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Prenatal zinc deficiency-dependent epigenetic alterations of mouse metallothionein-2 gene☆

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Abstract

Zinc (Zn) deficiency *in utero* has been shown to cause a variety of disease states in children in developing countries, which prompted us to formulate the hypothesis that fetal epigenetic alterations are induced by zinc deficiency *in utero*. Focusing on metallothionein (MT), a protein that contributes to Zn transport and homeostasis, we studied whether and how the prenatal Zn status affects gene expression. Pregnant mice were fed low-Zn (IU-LZ, 5.0 μg Zn/g) or control (IU-CZ, 35 μg Zn/g) diet *ad libitum* from gestation day 8 until delivery, with a regular diet thereafter. Bisulfite genomic sequencing for DNA methylation and chromatin immunoprecipitation assay for histone modifications were performed on the *MT2* promoter region. We found that 5-week-old IU-LZ mice administered cadmium (Cd) (5.0 mg/kg b.w.) have an elevated abundance of *MT2* mRNA compared with IU-CZ mice. Alteration of histone modifications in the *MT2* promoter region having metal responsive elements (MREs) was observed in 1-day-old and 5-week-old IU-LZ mice compared with IU-CZ mice. In addition, prolongation of MTF1 binding to the *MT2* promoter region in 5-week-old IU-LZ mice upon Cd exposure is considered to contribute to the enhanced MT2 induction. In conclusion, we found for the first time that Zn deficiency *in utero* induces fetal epigenetic alterations and that these changes are being stored as an epigenetic memory until adulthood.
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Keywords: DNA methylation; Epigenetics; Histone modification; Metallothionein; Zinc

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1. Introduction

The malnutritional status *in utero* has been shown to affect the progeny's health and disease states later in life in humans as well as in laboratory animals. Low-birth-weight babies resulted from prenatal malnutrition can be a risk factor for lifestyle-related diseases, such as ischemic heart disease and diabetes [1–4]. This hypothesis has been widely acknowledged and expanded to the concept, named ‘Developmental origins of health and disease (DOHaD)’ [5]. Recently, rats that were grown under low-protein nutritional conditions *in utero*, or had intrauterine growth retardation have been shown to develop hypertension or type 2 diabetes later in adulthood [6–9]. Moreover, a plethora of published works have shown that extrinsic conditions *in utero*, such as nutrition and environmental chemicals, affect the propensity of the fetus to develop disease states later in adulthood [10–12]. Although the underlying mechanism is still under intensive investigation, there is a widely-acknowledged view that epigenetic alterations, namely, DNA methylation (the covalent addition of a methyl group to the 5′-carbon of cytosine in the CpG dinucleotide) and histone modification (methylation, acetylation, phosphorylation, ADP-ribosylation, and ubiquitination), play a pivotal role in the expression of particular genes, which will subsequently alter the physiological status of the whole organism. Gene expression is suppressed by DNA methylation of the promoter region of a given gene [13], whereas histone modifications regulate chromatin structure and alter gene activity [14]. Such epigenetic alterations could be inherited by succeeding generations [15].

Experimentally, a few studies have shown that zinc (Zn) restriction during pregnancy induces disease states later in life. Rats grown under prenatal or postnatal Zn restriction have been reported to develop hypertension [16] and impairments of learning and memory [17,18] later in adulthood. Pregnant mice fed a Zn-deficient diet *in utero* have shown persistent immunodeficiency for three succeeding generations [19]. Zn is an essential trace element and a key component of approximately 300 enzymes in various types of tissues [20,21]. Zn deficiency induces various disease states in humans, such as immunodeficiency, developmental disorders, alopecia, dysgeusia, skin disorders and anemia. Vegetarians [22], elderly

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persons [23], habitual alcohol drinkers [24], infants and pregnant/parturient women [22] have the tendency to develop Zn deficiency. In addition, maternal Zn deficiency during pregnancy induces pregnancy complications, delayed delivery, and low-body-weight birth [25]. Zn deficiency is responsible for 4.4% of deaths of children aged 6–59 months in developing countries [26].

Metallothionein (MT), a low-molecular weight protein, has been shown to be involved in the transport, metabolism and homeostasis of heavy metal ions, such as Zn and copper, in tissues and cells. One-third of their amino acid residues of this protein are cysteine residues without a disulfide bond. This characteristic enables MTs to play a role in the transport and inactivation/detoxification of metals [27,28]. Aside from the metabolism and homeostasis of heavy metals, MT has been known to protect cells from oxidative stress and inflammation elicited by various environmental stimuli including heavy metals. Among the four MT isoforms known so far, MT1 and MT2 exist in nearly all types of cells in the body. It has been established that the expression of *MT1/2* genes is induced by metal ions, such as Cd, Zn, Cu and Hg [27]. For the up-regulation of *MT1/2* transcription upon exposure to these metal ions, metal responsive elements (MREs) located in the promoter region are essential. A limited line of experimental evidence showed that metal transcription factor 1 (MTF1) [29] will bind to the MRE motif upon exposure to at least Zn ions, and the Zn-ion-bound MTF1 forms a complex with p300 and Sp1, and then this complex is recruited to MREs of the *MT1* promoter region [30].

The effects of prenatal zinc deficiency on MT regulation have been studied. Pregnant mice were fed either a control diet (100 μg Zn/g) or a low-Zn diet (5.0 μg Zn/g) from gestation day 7 to delivery, and both groups of dams were given the control diet after delivery. Although the Zn and MT levels in pups born to these two groups of dams were similar at postnatal day 3, serum IgM concentrations were significantly lower in adulthood in the mouse offspring born to dams given the low-Zn diet than in the offspring born to control dams. Moreover, when the mouse offspring was given Zn injections to stimulate MT synthesis, the mice deprived of Zn while *in utero* had markedly higher MT levels in the liver than control mice later in adulthood [31].

Collectively, prenatal Zn deficiency has been shown to induce disease states, which is presumably due to epigenetic alterations. However, nearly no studies to elucidate the molecular basis of disease states induced by prenatal Zn deficiency are available. Thus, we have hypothesized that fetal epigenetic alterations can be induced by Zn deficiency *in utero* and alter the physiological conditions that will lead to the onset of disease conditions later in adulthood. In this study, we developed an experimental animal model of prenatal Zn deficiency and studied whether Zn deficiency *in utero* exerts fetal epigenetic alterations in *MT1/2* genes.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from the manufacturers described in parentheses: RNase A, mouse monoclonal anti-β-actin IgG1 and CellLyticNuCLEARExtraction kit (Sigma-Aldrich, St Louis, MO, USA); RNeasy Mini kit, QIAquick PCR Purification kit, QIAquick Gel Extraction kit and QIAprep Spin Miniprep kit (Qiagen K.K., Tokyo, Japan); Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); Wizard DNA Clean-Up system, pGEM-T Easy Vector, pGL4.0 Luciferase Reporter Vector, phRL-TK Vector and Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA); proteinA agarose/salmon sperm DNA, rabbit polyclonal anti-acetylated histone H3 IgG, rabbit polyclonal anti-acetylated histone H4 IgG, rabbit polyclonal anti-acetylated histone H3 lysine14 IgG and Immobilon-P transfer membrane (MilliPore, Billerica, MA, USA); proteinase K, Blocking One and Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan, USA). *Bam* HI, *Aci* I, *Kpn* I, *Xho* I and *Dpn* I were purchased from New England Biolabs Japan (Tokyo, Japan); rabbit polyclonal anti-MTF1 IgG and goat polyclonal anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA); Immunopure goat anti-rabbit IgG, F(ab′)2, peroxidase conjugated, ImmunoPure goat anti-mouse IgG, F(ab′)2, peroxidase conjugated and Immuno Pure rabbit anti-goat IgG, F(ab′)2, peroxidase conjugated (Thermo Fisher Scientific, Rockford, IL, USA); rabbit polyclonal anti-acetylated histone H3 lysine9 IgG and rabbit polyclonal anti-trimethylated histone H3 lysine4 IgG (Cell Signaling Technology, Danvers, MA, USA). LightCycler480 SYBR Green I Master (Roche Diagnostics Japan, Tokyo, Japan); Minisart SRP 15 (Sartorius Stedim Biotech, Goettingen, Germany). IGEPAL-CA630 (Wako Pure Chemical, Osaka, Japan); PrimeScript RT reagent kit, SYBR Premix Ex Taq, TaKaRa Ex Taq, LA Taq and T4 polynucleotide kinase (TaKaRa BIO, Otsu, Japan). *Not* I, DH5α and KOD -Plus (Toyobo, Osaka, Japan); Ligation Convenience kit and ISOGEN (Nippon Gene, Tokyo, Japan); all other reagents of analytical grade (Sigma-Aldrich, Invitrogen and Wako Pure Chemical); all oligonucleotides (Hokkaido System Science, Sapporo, Japan).

