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Methionine-dependent histone methylation at developmentally important gene loci in mouse preimplantation embryos☆

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Abstract

The involvement of specific nutrients in epigenetic gene regulation is a possible mechanism underlying nutrition-directed phenotypic alteration. However, the involvement of nutrients in gene-specific epigenetic regulation remains poorly understood. Methionine has been received attention as a possible nutrient involved in epigenetic modifications, as it is a precursor of the universal methyl donor for epigenetic methylation of DNA and histones. In the present study, the disruption of methionine metabolism by ethionine, an antimetabolite of methionine, induced abnormally higher expression of genes related to cell lineage differentiation and resulted in impaired blastocyst development of mouse preimplantation embryos *in vitro*. These effects were mitigated by the presence of methionine. Importantly, ethionine treatment induced lower trimethylation of histone H3 lysine 9 but did not affect methylation of DNA in the promoter regions of the examined genes. These results demonstrated that intact methionine metabolism is required for proper epigenetic histone modifications and normal expression of developmentally important genes during preimplantation development.

Keywords: Methionine; Methylation; Histone; Epigenetics; Preimplantation; Embryo

1. Introduction

In living organisms, environmental factors can lead to long-term changes in the genome through mechanisms that do not involve the primary sequence of DNA, a process referred to as *epigenetic modification*. Mammalian preimplantation development is a vulnerable period in terms of its susceptibility to environmental epigenetic modifiers [1]. Among the various environmental factors involved, specific nutrients have been implicated in epigenetic processes that are important for normal preimplantation development and developmental programming during this period [2]. Methionine is a dietary amino acid involved in one-carbon metabolism that produces the universal methyl donor *S*-adenosylmethionine (SAM) for epigenetic methylation of DNA and histones [3]. Therefore, methionine, along with the other nutrients that contribute to one-carbon metabolism (folate, choline, betaine, vitamin B12, *etc.*), has received attention as a nutritional factor that can cause and/or predispose an organism to developmental changes during the periconceptional period [2,4,5].

There is increasing evidence that one-carbon metabolism is crucial for normal preimplantation development [6–9]. For example, the disruption of methionine metabolism by ethionine, an antimetabolite of methionine, induces abnormally high expression of specific genes and impaired blastocyst development in bovine preimplantation embryos *in vitro*[6]. Recently, Zhang *et al.*[9] disrupted one-carbon metabolism in mouse preimplantation embryos in a different way (folate cycle inhibition and knock-down of betaine-homocysteine methyltransferase), and they also observed impaired blastocyst development. Although both studies showed decreased global DNA methylation by their respective interventions, the involvement of these nutrients in epigenetic regulation at specific gene loci has not been documented in preimplantation embryos. The elucidation of interactions between a certain nutrient and the epigenetic regulation of specific genes may yield a better understanding of the mechanism of nutritional epigenetics [10,11] and its application to promote human and animal health via modulation of dietary exposures [12,13].

The objective of the present study is to elucidate the roles of methionine in the epigenetic modification of specific genes by using mouse preimplantation embryos as a model. Here, we show that methionine is involved in regulating histone methylation rather than DNA methylation at developmentally important gene loci during preimplantation development.

2. Materials and methods

2.1. Embryo culture

All animal experiments were approved by the Animal Research Committee of Kyoto University (Permit Number 25–27 and 26–27) and performed in accordance with the

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Regulation on Animal Experimentation at Kyoto University (Animal Research Committee, Kyoto University, Revised 2007). Female 3- to 7-week-old ICR mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (ASKA Pharmaceutical); 46–48 h later, they were injected with 5 IU of human chorionic gonadotropin (hCG; Yell Pharmaceutical). After the injection of hCG, female mice were mated with male BDF1 mice overnight. After the observation of a vaginal plug on the subsequent morning, female mice were euthanized by carbon dioxide (CO_2) inhalation and dissected to obtain their oviducts at 18 h after hCG administration. One-cell embryos were collected in M2 medium (Sigma Aldrich, St. Louis, MO, USA) containing 0.3-mg/ml hyaluronidase, freed from the attached cumulus cells and washed in potassium simplex optimization medium (KSOM; which does not contain methionine) [14]. After the washing, approximately 11 embryos as a group were allocated to one of the following experimental groups: KSOM with no additive (control), KSOM with 10 or 25-μM ethionine (methionine antagonist) or KSOM with both 10-μM ethionine and 250-μM methionine. The embryos were cultured until 114 h post-hCG at 37.5°C under 5% CO_2 with high humidity, and blastocyst development was then evaluated under a stereomicroscope at the end of culture.

