MEHP, < 1.0 ng/mL for MINP and < 5.0 ng/mL for MBzP. The limit of quantification (LOQ) for each of the five phthalate monoesters was calculated as $10S_0$, where S_0 is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as $10S_1$, where S_1 is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of the standard sample. The LOQ for each of the five phthalate monoesters is summarized in Table 2. With careful control of contamination, as described in the Materials and Methods section, sample analysis was achieved with a low-level background, which allowed us to evaluate precisely the concentration of phthalate monoesters.

This method was then applied to the analysis of urine samples obtained from 36 volunteers (Table 3). MBP and MEP were detected in all samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINP was not detected in most samples

Table 3 Concentrations of phthalate monoesters in urine

Analyte	No. of Positives	Concentration			
		Measured (ng/mL)		Creatinine-adjusted ($\mu\text{g/g}$ creatinine)	
		Median	Range	Median	Range
MBP	36 (100) ^a	60.0	9.09–194	58.7	22.8–554
MEP	36 (100)	10.7	1.36–1,350	13.7	3.06–944
MBzP	27 (75)	10.9	<LOQ–40.6	11.4	<LOQ–39.4
MEHP	20 (56)	5.75	<LOQ–29.6	7.76	<LOQ–56.2
MINP	2 (6)	<LOQ	<LOQ–3.00	<LOQ	<LOQ–6.38

^a Numbers in parentheses indicate percentages

(detectable in 6%). The concentrations of MBP and MEP showed larger inter-individual variation: 9.09–194 ng/mL for MBP and 1.36–1,350 ng/mL for MEP, whereas those of MBzP, MEHP and MINP showed less inter-individual variation; $< \text{LOQ}$ –40.6 ng/mL for MBzP, $< \text{LOQ}$ –29.6 ng/mL for MEHP and $< \text{LOQ}$ –3.00 ng/mL for MINP. We speculate that these results may reflect the differences in the individuals' use of products containing the parent phthalate diesters. DBP and diethyl phthalate (DEP) are used mostly in consumer products (detergents, soaps, cosmetics, shampoo, and perfumes), while BBzP, DEHP and DINP are used primarily for industrial purposes as plasticizers (Silva et al. 2004; Mortensen et al. 2005). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP (114 and 110 $\mu\text{g/g}$ creatinine) than did men (58.7 and 12.6 $\mu\text{g/g}$ creatinine). The fact that women had higher concentrations of MBP and MEP than men was most likely attributable to women's increased use of personal care products, such as hair care products, cosmetics, and perfumes as indicated by Silva et al. (2004). Urinary levels of phthalate monoesters in approximately 2,540 samples collected from participants in the National Health and Nutrition Examination Survey (NHANES), 1999–2000 in the USA have been reported (Silva et al. 2004). The median concentration of MBP and MEHP was 2.7- and 2.5-fold lower in the NHANES 1999–2000 population (21.9 and 3.08 $\mu\text{g/g}$ creatinine) than in the present study (58.7 and 7.73 $\mu\text{g/g}$ creatinine), whereas that of MEP in the NHANES 1999–2000 population (141 $\mu\text{g/g}$ creatinine) was almost tenfold higher than that in the present study (13.7 $\mu\text{g/g}$ creatinine). The median concentrations of MBzP were almost the same in both studies (13.3 $\mu\text{g/g}$ creatinine for NHANES 1999–2000 population and 11.4 $\mu\text{g/g}$ creatinine for the present study). Despite the limited numbers of samples in the present study, these results may represent the differences in the use of phthalate diesters between the USA and Japan.

Human exposure to phthalate diesters has been estimated by calculating the daily intake using the concentrations of the phthalate monoesters in urine (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005). Estimates of the intake of four phthalate diesters excluding MINP are shown

Table 4 Comparison of the intake estimates for four phthalate diesters

	Phthalate diester	Median intake value ($\mu\text{g}/\text{kg}/\text{day}$)				TDI ($\mu\text{g}/\text{kg}/\text{day}$)
		This study (n = 36)	Kohn et al. (n = 289)	Koch et al. (n = 85)	Itoh et al. (n = 36)	
^a Numbers in parentheses indicate the recalculated intake value by using the fractionary urinary excretion factor of 0.024 reported by Koch et al. (2003)	DEHP	5.69	0.71 (3.14) ^a	10.3	1.8 (5.48)	40–140
	DBP	1.47	1.5	5.22	1.3	100
	DEP	0.29	12	2.32	–	5,000
	BBzP	0.27	0.88	0.60	–	200