2.2. Animals

C57BL/6J strain pregnant ($n=44$) and male mice ($n=18$) were purchased from CLEA Japan. The mice were housed in a room with temperature at 23±1°C and humidity at 50±10% on a 12/12-h light–dark cycle. We used three kinds of rodent chow. Laboratory rodent chow (50 μg Zn/g; Labo MR Stock, Nosan) was given to mice unless specifically described. Low-Zn diet (5.0 μg Zn/g) or control diet (35 μg Zn/g) (CLEA Japan) was used in Zn-deficiency experiments. According to the previous studies [32,33], zinc concentration in diet (35 μg Zn/g) was found to be high enough to be used for a control diet group. These chows and deionized water were provided *ad libitum*. For this study, male mice were used unless specifically described. The experiments protocols using mice were approved by the Animal Care and Use Committee of the Graduate School of Medicine, the University of Tokyo.

2.3. Experiments on Zn deficiency in utero

Pregnant mice were fed a Labo MR Stock rodent chow until gestation day 7, and the chow was replaced with a low-Zn diet or a control diet thereafter until delivery. On the day of birth, two to three male pups per dam were randomly adopted from fourteen dams to minimize possible litter effects and to make two groups: (1) *in utero* low-Zn (IU-LZ) mice and (2) *in utero* control (IU-CZ) mice. The pups were decapitated by scissors, and their livers were harvested. All the liver tissues except those used for chromatin immunoprecipitation (ChIP) assay were immediately frozen in liquid nitrogen, and kept at −80°C until analyses. Livers used for the ChIP assay were immediately minced by scissors and subjected to the subsequent processes as described in the ChIP assay section below. The number of pups for each dam was adjusted to be 6 to 7 pups by adoption from other dams on the day of birth. The dams were given Labo MR Stock rodent chow from the delivery to weaning. After weaning, male pups were given Labo MR Stock rodent chow thereafter.

When IU-CZ mice and IU-LZ mice became 5 weeks old, they were administered orally with a single dose of cadmium (Cd) (5.0 mg kg^{-1} b.w.). Mice were sacrificed by cervical dislocation, and livers were harvested 0, 1 and 6 h post Cd administration (Fig. 1A).

2.4. Experiment on Zn deficiency in adulthood

Male mice aged 10 weeks were fed a low-Zn(AD-LZ) diet or a control (AD-CZ) diet *ad libitum* for 12 days, and then sacrificed by cervical dislocation to harvest the liver. Other mice were fed Labo MR Stock rodent chow for another 30 days, and administered orally a single dose of Cd (5.0 mg kg^{-1} b.w.). Livers were collected 6 h after Cd administration (Fig. 1B).

2.5. Measurement of Zn and Cd concentrations

Livers (approx. 0.1 g) and blood (approx. 0.2 g) specimens were digested in 2 ml of concentrated nitric acid in glass test tubes. The temperatures were kept at 80°C for 1 h, with a gradual increase with 10°C for 1 h each to 130°C. When the acid-digested specimens were became transparent, they were diluted with 1% $HNO_3$and filtered with Minisart SRP 15 and determined for Zn and Cd concentrations by inductively coupled plasma mass spectrometer (Agilent 7500ce; Agilent Technologies).

2.6. RNA isolation and reverse transcription

Total RNA was isolated using RNeasy Mini Kit and then reverse-transcribed using PrimeScript RT reagent Kit, according to the manufacturer's instructions.

2.7. DNA isolation

DNA was isolated using ISOGEN, according to the manufacturer's instructions and purified by phenol/chloroform extraction method.

2.8. Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) analysis was performed using SYBR Premix Ex Taq and amplified by LightCycler under the following conditions: 95°C/10 s ×1 cycle; 95°C/5 s, 60°C/30 s, ×45 cycles or using LightCycler480 SYBR Green I Master and amplified by LightCycler480 under the following conditions: 95°C/5 min ×1 cycle; 95°C/15 s, 60°C/10 s, 72°C/30 s ×45 cycles. Primers used in the qPCR analysis

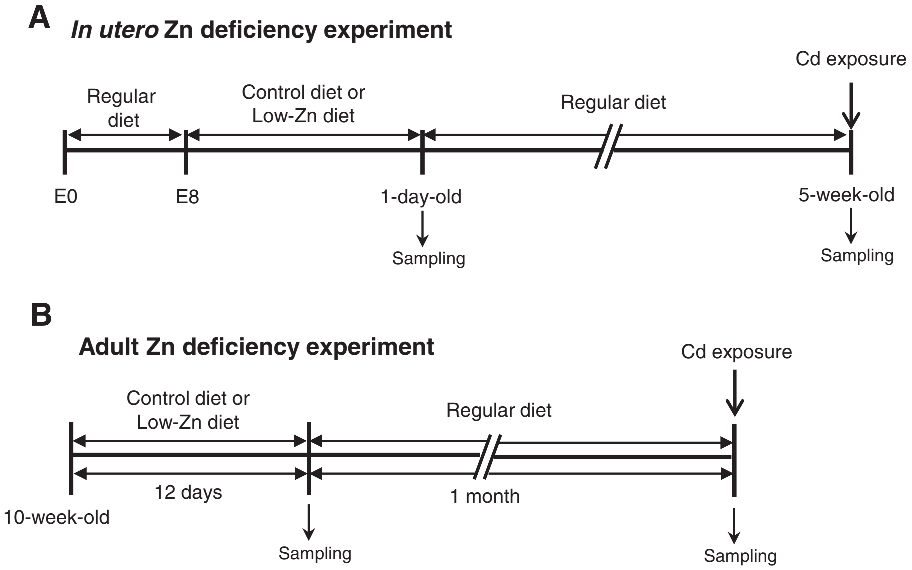

Fig. 1. Study design. (A) Pregnant mice were fed a regular diet until Gestation Day 7, and the chow was replaced with a low-Zn diet or control diet thereafter until delivery. On the day of birth, male pups were divided into two groups: (1) *in utero* low-Zn (IU-LZ) mice and (2) *in utero* control-Zn (IU-CZ) mice. Livers were harvested from a group of 1-day-old male pups. The dams were given Labo MR Stock rodent chow from the delivery to weaning. After weaning, male pups were given Labo MR Stock rodent chow thereafter. When IU-CZ mice and IU-LZ mice became 5 weeks old, they were administered orally a single dose of Cd (5.0 mg kg^{-1} b.w.) and sacrificed by cervical dislocation, and livers were collected 0, 1 and 6 h after Cd administration. (B) Male mice aged 10 weeks were fed a low-Zn diet (AD-LZ) or control diet (AD-CZ) ad libitum for 12 days and then sacrificed by cervical dislocation, and livers were collected. Other mice were fed a regular diet for another 30 days and administered orally a single dose of Cd (5.0 mg kg^{-1} b.w.). Livers were collected 6 h after Cd administration.

are described in Table S1. All primer sets were designed by using Primer3 [34]. All quantitative data were calculated by dividing the copy number of targets by the original RNA concentration, according to a previous study [35].

2.9. Bisulfite genomic sequencing

Mouse genomic DNA was digested with *Not* I and bisulfite conversion reaction was performed as previously described [36]. The bisulfite-treated DNA was cleaned up using Wizard DNA Clean-Up system and amplified by nested PCR method using Ex Taq under the following conditions for the first and second PCRs: 94°C/2 min ×1 cycle; 94°C/2 min, 50°C/2 min, 72°C/3 min, ×5 cycles; 94°C/2 min, 50°C/2 min, 72°C/30 s, ×5 cycles; 72°C/5 min ×25 cycles. Each primer set for the nested amplification is shown in Table S2. All primers were designed using Methyl Primer Express Software v1.0 (Applied Biosystems). The amplified DNA was purified using QIA quick PCR Purification kit and ligated into pGEM-T Easy Vector and transformed into DH5α. Colony PCR was performed to amplify target DNAs using Ex Taq and M13 primers. The PCR products were sequenced using Big Dye Terminator v3.1 Cycle Sequencing kit and analyzed using 3730 DNA Analyzer (Applied Biosystems).

