Expression of Histone Methylases and Demethylases during Preimplantation Development in Mice

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Abstract: *The transition from germ cell to embryo is an important event in the creation of new life. During this period, cells change their characteristics through changes in gene expression patterns. Histone methylation is considered to be a stable and important epigenetic marker regulating gene expression. In the present study, we investigated the expression of histone methyltransferases (HMTs) and histone demethylases (HDMs) during preimplantation development. Analysis by reverse transcription-PCR (RT-PCR) revealed that many of these enzymes are abundantly expressed in MII stage oocytes and that their expression levels change dynamically during preimplantation development. These results suggest that HMTs and HDMs are involved in the regulation of gene expression during preimplantation development.*

Key words: *Histone demethylase, Histone methylase, Preimplantation embryo*

Introduction

The transition from oocyte to zygote is an important process in generating new life and entails the reprogramming of gene expression. A dynamic alteration of the gene expression pattern occurs during oogenesis and preimplantation development [1–4]. However, little is known about the mechanisms regulating these changes in gene expression. Recent studies have revealed that epigenetic modifications, such as histone modifications, play important roles in the regulation of chromatin structure and gene expression [5–9]. These modifications are altered genome-wide when cells change characteristics, e.g., during differentiation or cancer development.

Among the various types of epigenetic modifications, histone lysine methylations are considered relatively stable and important epigenetic modifications for the long-term regulation of gene expression. A number of lysine residues are methylated in histones. It has been suggested that methylation of H3 at lysine 9 (H3K9) is associated with gene silencing [9, 10]. Conversely, methylation of lysine 4 on histone H3 (H3K4) is tightly linked to active gene expression [5, 11–13]. Thus, histone lysine methylation may be a marker determining gene expression patterns in cells.

Histone methylation is known to be catalyzed by histone methyltransferases (HMTs) that contain a SET domain. One exception is Dot1l, which does not contain this domain [14, 15]. HMTs that act on H3K4 and H3K9 have previously been identified. For example, *Set7*, *Smyd3* and *Mll* are known to increase methylation of H3K4 [16–18]. Specifically, *Set7* is associated with the dimethylation, but not trimethylation, of H3K4, while *Smyd3* and *Mll* are both involved in the trimethylation of H3K4. *Suv39h*, *Eset*, *G9a*, and *GLP* (*G9a* homolog) are known to be H3K9 methylases [6, 11, 19]. Both *Suv39h* and *Ezh2* are also involved in H3K27 methylation [6, 20]. Although the functions of several HMTs are presently known, the targets of several HMTs that possess the SET domain are not.

Recently, it was reported that histone demethylation is catalyzed by histone demethylases (HDMs) that contain the AO domain [21]. Known AO domain-containing genes include *LSD1* and *AOF1*. *LSD1* has been shown to be involved in the demethylation of H3K4 and H3K9 [11, 22], whereas the target of *AOF1*

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has not been clearly identified.

Histone methylation is dynamically altered during preimplantation development. The levels of H3K9 methylation are unequal in the male and female pronucleus of 1-cell embryos; H3K9 methylation is higher in the female pronucleus. H3K9 is demethylated at the 2-cell stage and increases during preimplantation development [23, 24]. Additionally, H3K4 methylation increases following fertilization [23, 25]. Thus, these changes in histone methylation appear to be involved in the alteration of gene expression during preimplantation development. It has previously been reported that mice lacking *G9a*, *Suv39h*, *Eset*, or *GLP* show abnormal histone methylation and early embryonic lethality [26–29]. Mice lacking any of these genes died after implantation with the exception of *Eset*-deficient mice, which died before implantation. Although these reports demonstrate the importance of histone methylation in early development, the mechanism regulating histone methylation and their role in preimplantation development remain to be elucidated.

To better understand the mechanisms regulating histone methylation in preimplantation embryos, it is necessary to outline the expression patterns of various HMTs and HDMs during this developmental period. Therefore, in addition to known HMTs and HDMs, we first chose genes containing SET and AO domains as the candidates for HMTs and HDMs, respectively, by *in silico* screening, and then investigated mRNA expression of these genes during preimplantation development in mice.