in Table 4 together with other previous assessments. The median values in the present study were highest for DEHP intake and decreased in the order DEHP, DBP, DEP, and BBzP. The median values of DEHP intake were higher in the present study (5.69 $\mu\text{g}/\text{kg}/\text{day}$) than those reported by Kohn et al. (2000) (0.71 $\mu\text{g}/\text{kg}/\text{day}$) and Itoh et al. (2005) (1.8 $\mu\text{g}/\text{kg}/\text{day}$), and were lower than those reported by Koch et al. (2003) (10.3 $\mu\text{g}/\text{kg}/\text{day}$). However, previous assessments used different fractional excretion values, suggesting that the calculated daily intake values have inherent uncertainties. When DEHP intake values are recalculated using the fractional urinary excretion factor (0.024) reported by Koch et al. (2003), these values range from 3.14 to 10.3 $\mu\text{g}/\text{kg}/\text{day}$ (Table 4) which are comparatively similar values. The establishment of a method for calculating the intake estimates remains to be elucidated.

In summary, we have developed a gas chromatography–mass spectrometry method to determine the concentration of five phthalate monoesters in human urine. Using this method we were able to measure precisely the level of phthalate monoesters in human urine obtained from 36 volunteers. This method will help to evaluate human exposure to phthalates.

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8. 子宮筋腫のゲノムワイド DNA メチル化プロファイル解析

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われわれは、子宮筋腫の発生・進展機序の解明を目的として、子宮筋腫はエピゲノム異常により発生するという新規の仮説に基づいて基礎的研究に取り組んでいる。まず、子宮筋腫のDNAメチル化プロファイルをゲノムワイドに解析したところ、子宮筋腫では、ゲノムワイドに多くのメチル化異常が生じていることが明らかになった。また、子宮筋腫はエストロゲンに対する感受性が高い腫瘍であるが、DNAメチル化異常などのエピジェネティックな機構がエストロゲン作用を修飾し、筋腫の発生・発育に関与する可能性も示された。子宮筋腫研究の新しい視点として、エピジェネティクスの重要性が確認された。

はじめに

子宮筋腫（子宮平滑筋腫）は、主に子宮平滑筋細胞に由来する良性の腫瘍である。女性生殖器に発生する腫瘍性疾患のなかで最も頻度が高く、性成熟期女性の20～30%に子宮筋腫が認められる。良性疾患ではあるが、月経痛や過多月経などの症状から治療を要する場合も多い他、不妊症や流産の原因にもなる。治療としては、腫瘍の縮小や症状の軽減のため薬物療法も行われるが、根治のためには外科的な子宮摘出術や子宮

筋腫核出術が必要である。子宮筋腫の頻度の高さから考えれば、女性のquality of lifeを著しく損なうだけでなく、社会活動の制限や治療費など社会的損失はきわめて大きい。したがって、子宮筋腫の発症予防や、低侵襲でより効率的な治療法の開発が望まれる。そのためには、子宮筋腫の発生過程や増殖機構の解明が重要となる。

1 子宮筋腫とエピジェネティクス

1) 筋腫細胞における遺伝子異常

子宮筋腫はモノクローナル発生であることが知られている。つまり、筋腫の発生母地（子宮平滑筋細胞、組織幹細胞）と考えられる細胞に何らかの異常が生じて筋腫細胞となり、この1個の筋腫細胞が増殖して腫瘍を形成するという過程が推察される。細胞の腫瘍化にはいくつかの遺伝子異常が関与すると考えられており、その1つに染色体異常によるものがあげられる。筋腫細胞ではその約40%に転座や逆位、染色体の欠失などの染色体構造異常がみられる。しかし、残りの多

【キーワード&略語】

子宮筋腫, DNAメチル化, エストロゲン・レセプター

DNMT: DNA methyltransferase

D-REAM: T-DMR profiling with restriction-tag mediated amplification

ER α : estrogen receptor α

RLGS: Restriction Landmark Genomic Scanning

Genome-wide DNA methylation profile of uterine leiomyomas

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くの筋腫細胞には染色体異常を認めないことなどから、染色体異常は子宮筋腫の発生に関与する可能性は少ないと考えられている^{11)~13)}。つぎに、突然変異を含めて何らかの原因で遺伝子異常が起こることは十分考えられる。実際に、正常子宮筋組織と筋腫組織での遺伝子やタンパク質の発現プロファイルの違いをマイクロアレイなどの実験手法を用いて多くの研究がなされてきた。これまでに、子宮筋腫では、EGF, IGF, TGF- β などの増殖因子やcyclin E, cdc2, cdk2などの細胞周期調節因子の発現が亢進していることが報告されている。これらは、筋腫細胞の増殖機序に大きく関与していると考えられるが、これらの発現異常を引き起こす原因や筋腫の発生機序については依然として不明である。