2.10. Methylation frequency analysis

Purified DNA was digested with *Bam* HI. The digested DNA solution was divided into two portions. One portion was digested with the methylation-sensitive *Aci* I, whereas the other was kept as it was. These *Aci* I-digested and non-digested DNA was subjected to qPCR using SYBR Premix Ex Taq and amplified by LightCycler under the following conditions: 95°C/10 s × 1 cycle; 95°C/5 s, 60°C/15 s, 72°C/20 s, ×40 cycles. Ch-R1 primer sets described in Table S3. The DNA methylation frequency was represented as copy numbers of *Aci* I-digested DNA/copy numbers of non-digested DNA.

2.11. Chromatin Immunoprecipitation assay

The ChIP assay was performed by the essentially same method as previously described [37], with some modifications: the minced mouse liver was cross-linked with 1% formaldehyde for 10 min at room temperature, followed by addition of glycine to be a final concentration of 125 mM and by incubation for 5 min. The cross-linked liver specimen was homogenized using a Dounce homogenizer (catalog# 432–1273, Wheaton) and washed with phosphate-buffered saline three times. The pellet was dissolved in lysis buffer (5.0 mM Tris–HCl, pH 8.1, containing 1.0% sodium docecyl sulfate [SDS], 10 mM EDTA). It was subjected to sonication by Bioruptor UCD-250HSA (Cosmo Bio) to make an average chromatin fragment size to be 200 to 1000 bp. ProteinA agarose and salmon sperm DNA were added to the fragmented DNA solution, and it was incubated for 1 h at 4°C to eliminate nonspecific substances. An aliquot of the sample was diluted by adding dilution buffer (16.6 mM Tris–HCl, pH 8.1, containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 167 mM NaCl) and incubated with an antibody at 4°C overnight. The sample was immunoprecipitated with ProteinA agarose and salmon sperm DNA and washed consecutively with the following four kinds of buffers: low-salt immune complex buffer (20 mM Tris–HCl, pH 8.1, containing 0.1% SDS, 1.0% Triton X-100, 2.0 mM EDTA, and 0.15 M NaCl), high-salt immune complex buffer (20 mM Tris–HCl, pH 8.1, containing 0.1% SDS, 1.0% Triton X-100, 2.0 mM EDTA, and 0.5 M NaCl), LiCl immune complex buffer (10 mM Tris–HCl, pH 8.1, containing 1.0% IGEPAL-CA630, 1.0 mM EDTA, 0.25 M LiCl, 1.0% deoxycholic acid), and TE buffer. The immunoprecipitated DNA was eluted with buffer (0.1 M $NaHCO_3$, pH 8.5, containing 1.0% SDS and 10 mM DTT). Cross-link was removed by an addition of 5.0 M NaCl with incubation at 65°C overnight. The resultant DNA was treated with RNase A for 30 min and proteinase K for 1 h, and the DNA was purified by using QIA quick PCR Purification kit. Quantitative real time PCR was carried out with the DNA using SYBR Premix Ex Taq and amplified by LightCycler under the following conditions: 95°C/10 s × 1 cycle; 95°C/5 s, 60°C/15 s, 72°C/20 s, ×60 cycles or using LightCycler480 SYBR Green I Master and amplified by LightCycler480 under the following conditions: 95°C/5 min ×1 cycle; 95°C/15 s, 60°C/10 s, 72°C/30 s ×65 cycles. Primers used in this assay are described in Table S3.

2.12. Western blotting

Nuclear and cytosolic proteins were extaracted from mouse liver using CellLyticNuCLEARExtraction kit according to the manufacturer's instructions. The protein specimens (25 μg protein/lane) were separated on a 10% SDS-polyacrylamide gel and blotted on an Immobilon-P transfer membrane. The blotted membrane was blocked with Blocking One at room temperature for 1 h. Primary antibody was applied at 4°C overnight, with a dilution factor as described in parentheses: anti-MTF IgG (1:10,000), β-actin IgG1 (1:4,000) and anti-laminB IgG (1:1,000). The 5,000-fold diluted the secondary antibody was applied at room temperature for 1 h. The antigen-antibody complexes were visualized by using Chemi-Lumi One. For quantitative analysis, the chemiluminescence intensity of respective bands was quantified using CS analyzer ver.2.02b (ATTO).

2.13. Plasmid constructs

The DNA fragment containing *Kpn* I and *Xho* I restriction sites of −2,166 to −17 in the *MT2* promoter was amplified from mouse hepatic DNA by PCR method using LA Taq under the following conditions: 95°C/1 min ×1 cycle; 95°C/30 s, 60°C/1 min, 72°C/2 min, ×30 cycles; 72°C/10 min ×1 cycle. Primers used for the qPCR are described in Table S4. This fragment was inserted into pGEM-T Easy Vector using Ligation Convenience kit. This plasmid was transformed to DH5αand cloned. The cloned plasmid was digested by *Kpn* I and *Xho* I. The inserted fragment was separated by electrophoresis in agarose gel and purified by QIAquickGel extraction kit. This purified fragment was inserted into pGL4.0 Luciferase Reporter Vector digested by *Kpn* I and *Xho* I. This construct was named as *pGL4MT2 -2166*. The *MT2* MRE-deletion constructs

(Fig. 4B) were made from *pGL4MT2 -2166* as a template by inverse PCR method as follows. The MRE-deletion fragments were amplified from *pGL4MT2-2166* construct by PCR method using KOD-Plus under the following conditions: 94°C/2 min ×1 cycle; 98°C/10 s, 68°C/2 min, ×10 cycles. Primers used are described in Table S4. The remaining pGL4MT2 construct was digested by *Dpn* I. The 5′-prime of MRE-deletion fragments was phosphorylated by T4 polynucleotide kinase. These fragments were self-ligated by Ligation Convenience kit and transformed to DH5α. The cloned MRE-deletion constructs were purified by QIAprep Spin Miniprep kit.

2.14. Transfection and luciferase reporter assay

Hepa1c1c7 cells purchased from American Type Culture Collection were maintained in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, 0.01% streptomycin, 0.01% sodium pyruvate, 0.03% L-glutamine, and 55 μM 2-mercaptethanol at 37°C under 5.0% CO_2 condition. In the first experiment, to examine induction of *MT2* mRNA by Cd exposure, cells were seeded at a density of 4.4×10^5 cells per well in a six-well multiplate. After 24 h, cells were exposed to 5.0 μM Cd for a specified time and harvested for qPCR analysis. In the second experiment, to study transcription activity of *MT2*, cells were seeded and incubated at a density of 4.4×10^5 cells per well in a 48-well multiplate for 24 h, followed by co-transfection with phRL-TK Vector and each MT2 reporter construct using Lipofetamine2000 for another 24 h. Then, the cells were exposed to 5.0 or 10.0 μM Cd for 24 h, and reporter assays were conducted using the Dual-Luciferase Reporter Assay System, following the manufacturer's instructions.

2.15. Statistical analysis

All results are expressed as mean±standard errors. Statistical analysis was performed using IBM SPSS Statistics ver. 19.0 (IBM). A two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test was performed to compare means of mRNA expression, histone modification, and protein expression among IU-LZ and IU-CZ or AD-LZ and AD-CZ groups of 5-week-old mice as well as reporter gene assay data. Student's *t* test was used for other analyses to compare between IU-LZ and IU-CZ groups or AD-LZ and AD-CZ groups. A *P*-value of less than .05 was considered to be statistically significant.

3. Results

3.1. Zn and Cd concentrations in liver

Pregnant mice fed the low-Zn diet had a significantly lower blood Zn concentration than those fed the control diet (3.98±0.04 μg/g, $n=3$ vs. 4.92±0.10 μg/g, $n=6$). As for the mouse progeny, no significant difference in body weight was observed between IU-LZ and IU-CZ mice on postnatal days 1, 27, and 35 (data not shown). The hepatic Zn concentration was significantly lower in 1-day-old IU-LZ mice than in IU-CZ mice (20.4±1.8 μg/g tissue, $n=13$ vs. 37.3±2.9 μg/g tissue, $n=18$), but they became similar by postnatal week 5 (Table 1). No significant changes in hepatic Zn and Cd concentrations were observed between IU-LZ and IU-CZ mice 6 h after Cd administration (Table 1).