2.2. Cell counting of blastocysts

Inner cell mass (ICM) and trophectoderm (TE) of blastocysts were differentially stained with CDX2 immunolabeling according to the method of Wydooghe *et al.*[15] with some modifications. Briefly, blastocysts were fixed in 10% (v/v) formalin neutral buffer solution (Wako Pure Chemical, Osaka, Japan) for 1 h, washed in PBS containing 0.05% (v/v) Tween 20 (PBST) for 1 h and subsequently permeabilized with 0.5% (v/v) Triton X-100 in PBS for 1 h at room temperature. After washing in PBST for 1 h, the samples were treated with blocking solution [PBST supplemented with 1% (w/v) bovine serum albumin] for 1 h at room temperature and subsequently incubated in ready-to-use primary CDX2 antibody solution (Biogenex, Fremont, CA, USA) overnight at 4°C. After washing with blocking solution for 1 h, samples were incubated for 3 h at room temperature in the presence of Alexa Fluor 546-conjugated goat antimouse IgG (1:1000; Life Technologies, Carlsbad, CA, USA). Nuclei were then counterstained with 10-μg/ml Hoechst 33342 in PBST for 20 min. The samples were washed with PBST, mounted on slides with a droplet of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and flattened with a coverslip. The slides were examined under a fluorescence microscope. The total number of cells was counted based on the Hoechst image, and the number of TE cells, which had been stained by both CDX2 antibody and Hoechst, was determined based on the merged images. The number of ICM cells was calculated by subtracting the number of TE cells from the total number of cells.

2.3. Gene expression analysis of blastocysts by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Blastocysts in each experimental group were collected (n=20) in small volumes of RNAlater (Sigma). Total RNA was extracted from the samples by using the RNeasy Micro kit (Qiagen, Hilden, Germany) and reverse transcribed in a 31.5-μl volume with SuperScript III reverse transcriptase and the oligo (dT)20 primer (Life Technologies). The synthesized cDNA (2 μl) was used as a template in a quantitative PCR (qPCR) reaction with 0.2 μM of each primer and 1× SYBR Select Master Mix (Life Technologies) in a volume of 20 μl. qPCR was performed using the StepOnePlus Real-time PCR system (Life Technologies) with the following cycle parameters: 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis and agarose gel electrophoresis were performed after the amplification to confirm the specificity of the PCR products. Quantification of *Oct4*, *Nanog*, *Cdx2* and *Tead4* transcripts were performed by the ΔΔCt method using β-actin gene (*Actb*) transcripts as an internal control. The data analysis was performed with the StepOne Software V 2.3. The primer pairs are listed in Table 1. The experiments were repeated four times for each gene.

2.4. Micro chromatin immunoprecipitation (μChIP)

Chromatin immunoprecipitation for small cell numbers, μChIP [16] was performed with the True MicroChIP kit (Diagenode, Liege, Belgium) according to the manufacturer's protocol with some modifications. All ChIP steps were implemented in Eppendorf 1.5-ml DNA LoBind Tubes (Eppendorf, Hamburg, Germany). To equalize the number of cells among the experimental groups, approximately 30 (control and ethionine + methionine group) and 45 (ethionine) blastocysts per treatment were collected and cross-linked for 10 min with 1% formaldehyde in PBS containing 20-mM Na-butyrate (PBS-NaBu) and then quenched with 125-mM glycine for 5 min. The cells were pelleted by centrifugation at 470× g for 10 min and washed with PBS-NaBu containing protease inhibitor cocktail (PBS-NaBu-PIC). After centrifugation, the supernatant was discarded, and the cell pellet (10 μl) was stored at −80°C. The cells were resuspended in lysis buffer to a final volume of 37.5 μl, and the lysate was mixed with 82.5 μl of PBS-NaBu-PIC. The sample was sonicated to shear chromatin using a Bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan) for 5×30 s with 30-s pauses in ice-water. The sample was centrifuged for 10 min at 14,000×g, and the supernatant (110 μl) was transferred to a new tube and mixed with 110 μl of ChIP Buffer. From each 220-μl sample of sheared chromatin, 20 μl was set aside at 4°C as the "Input," and the rest (200 μl) was mixed with 2.5 μg of antitrimethylated histone H3 at lysine 9 (H3K9me3) antibody (07-442, Merck Millipore, Billerica, MA, USA) and incubated for 16 h at 4°C with rotation at 40 rpm. Negative control was set by using a nonspecific rabbit IgG. Protein A-coated magnetic beads (10 μl) were added to the sample, which was then rotated at 40 rpm for 2 h at 4°C. The DNA was eluted from the immunoprecipitate and decross-linked overnight and subsequently purified with MicroChIP DiaPure columns (Diagenode), which resulted in 12 μl of ChIPed DNA. The same DNA purification method was also applied to the input sample. From the 12 μl of ChIPed or input DNA, 4 μl was used as a template in a qPCR reaction. The PCR conditions were as described above except that the cycling consisted of 45 cycles at 95°C for 30 s, 60°C (*Oct4* and *Tead4*) or 58°C (*Nanog*) for 30 s and 72°C for 30 s. The primer pairs used are listed in Table 1. The amount of ChIPed DNA was calculated as the percentage of the input (%input) from Ct values. The experiments were repeated three to four times for each gene.