子宮筋腫の発生起源についても未だ不明である。子宮筋腫発症のリスク因子として、人種（アフリカ系）、高 body mass index (BMI)、早期の初経開始、高血圧、骨盤内炎症性疾患の既往、肉食などがあげられ、一方、リスクを下げる因子として、経口避妊薬の服用、喫煙、多産、菜食などがあげられている。このような疫学的情報を見ると、個人の遺伝的背景も関与すると考えられるが、後天的な因子であるホルモン環境、栄養、生活環境が大きく影響していることが予想される。すなわち、エピジェネティックな影響が関与していても何ら不思議はない。

2) 子宮筋腫とDNAメチル化異常

エピジェネティックな影響として、DNAメチル化やヒストン修飾があげられる。DNAメチル化はヒストン修飾と密接に絡んでいる。DNAメチル化が変化するとヒストン修飾が変化し、逆にヒストン修飾がDNAメチル化にも影響する。ヒストン修飾にはアセチル化、メチル化、リン酸化、スモリル化などがある。さまざまな化合物（薬物、食品添加物、環境汚染物質）、栄養因子、ウイルスなどがこれらのいずれかに作用すれば、ゲノムワイドにエピジェネティックな変化が引き起こされる可能性がある。環境が遺伝子に作用することで表現型に異常が引き起こされる。まさにエピジェネティクスがさまざまな疾患の理解には欠かせない。

われわれ人類は、種々の化学物質に暴露されているが、興味深いことに、有機リン系農薬の一つである dimethyl sulfoxide が胚性幹細胞において多くの遺伝

子にDNAメチル化異常を引き起こすことが明らかにされている¹⁴⁾。DNAメチル化は細胞分裂に際して忠実に保存される仕組みで、DNAメチル化によるがん抑制遺伝子の silencing が発がんに関与することが多くのがん種で知られている。さらに、重金属やダイオキシンの暴露と子宮筋腫・子宮内膜症発症との関連性も示唆されている^{15)~17)}。ダイオキシンの受精卵への暴露はインプリント遺伝子のDNAメチル化プロファイルを変化させる¹⁸⁾。一方、子宮筋腫は、初経前に発症することはなく、臨床的にも実験的にもエストロゲンやプロゲステロンの作用で増大することから、性成熟期のホルモン環境、また月経（子宮収縮など）が子宮筋腫の発生や発育に与える影響が大きいと考えられている。実際に、われわれは、エストロゲンとプロゲステロンが培養子宮内膜細胞において、DNAメチル化に関与する DNA methyltransferase (DNMT) mRNA 発現に影響を及ぼすことを見出している¹⁹⁾。このような背景から、われわれは、子宮筋腫の発生や進展にDNAメチル化異常が関与している可能性を考えている。そこで、子宮筋腫のDNAメチル化プロファイルをゲノムワイドに解析した。

2 子宮筋腫におけるDNMTの発現

まず、手術により摘出した子宮から正常子宮筋組織と筋腫組織を採取し（同一の患者において）、DNMT1, DNMT3a, DNMT3bの発現を比較した（表）。DNMT1は細胞分裂におけるメチル化の維持、またDNMT3aとDNMT3bは*de novo*のメチル化に関与する。筋腫組織でのDNMT1とDNMT3aのmRNA発現は、正常子宮筋組織に比べ有意に高い発現を示しており、DNAメチル化状態が広範に影響を受けていることが推測された¹⁹⁾。一方、Liら¹¹⁾は、DNMT1についてはわれわれと同様に筋腫組織で高発現であったが、DNMT3a, DNMT3bは、ともに筋腫で低発現であったと報告している。子宮筋腫は人種などによっても発症率が大きく異なるとの報告があり、被験者の人種の違いが影響しているかもしれない。いずれにせよ、DNMTの発現異常からは子宮筋腫でのゲノムワイドなDNAメチル化異常が生じていることが推測される。

表 正常子宮筋と子宮平滑筋腫における DNMT mRNA 発現

	症例数	正常子宮筋	子宮平滑筋腫	no. of myo < leio
DNMT1	20	92.6±5.0	118.6±14.2 ^b	15 (75%)
DNMT3a	20	110.5±12.5	161.9±19.8 ^a	17 (85%)
DNMT3b	20	115.2±8.6	97.2±6.9	6 (30%)