3.2. Induction of MT1 and MT2 mRNAs upon Cd exposure in the liver of mice fed a Zn deficient-diet

The abundances of *MT1* and *MT2* mRNAs in the liver were examined in 5-week-old mice with and without Cd exposure. The abundances of *MT1* mRNAs in IU-LZ and IU-CZ mice at 6 h after Cd administration were significantly higher than those in the corresponding groups of mice at 0 h (Fig. 2A). Similar results to *MT1* mRNA were observed for *MT2* mRNA although no statistically significant observations were obtained for the abundance of *MT2* mRNA in IU-CZ before and after Cd administration (Fig. 2B). Without Cd exposure, the abundances of *MT1*and *MT2* mRNAs of IU-LZ mice and those of IU-CZ mice were not different from each other (Fig. 2A, B). Six hours after Cd administration, the abundances of *MT1* mRNA was similar between IU-LZ and IU-CZ mice (Fig. 2A), whereas the abundances of *MT2* mRNA was higher in IU-LZ mice than in IU-CZ mice (Fig. 2B). Although the elevated expression of *MT* genes has been shown to be mediated by MTF1 upon Zn administration [30], no significant alterations in *MTF1* mRNA abundance were found between IU-LZ and IU-CZ mice after Cd administration (Fig. 2C).

Table 1
Zn and Cd concentrations in the liver of 5-week-old mice born to dams given a low-Zn diet or a control diet and those before or after Cd administration

Experimental group	Zn (μg/g tissue)		Cd (μg/g tissue)	
	Unexposed	Cd-exposed	Unexposed	Cd-exposed
IU-CZ	32.2±0.5 (5)	42.8±1.2 (6)	n. d. (5)	0.71±0.13 (6)
IU-LZ	32.6±0.5 (9)	42.1±0.8 (11)	n. d. (9)	0.89±0.11 (11)

Data are expressed as mean±S.E.M. with a number of animals in parentheses.
n.d., not detectable.

3.3. Methyl-CpG status of MT2 gene altered by prenatal Zn deficiency

We hypothesized that the *MT2* mRNA abundance in IU-LZ mice is enhanced by epigenetic alterations. Then, we analyzed the DNA methylation frequency by bisulfite sequencing with a special reference to the methyl-CpG status in the *MT2* promoter region and

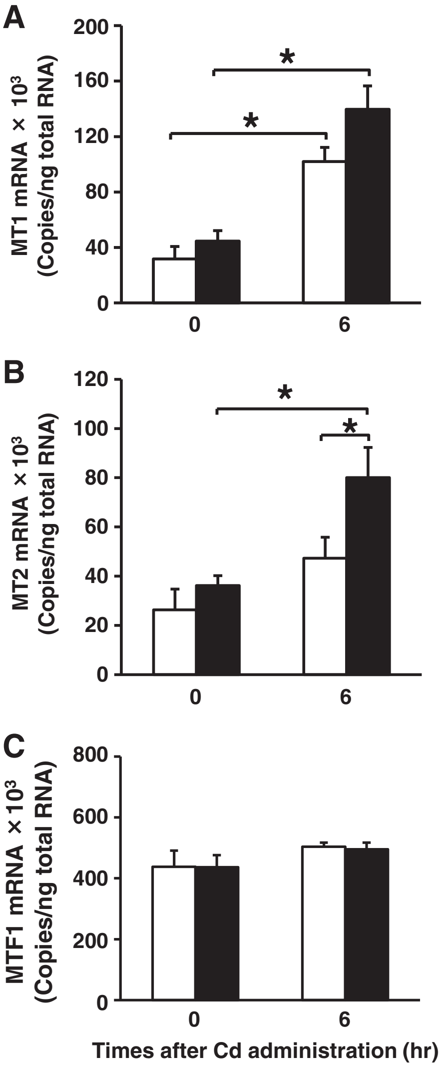

Fig. 2. Abundances of *MT1, MT2* and *MTF1* mRNA upon Cd exposure in liver of 5-week-old pups fed a low-Zn diet: (A) *MT1* mRNA, (B) *MT2* mRNA and (C) *MTF1* mRNA. IU-CZ (open) and IU-LZ (closed) groups. Data are expressed as mean±SEM (IU-CZ 0 h, $n=5$; IU-LZ 0 h, $n=9$; IU-CZ 6 h, $n=6$; IU-LZ 6 h, $n=11$). Statistically significant difference was determined by two-way ANOVA, followed by *post hoc* Bonferroni's test (*$P<.05$).

compared the DNA methylation frequency between IU-CZ and IU-LZ mice in 5-week-old mice. The locations of the transcription start site, MREs, TATA box, and target regions amplified by primer sets are shown in Fig. 3A. The CpG islands, from the −480 to +140 bp region (BS-R4 and BS-R5) of the *MT2* gene, which include MREs, were not methylated in IU-CZ and IU-LZ mice. Although the vast majority of CpG sites in the promoter region showed absolutely no methylation in either animal groups, the −834 and −820 bp CpG sites in IU-LZ mice were more frequently methylated than those in IU-CZ mice. Since the −820 bp CpG site (Fig. 3B; arrow) has the CCGC sequence that can be recognized by the methyl-sensitive restriction enzyme *Aci* I, methylation frequency analysis using *Aci* I was performed at the −820 CpG

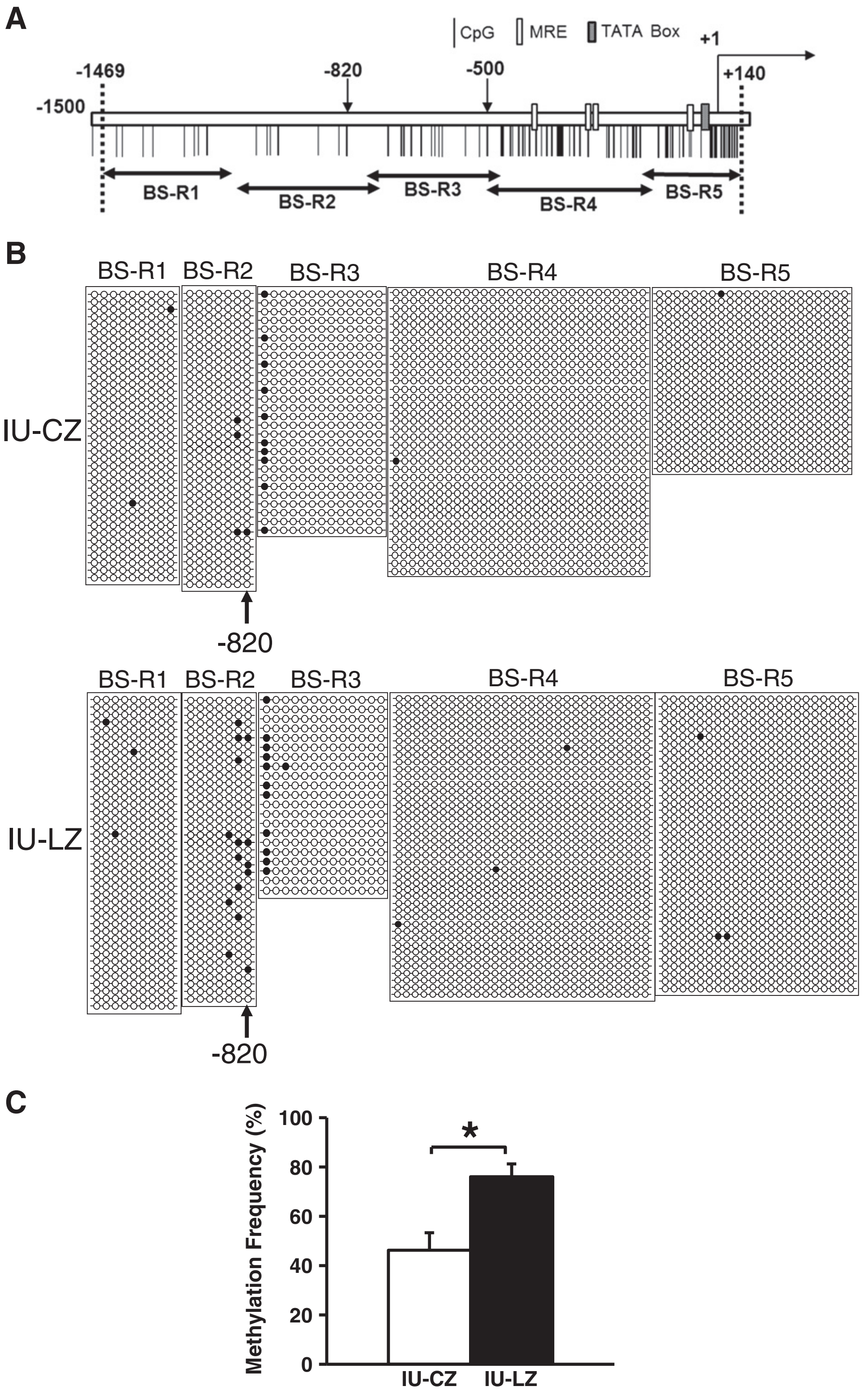

Fig. 3. Comparison of DNA methylation status and frequency of 5′-flanking region of *MT2* gene in livers of 5-week-old mice grown under perinatal Zn deficiency. (A) Target regions of *MT2* gene analyzed by bisulfite genomic sequencing. The locations of transcription start site, MREs and TATA box are shown in a previous study [50]. (B) DNA methylation status and frequency of 5′-flanking region of *MT2* of the control (IU-CZ: opened) and Zn deficiency (IU-LZ: closed) mice: CpG dinucleotides are represented by circles (●, methylated cytosine; ○, unmethylated cytosine). (C) Methylation frequency of -820 bp CpG determined by quantitative PCR with methylation-sensitive restriction enzyme. Open (IU-CZ mice) and closed (IU-LZ mice) columns are presented. Data are expressed as mean±S.E.M. (IU-CZ, $n=5$; IU-LZ, $n=9$). Statistically significant difference was determined by Student's *t* test (*$P<.05$).