Table 1
PCR primers used for RT-qPCR, μChIP and bisulfite sequencing analyses

Analysis	Gene	Primer sequence (5'-3')	Product length (bp)	Ref.[a]
RT-qPCR	*Oct4*	GGCGTTCTCTTTGGAAAGGTG AGTTCGCTTTCTCTTCCGGG	192	
	Nanog	ACCTGAGCTATAAGCAGGTTAAGA AAGGCTTCCAGATGCGTTCA	185	
	Cdx2	TGTACACAGACCATCAGCGG ACCAAATTTTAACCTGCCTCTCG	126	
	Tead4	GCACCATTACCTCCAACGAG GATCAGCTCATTCCGACCAT	227	[40]
	Actb	AAGATCAAGATCATTGCTCCTCCT CAGCTCAGTAACAGTCCGCC	172	
μChIP	*Oct4*	CTGTAAGGACAGGCCGAGAG CAGGAGGCCTTCATTTTCAA	139	[41]
	Nanog	TCACACTGACATGAGTGTGG TCTGTGCAGAGCATCTCAGT	156	[41]
	Tead4	GGAAAGCGGTGGCGGATCT GCTGCTACGGAGGACACTCG	78	
Bisulfite sequencing	*Nanog*	GATTTTGTAGGTGGGATTAATTGTGAATTT ACCAAAAAAACCCACACTCATATCAATATA	367	[21]
	Tead4	TTTAAGGGATAAGGAAAGAATTGAAA AAAACAAAAAAAACTATTCCCTTCC	160	

[a] The primer pairs without reference were designed by authors.

2.5. Bisulfite sequencing

Blastocysts from the control and ethionine-treated groups (50–60 blastocysts per group) were snap frozen in liquid nitrogen and stored at −80°C until DNA extraction. Genomic DNA was extracted from the blastocysts with the DNA Micro kit (Qiagen) and was then treated with bisulfite with the EpiTect Bisulfite kit (Qiagen). Bisulfite-treated DNA was eluted in 40 μl of elution buffer. PCR was performed in a 50-μl Platinum PCR SuperMix (Life Technologies) reaction containing 10 μl of bisulfite-treated DNA and 0.2 μM of each primer indicated in Table 1. The PCR cycles consisted of an initial denaturation at 94°C for 1.5 min, followed by 45 cycles of 94°C for 30 s, 52°C (*Nanog*) or 56°C (*Tead4*) for 30 s and 72°C for 30 s. The PCR products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and then were transfected into DH5α competent cells (Toyobo, Osaka, Japan). At least 10 clones for each sample were sequenced. Six and 8 CpGs were examined for *Nanog* and *Tead4*, respectively.

2.6. Statistical analyses

The embryonic development expressed as a percentage and results of RT-qPCR and μChIP were subjected to a general linear model in which treatment was taken as a fixed variable. When multiple comparisons were made, Tukey–Kramer test was used. These data are presented as means±standard error of the mean. The data for bisulfite sequencing were analyzed by chi-square test. All analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Significance was accepted at P<0.05.

3. Results

3.1. Methionine antagonist decreases mouse blastocyst development

We first examined the effects of ethionine, an antimetabolite of methionine [17], on the blastocyst development of mouse preimplantation embryos *in vitro*. Treatment with 10-μM ethionine halved the rate of blastocyst development compared with the control (P<0.01), and this effect was almost lethal at 25-μM ethionine (Table 2 and Fig. 1). Treatment with 10-μM ethionine also decreased the total