20症例から得られた正常子宮筋と子宮平滑筋腫組織における DNMT1, DNMT3a, DNMT3b の mRNA レベルを RT-PCR 法で解析した¹⁰⁾。最右列には正常子宮筋 (myo) に比べ筋腫 (leio) で発現が高かった症例数 (%) を示した。Mean±SEM, a: p<0.01, b: p<0.05

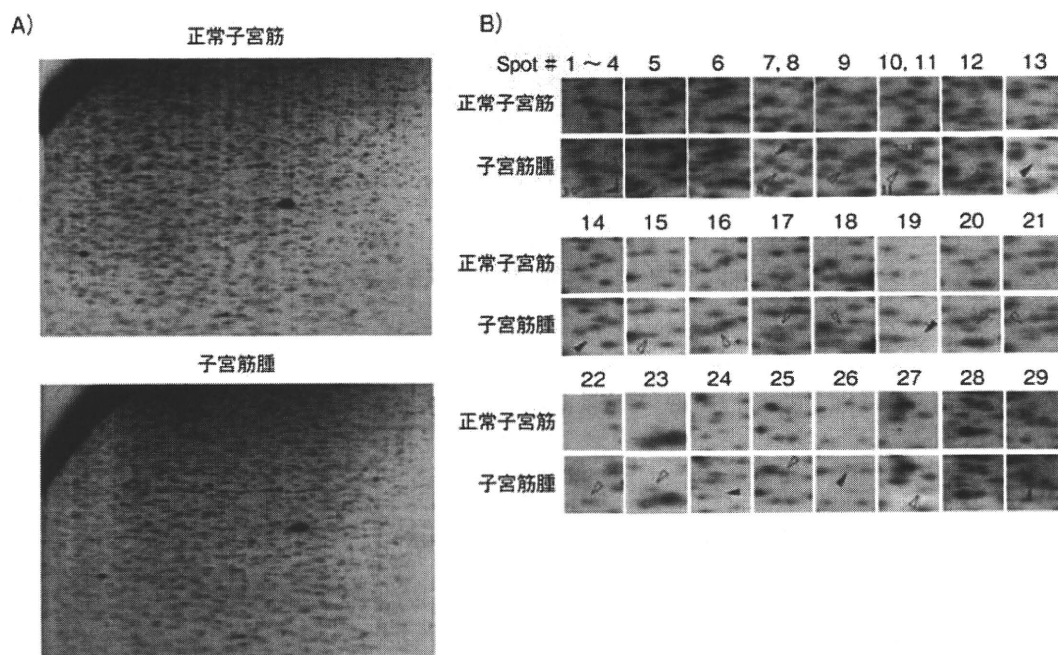


図1 RLGS法による正常子宮筋と子宮筋腫のメチル化プロファイルの比較

A) 正常子宮筋と子宮筋腫の RLGS プロファイル, B) 両者の間に相違のあった 29カ所の spot の拡大像, 上段に正常子宮筋, 下段に子宮筋腫を示している。矢頭で示すように, 子宮筋腫では正常子宮筋に比べ, 19個の遺伝子領域が低メチル化 (▷) に, 10個の領域が高メチル化 (▶) となっていた (文献10より転載)

3 子宮筋腫におけるゲノムワイド DNA メチル化異常の検索

1) Restriction Landmark Genomic Scanning (RLGS)

子宮筋腫におけるゲノムワイドな DNA メチル化異常を評価するため, われわれは RLGS 法による解析を行った。同一患者の子宮筋腫組織と正常子宮筋組織から抽出したゲノム DNA を用いて, 両者の DNA メチル化パターンを比較した。RLGS により約 1,800 個から

なる DNA 断片が展開された (図 1 A)。子宮筋腫では正常子宮筋に比し, 19 個の遺伝子領域が低メチル化に, 10 個領域が高メチル化となっていた (図 1 B)。すなわち, 子宮筋腫では多くの遺伝子領域に DNA メチル化異常が起こっていることが明らかとなった¹⁰⁾。

さらに, 正常子宮筋と筋腫の間でメチル化パターンが異なっていた遺伝子領域を virtual RLGS という方法で同定したところ, 正常子宮筋に比べ筋腫で低メチル化状態となっていた 1 つの遺伝子領域を同定することができた¹⁰⁾。なお, この遺伝子領域は 15 番染色体上