site in the IU-CZ (n=6) and IU-LZ (*n*=9) mouse samples. A significant increase in DNA methylation frequency was observed at the −820 CpG site in IU-LZ mouse samples, suggesting the association of prenatal Zn deficiency with a high methylation status (Fig. 3C), which is consistent with the result of bisulfite sequencing (Fig. 3B).

3.4. MT2 promoter analysis

No data on *MT2* promoter functional analysis in various animal species is available. Thus, using an *MT2* gene-promoter-driven luciferase reporter gene assay, we confirmed that *MT2* mRNA abundances were significantly induced in Hepa1c1c7 cells as early as 3 h after Cd addition to the medium (Fig. 4A). For deletion analysis, we made six deletion constructs from an original construct connected with a 2,166 bp flanking region to the pGL4 vector (*pGL4MT2-2166*), on the basis of the strategy that the possible functionalities of the four MREs can be evaluated (Fig. 4B). Hepa1c1c7 cells that were transfected with either of the *pGL4MT2-2166, pGL4MT2-397, pGLMT2-307* or *pGLMT2-287*construct were found to have significantly elevated transcription activity upon exposure to 5.0 or 10.0 μM Cd (Fig. 4B). However, almost no additional induction above a constitutive level was observed by Cd treatment in the following three transfected cell lines: cells transfected with *pGL4MT2Δ-397-37* construct from which all four MRE motifs were deleted, those transfected with *pGL4MT2-65* having an MRE motif and those transfected with *pGL4MT2-37* having no MRE motif (Fig. 4B). Collectively, only one MRE located between a −287 to −65 bp region are suggested to play a crucial role in the induction of *MT2* mRNA by Cd in these cells.

3.5. Histone modifications of MT2 gene altered by prenatal Zn deficiency

We applied the ChIP assay to the four target regions (Ch-R1, Ch-R2, Ch-R3 and Ch-R4) of the *MT2* promoter regions, in 5-week-old mice, which contain MREs or −820 CpG, to examine whether histone modifications are altered by the Zn status in the prenatal period (Fig. 5A). We found that the basal amounts of AcH3 at Ch-R1 and Ch-R3, AcH4 at Ch-R1 and Ch-R2 and AcH3K14 at Ch-R3 in IU-LZ mice are significantly higher than those in IU-CZ mice (Fig. 5B, C and E). In Cd-exposed mice, histone modification levels in IU-LZ mice in comparison with those in IU-CZ mice were significantly increased: AcH3 (Fig. 5B) and AcH4 (Fig. 5C) levels in Ch-R2, Ch-R3, and Ch-R4; AcH3K9 (Fig. 5D) levels at Ch-R2 and Ch-R3; AcH3K14 levels in Ch-R1, Ch-R2, Ch-R3 and Ch-R4 (Fig. 5E); and H3K4me3 levels at Ch-R3 (Fig. 5F).

In IU-LZ mice, Cd administration significantly increased the AcH3K14 levels at Ch-R1 and Ch-R2 (Fig. 5E) and H3K4me3 levels at Ch-R3 (Fig. 5F), whereas it significantly decreased the AcH3 (Fig. 5B) and AcH4 (Fig. 5C) levels at Ch-R1. It is not known why Cd exposure reduced histone acetylation in the Ch-R1 region. However, this region is not considered to be responsible for transcription. Next, to determine whether these histone modifications occurred in the newborn livers, we analyzed *MT2* promoter regions in the liver from the 1-day-old pups born to dams given a Zn deficient diet during gestation. The abundances of *MT1, MT2* and *MTF1* mRNAs in IU-LZ

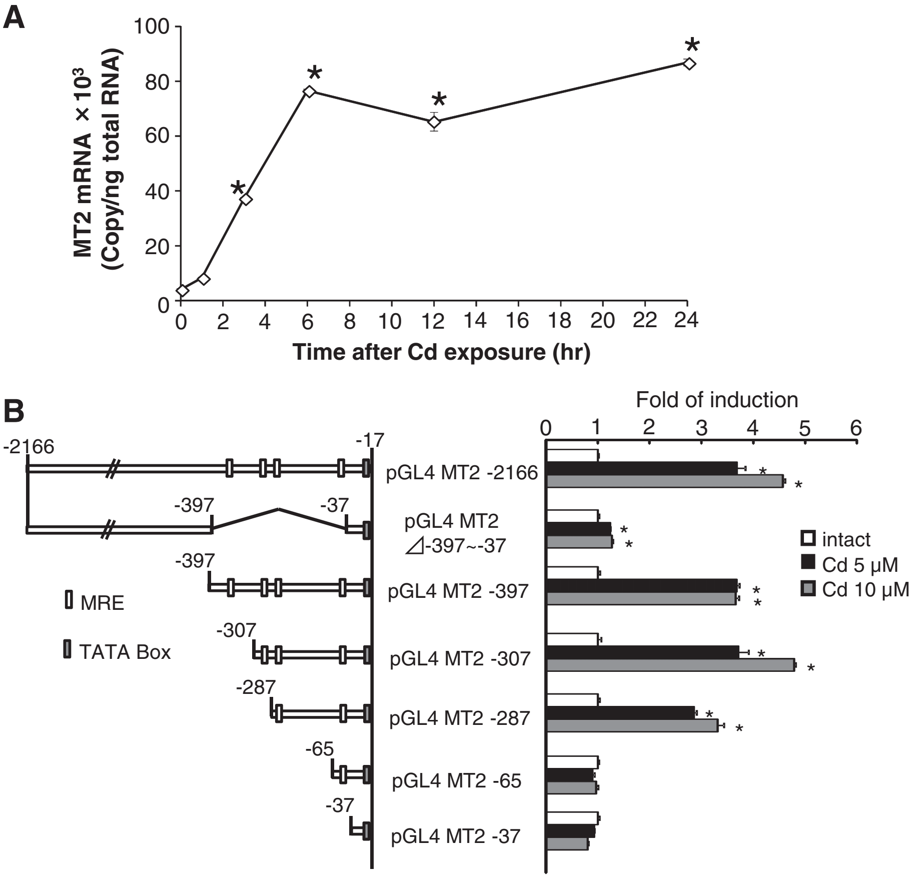

Fig. 4. *MT2* promoter analysis using reporter gene assay. (A) Abundance of *MT2* mRNA in Hepa1c1c7 cells at 0, 3, 6, 12 and 24 h after 5.0 μM Cd treatment. Data are expressed as mean±S.E.M. (*n*=3 per group). Statistically significant difference was determined by one-way ANOVA, followed by post hoc Bonferroni's test (*$P<.05$ vs. 0 h). (B) Structure of MT2 MRE-deletion constructs (left). Reporter gene activity in Hepa1c1c7 cells transfected with MT2 MRE-deletion constructs at 24 h after 5.0 and 10.0 μM Cd treatment (right). Data are expressed as mean±S.E.M. (*n*=3 per group). Statistically significant difference was determined by one-way ANOVA, followed by post hoc Bonferroni's test at each construct (*$P<.05$ vs. intact).

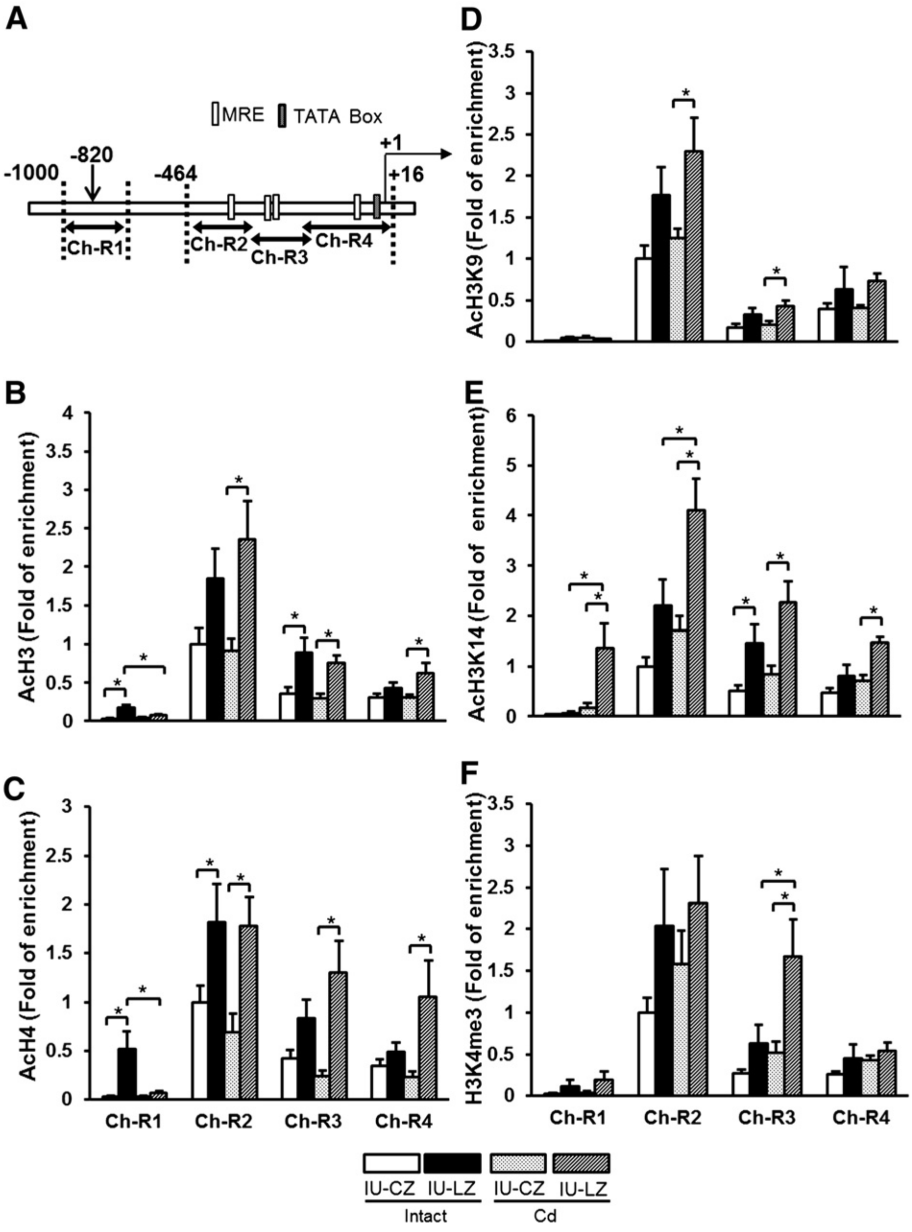

Fig. 5. Changes in histone modification levels in *MT2* promoter regions in 5-week-old mice grown under prenatal Zn deficiency. The livers were collected before and 6 h after Cd administration. (A) Target regions of *MT2* promoter for ChIP assay and localizations of transcription start site, MREs and TATA box are shown in a the previous study [50]. Changes in levels of (B) acetylated histone H3, (C) acetylated histone H4, (D) acetylated histone H3 lysine 9, (E) acetylated histone H3 lysine 14, and (F) tri-methylated histone H3 lysine 4. Open (IU-CZ intact mice), closed (IU-LZ intact mice), dotted (IU-CZ Cd-exposed mice), and diagonal (IU-LZ Cd-exposed mice) columns are presented. Data are expressed as mean±S.E.M. (n= 8 per group). Statistically significant difference was determined by two-way ANOVA, followed by post hoc Bonferroni's test at each region (*P<.05).

mice was not altered in comparison with those in IU-CZ mice (Fig. 6A).The AcH3 levels at Ch-R2 and Ch-R3 were significantly higher in IU-LZ mice than in IU-CZ mice (Fig. 6B). The AcH4 levels at Ch-R2 in IU-LZ mice tended to increase in comparison with those in IU-CZ mice (Fig. 6C). These results suggest that the histone acetylation level was already initiated to increase during the perinatal stage by the Zn deficiency *in utero*.

3.6. Zn deficiency in utero prolonged MTF1 binding to the MT2 promoter region upon Cd administration

Since the epigenetic alterations as shown in the previous section suggested the loosening of the chromatin structure in the *MT2* promoter region, we investigated whether Cd exposure *in vivo* affects the status of MTF1binding to a particular region (Ch-R1, Ch-R2, Ch-R3, and Ch-R4) in the *MT2* promoter by the ChIP assay (Table 2). The amounts of MTF1 bound to Ch-R2, Ch-R3 and Ch-R4 were greater at 1 h than those at 0 and 6 h after Cd administration in all the animals (Table 2). The amount of MTF1 bound to Ch-R1, which does not have an MRE motif, was not detected at these time points (data not shown).

The amounts of MTF1bound to Ch-R3 and Ch-R4, but not Ch-R2 were observed to be significantly higher in IU-LZ mice than in IU-CZ mice 6 h after Cd administration, whereas no difference in the amounts of MTF1 bound to these regions was found between the IU-CZ and IU-LZ mice 1 h after Cd administration, which suggests the prolongation of MTF1 binding to the MRE motif (Table 2). The amounts of MTF1 protein in both nucleus and cytosol were unchanged between the IU-LZ and IU-CZ mice and between before

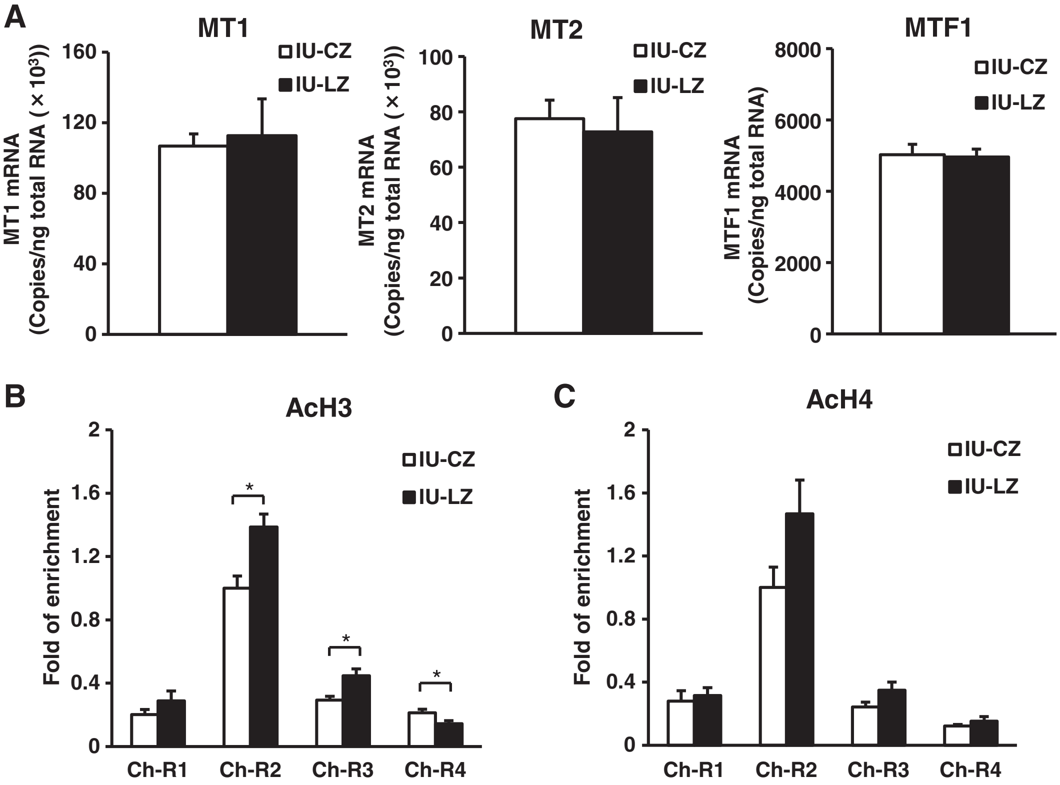

Fig. 6. (A) Abundances of *MT1*, *MT2* and *MTF1* mRNAs and (B) levels of acetylated histone H3 and (C) histone H4 in the *MT2* gene in the liver in 1-day-old male pups grown under Zn deficiency condition. The target regions for histone modifications analysis are shown in Fig. 5A. Data are expressed as mean±S.E.M. (n=6 per group). Statistically significant difference was determined by Student's t test at each region (*P<.05).

and 6 h after Cd administration (Fig. 7), suggesting that the total amounts of MTF1 protein in the liver were not altered by prenatal Zn deficiency or Cd administration.