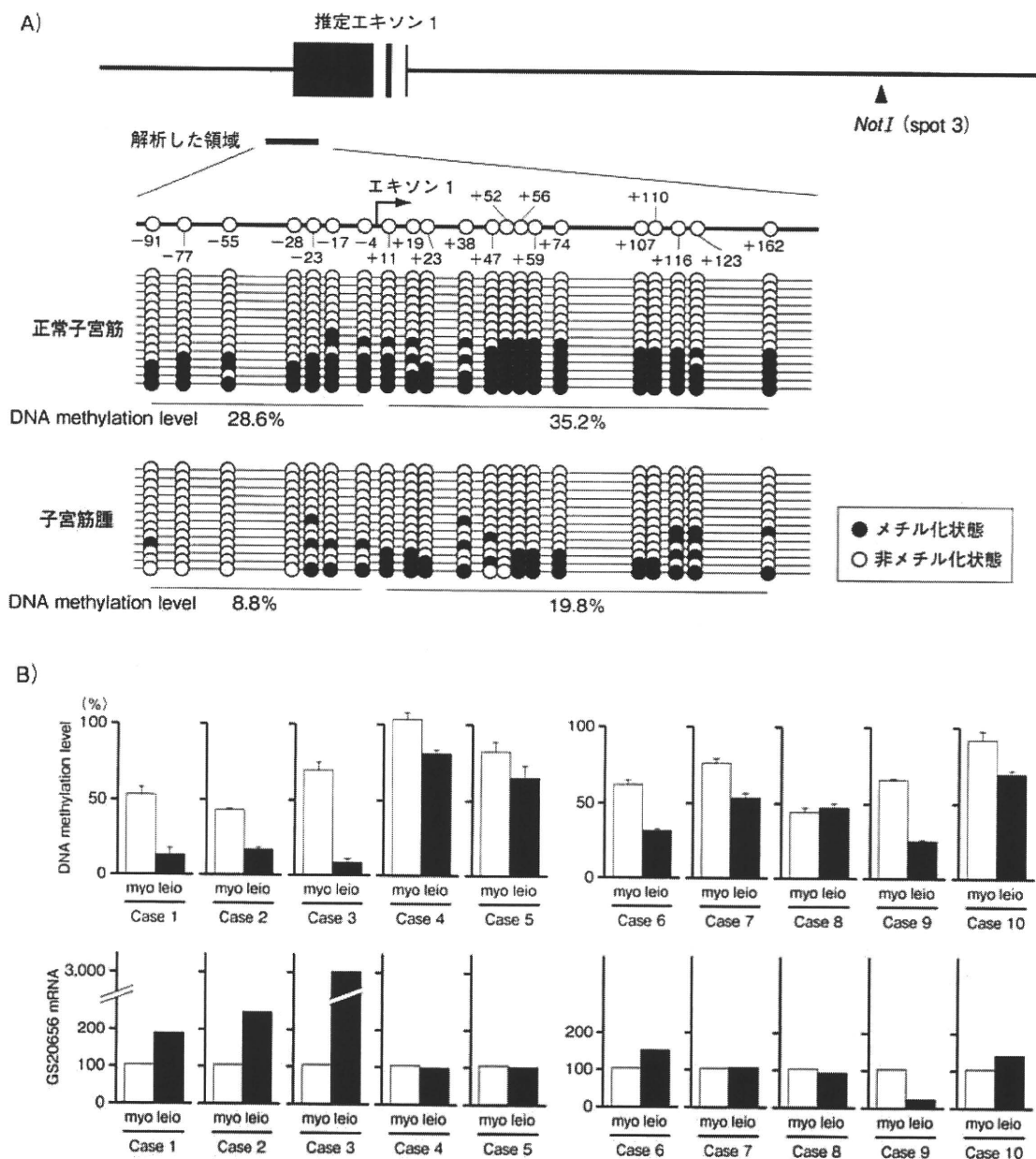


図2 RLSG法で同定されたメチル化異常遺伝子GS20656

A) GS20656のプロモーターからエクソン1領域のsodium bisulfite sequencing法によるメチル化解析。子宮筋腫(leio)では正常子宮筋(myo)に比べて低メチル化であった¹⁰⁾。B) GS20656の多数症例での検討。子宮筋腫ではメチル化レベルは症例間を通じて低い傾向であった(上段)。一方、mRNA発現レベルは子宮筋腫で高い傾向であった¹⁰⁾(下段)

にあり、既知の遺伝子ではなく、塩基配列から7個のエクソンからなる新たな遺伝子(GS20656)である可能性が考えられた。この遺伝子プロモーター領域か

らエクソン1領域を含む範囲についてCpG部位のメチル化状態をsodium bisulfite sequencing法で解析したところ、子宮筋腫で低メチル化であることが確認