3.7. Epigenetic alterations induced by Zn-deficiency in adulthood

To determine whether the epigenetic alterations induced by Zn deficiency are a temporally specific event, adult mice were fed a low-Zn diet or a control diet for 12 days, and the liver was collected just after the end of this period and subjected to the epigenetic analyses. In the AD-CZ and AD-LZ mice, no significant differences in the mRNA abundances of *MT1*, *MT2* and *MTF1* were found (Fig. 8A).Although the levels of AcH3 (Fig. 8B) and AcH3K14 (Fig. 8E) at Ch-R1 in the *MT2* promoter of AD-LZ mice were significantly higher than those of AD-CZ mice, the differences were not conspicuous. No significant differences in the amounts of other histone modifications in AcH4 (Fig. 8C), AcH3K9 (Fig. 8D) and H3K4me3 (Fig. 8F) were found between AD-LZ and AD-CZ mice. In addition, the DNA methylation frequency at a −820 bp CpG site in the *MT2* promoter of AD-LZ mice was not different from that of the AD-CZ mice (Fig. 8G).

Next, to study the possible involvement of Zn deficiency in the inducibilities of *MT1/2* mRNAs, the Zn-deficient diet was replaced with a regular diet, and the inducibilities of *MT1* and *MT2* mRNAs by Cd administration were examined one month later. Under this condition, no difference in *MT1* and *MT2* mRNA abundances was observed between the AD-LZ and AD-CZ mice 6 h after Cd administration (Fig. 8H). The *MTF1* mRNA abundance was significantly lower in AD-LZ mice than in the AD-CZ mice (Fig. 8I).

Collectively, epigenetic alterations of the *MT2* gene were found to be caused by Zn deficiency during the prenatal period, but not in adulthood.

4. Discussion

A remarkable finding of this study is that epigenetic alterations of the promoter of the *MT2* gene under prenatal Zn deficiency condition are associated with a significant enhancement of Cd-dependent induction of *MT2* mRNA in the liver of mouse progeny later in adulthood. The first question that was addressed is when such epigenetic alterations occur and how long they last. In the 5-week-old

Table 2
Amounts of MTF1 bound to MT2 promoter post Cd administration in the liver of 5-week-old mice born to dams fed a low-Zn diet or a control diet

Target region	Ch-R2			Ch-R3			Ch-R4		
Time post Cd administration (h)	0	1	6	0	1	6	0	1	6
	Fold of enrichment								
IU-CZ	1.00±0.29	9.13±3.44	0.74±0.23	1.00±0.45	6.21±1.60	0.65±0.18	1.00±0.31	3.24±1.08	0.32±0.06
IU-LZ	0.69±0.25	9.17±2.76	0.45±0.10	0.54±0.20	8.00±3.69	1.54±0.25*	0.78±0.19	3.30±1.25	1.16±0.30*

Each target regions are the ones shown in Fig. 5A. Data are expressed as mean±S.E.M. (n=6 per group).
Statistically significant difference between IU-CZ and IU-LZ mice by Student's t-test (*P<.05).

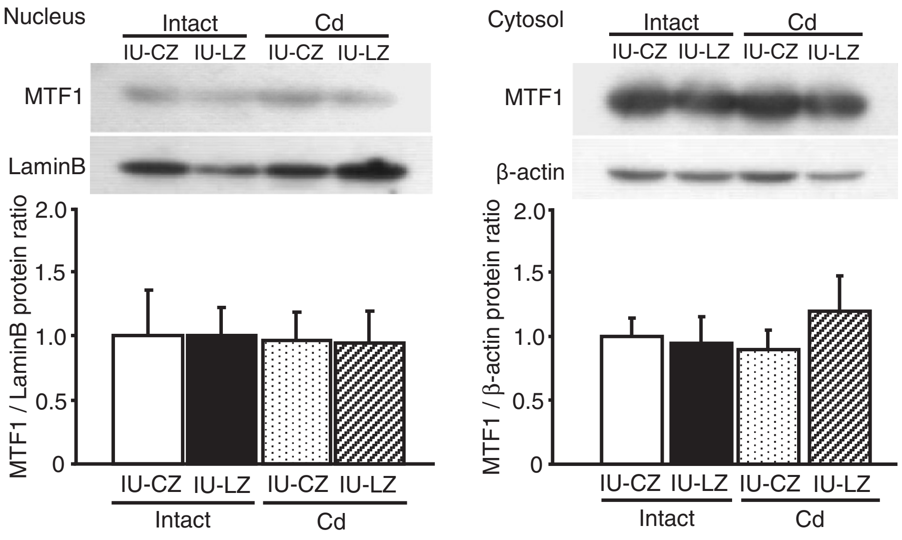

Fig. 7. Amounts of hepatic MTF1 protein in 5-week-old pups grown under Zn deficiency condition. The livers were collected before and 6 h after Cd administration. Scanning densitometry was used for semi quantitative analysis. Data are expressed as mean±S.E.M. (n=5 per group).

IU-LZ mice, significant increases in DNA methylation frequency at the −820 CpG site and histone modifications were demonstrated in comparison with those in IU-CZ mouse progeny. However, this region is not considered to be responsible for transcription. To examine when these histone modifications were caused by Zn deficiency, we analyzed the *MT2* promoter region in the liver from the 1-day-old pups born to dams given a Zn-deficient diet during gestation. Collectively, the IU-LZ mice had significantly elevated AcH3 levels at Ch-R2 and Ch-R3 and showed a tendency of AcH4 levels to increase at Ch-R2 in comparison with IU-CZ mice, suggesting that histone acetylation levels were increased during the prenatal stage. Taken together, zinc deficiency *in utero* alters fetal histone modifications, and these changes are being stored as an epigenetic memory until adulthood (Supplemental Fig. 1). In this study, we could not provide direct evidence on molecular mechanisms by which histone modification enhance Cd-induced *MT2* mRNA expression in IU-LZ mice. However, the elevation in histone acetylation observed in the IU-LZ mice is considered to keep the nucleosome in an “open chromatin” state [38]. Therefore, it is likely that p300 and Sp1 that are known to be recruited by MTF1 [30] will easily make an access to the *MT2* promoter region under the open chromatin condition and that Cd-induced *MT2* mRNA abundance is enhanced in IU-LZ mice. Further studies will be required to reveal the molecular mechanisms how prenatal Zn deficiency regulates not only gene expression via histone modifications, but also maintain histone modifications.

The next question was whether the epigenetic alterations are more specifically induced by Zn deficiency *in utero* rather than in adulthood. When adult mice fed a low-Zn diet (AD-LZ) or those fed a control diet (AD-CZ) were compared, the levels of AcH3/AcH3K14 of the *MT2* gene at Ch-R1 in AD-LZ mice were significantly different from those in AD-CZ mice. However, these changes did not seem to contribute to the alteration of the induction of *MT2* transcription following Cd exposure, because the Ch-R1 region is considered to be irrelevant to transcription (Fig. 4). In addition, the induction of *MT2* mRNA by Cd exposure in AD-LZ mice was not different from that in AD-CZ mice (Fig. 8H). Therefore, it is not likely that the alteration of histone modifications induced by Zn deficiency played a significant role to induce *MT2* mRNA upon Cd exposure in AD-LZ mice. Our results clearly show that mouse fetuses are more responsive to Zn deficiency resulting in epigenetic alterations than adult mice. On the other hand, epigenetic alterations are known to be caused by environmental factors in adulthood as well. Examples of this are an increase in DNA methylation levels of tumor-suppressor genes, such as p16, in the stomach following infection by *Helicobacter pylori* in humans [39,40], DNA hypomethylation of *Pparγ* in mice fed a high-fat diet [41] and DNA hypermethylation of *PPIc* and DNA hypomethylation of *fosB* in nucleus accumbens in cocaine-administered mice [42]. In the case of the nutritional status of Zn, significant epigenetic alterations might occur when adult animals are exposed to extremely low Zn levels for a longer time than under the present experimental conditions.

The other question is how Cd administration enhanced the *MT2* induction in IU-LZ mice compared with IU-CZ mice later in life. A plausible explanation is that the prenatal Zn deficiency induced enhanced histone modifications in the *MT2* promoter region that includes MREs, and that MTF1 or yet-unidentified transcription factors may have easy access to the MRE motif to activate the *MT2* gene expression upon Cd or Zn exposure. This conjecture was supported by the elevated levels of acetylated histones and methylated H3K4 in the *MT2*-400 bp 5′ flanking region in the IU-LZ mice compared with those in the IU-CZ mice (Fig. 5). It is reasonable to think that such an open-chromatin structure persists into adulthood and allows transcription to be activated, as has been reported for other genes such as *Hoxa10* or *Gfap* [43,44].On the other hand, because of the lack of MRE motifs, it is less likely that the change in DNA methylation frequency at −820 bp CpG in the *MT2* promoter region in the IU-LZ mice is associated with the enhanced *MT2* gene expression later in life (Fig. 3).

MTF1 binding to the MRE motif in the *MT1* gene has been proposed to play an important role in the induction of this gene [30]. Although no data are available on the interaction of MTF1 with the *MT2* gene, our reporter gene assay (Fig. 4) and ChIP assay (Table 2) results suggested that MTF1 can bind to the MRE motif in the *MT2* gene. The significantly prolonged MTF1 binding 6 h after Cd administration in the IU-LZ mice (Table 2) may explain the enhanced *MT2* mRNA induction: That is, it is conceivable that the highly acetylated state of histones bound to the DNA fragment adjacent to MREs (Fig. 5) affects the three-dimensional interaction between the MTF1 protein and DNA, and that the net dissociation of MRE with MTF1 may be reduced owing to structural changes in the nucleosome. As described above, the possible removal or slide of histone H3 in *MT1* promoter by Zn treatment may have an interaction with MTF1, and the binding of MTF1 to the promoter is required to initiate the exclusion of histone [45]. Thus, it can be speculated that the

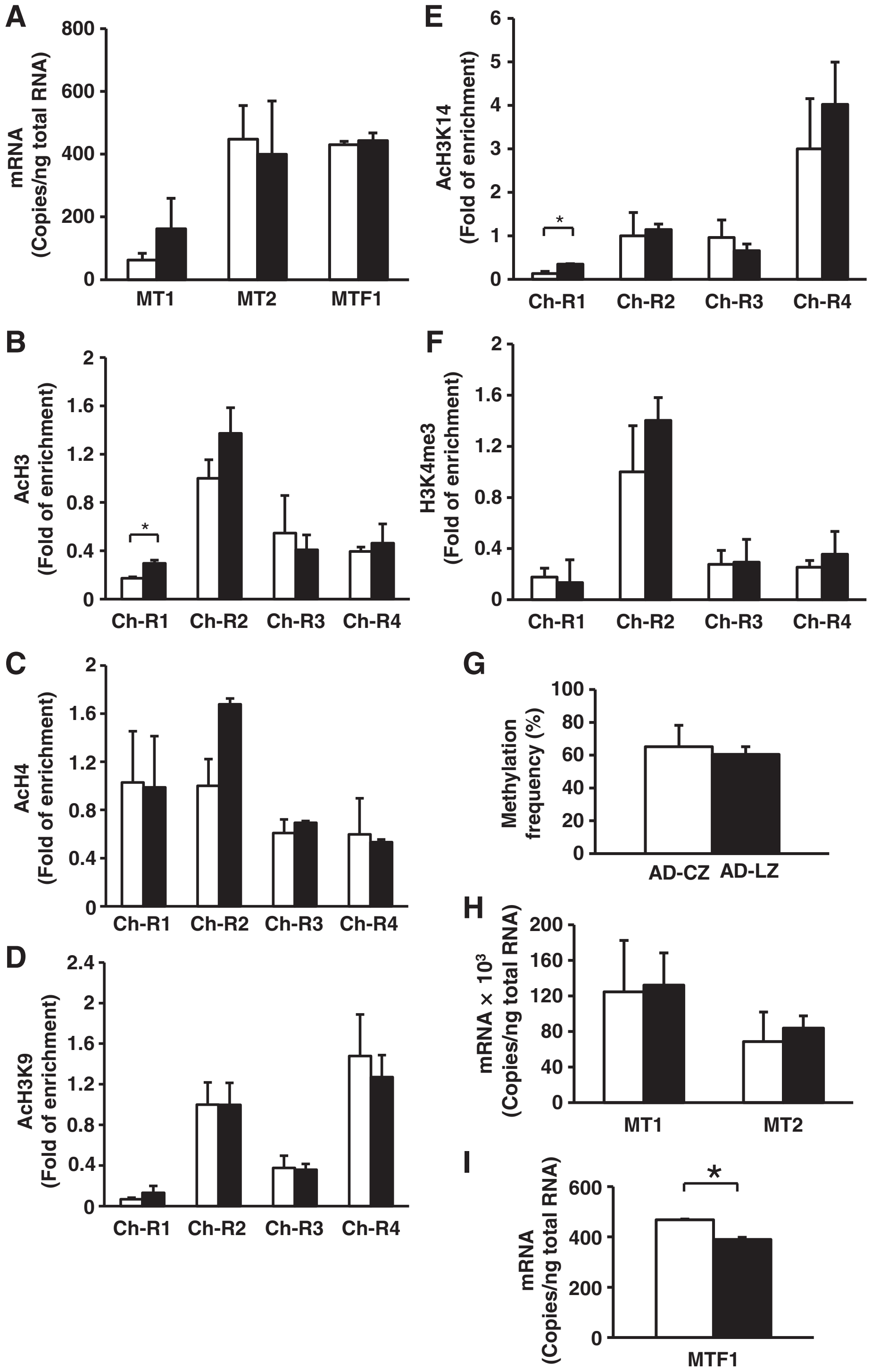

Fig. 8. Abundances of *MT1*, *MT2* and *MTF1* mRNAs, and histone modification in the *MT2* promoter of liver from mice fed a low-Zn diet for 12 days in adulthood (A-G), or those fed with a low-Zn diet followed by a regular diet for a month in adulthood (H-I). (A) *MT1*, *MT2* and *MTF1* mRNAs. (B) Acetylated histone H3. (C) Acetylated histone H4. (D) Acetylated histone H3 lysine 9. (E) Acetylated histone H3 lysine 14. (F) Tri-methylated histone H3 lysine 4. (G) Methylation frequency of −820 bp CpG included in *Aci* I site. See legend to Fig. 5A for target regions of histone modifications. Data on hepatic *MT1* and *MT2* (H) and *MTF1* mRNA (I) were obtained 6 h after oral administration of Cd at 5.0 mg kg^{-1} b.w. Open (AD-CZ mice) and closed (AD-LZ mice) columns are presented. Data are expressed as mean±S.E.M. (n=3 per group). Statistically significant difference was determined by Student's t test (*P<.05).

prolonged binding of MTF1 to *MT2* promoter in IU-LZ mice may be involved in the maintenance of the opened chromatin structure.

Another question to be addressed is what the enhanced *MT2* mRNA induction in adulthood caused by *in utero* Zn deficiency indicates. It has been reported that abnormal morphogenesis occurred in the *MT1/2*-null fetus grown under Zn deficiency *in utero* [46]. On the contrary, this effect was prevented in the transgenic mice over expressing the MT1 protein [47]. *MT1/2* null mice fed a low-Zn diet for 3 weeks from birth developed swollen Bowman's space in the kidney in comparison with wild-type mice [48]. These studies suggest that MT1/2 proteins protect against Zn deficiency. It can be speculated from our study that mice grown under prenatal Zn deficiency maintain the inducibility of *MT2* mRNA as an epigenetic memory in the genome. In this case, it is thought that mice can be prepared for Zn deficiency that they may encounter in the future, and that they will be able to efficiently response to the low-Zn condition. This idea could be supported by an analogy to the thrifty phenotype hypothesis in that neonates who experienced poor nutrition *in utero* have metabolic adaptations that emerge in anticipation of a low-quality adult breeding environment [49].

In conclusion, the present study demonstrates for the first time that prenatal Zn deficiency causes epigenetic alterations in the liver of offspring. The enhanced *MT2* gene induction by metal exposure after birth is considered to be due to epigenetic alterations, such as enhanced acetylation levels of histones bound to approximately −400 bp of the *MT2*-5′flanking region. Histone modifications caused by Zn deficiency during the early-developmental period may persist into adulthood as an epigenetic memory. The results of the present study results could also support the DOHaD hypothesis from the perspective that a particular nutrition factor such as an essential trace element during prenatal period can affect epigenome of children.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.05.013.

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