Materials and Methods

Media

Whitten's medium [30] was used for *in vitro* fertilization. It consisted of 109.51 mM NaCl, 4.78 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 22.62 mM $NaHCO_3$, 5.55 mM D-glucose, 0.31 mM sodium pyruvate, 1.49 mM calcium lactate, 0.075 mg/ml sodium penicillin-G, 0.05 mg/ml streptomycin sulfate and 3 mg/ml bovine serum albumin (BSA).

KSOM was used to culture embryos and consisted of 95 mM NaCl, 2.5 mM KCl, 0.3 mM KH_2PO_4, 0.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.2 mM D-glucose, 0.01 mM EDTA2Na, 0.2 mM sodium pyruvate, 10 mM DL-lactic acid (sodium salt, 60% syrup), 0.3 mM KH_2PO_4 and 3 mg/ml BSA.

Collection and culture of oocytes and embryos

Unfertilized oocytes were collected in Whitten's medium from 3-week-old BDF1 mice (SLC, Shizuoka, Japan) that had been superovulated by injection with 5 IU of human chorionic gonadotropin (hCG; Sankyo, Tokyo, Japan) following 5 IU of pregnant mares' serum gonadotropin (PMSG; Sankyo). Sperm were obtained from the cauda epididymis of mature male ICR mice (SLC) and incubated in Whitten's medium. The oocytes were inseminated with sperm that had been incubated for 2 h at 38°C. The embryos were washed with KSOM 3 h after insemination and then cultured in a humidified 5% CO_2/95% air atmosphere at 38°C.

Reverse transcription-PCR

Total RNA was isolated from 40 to 60 unfertilized oocytes and embryos at the 1-cell, 2-cell, and blastocyst stages (10, 28, and 90–92 h after insemination, respectively), using ISOGEN (Nippon Gene, Tokyo, Japan), as described previously [31]. As an external control, 50 pg of rabbit α-globin mRNA was added to each tube prior to the isolation of total RNA. The RNA was reverse-transcribed in 20 μl reaction mixture that contained 5 U ReverScript II (Wako, Osaka, Japan) and 0.5 μg oligo(dT) 12–18 primer (Invitrogen Corp., Carlsbad, CA, USA) at 42°C for 1 h, followed by 51°C for 30 min. The template mRNA was digested with 60 U RNase H (TaKaRa, Shiga, Japan) at 37°C for 20 min. After precipitation with ethanol, cDNA was dissolved in double-distilled water; cDNA derived from two oocytes or embryos was included in 2 μl water.

PCR was performed using an iCycler (Bio-Rad, Tokyo, Japan). The reaction mixture consisted of template cDNA, which was derived from four oocytes or embryos, 0.2 μM of each primer, 300 μM dNTPs, 3 mM $MgCl_2$, and 0.05 U/μl ExTaq DNA polymerase (TaKaRa). The list of genes examined and the sequences of the PCR primers used are shown in Table 1. PCR was performed for 32 cycles for rabbit α-globin, *ESET*, and *Smyd3*, and for 40 cycles for the other genes with denaturation at 95°C for 15 s, annealing at 61°C for 15 s, and extension at 72°C for 20 s.

Results

Histone methylation is catalyzed by proteins containing the SET domain, with the exception of Dot1l, which does not have this domain [14, 15]. Therefore, we searched for SET domain-containing genes in the Mouse Genome Informatics (MGI) database and found 30 genes containing the SET domain, which are listed in Table 1. We examined the expression of these 30 genes and Dot1l during preimplantation development.

Table 1. PCR primers

Name	Primer sequence	
	Sense	Antisense
Smyd4	5'-actgctggtgaaggaagatg -3'	5'-tcattctcagggcaacatgg -3'
Smyd5	5'-tctctctttgcccttgttgg -3'	5'-tcatctcatcccctagctct -3'
Suv420h1	5'-cagtgacagcaacctctatg -3'	5'-gtgctgtcctcctcatattg -3'
Suv420h2	5'-ggattacttgccccctttggc -3'	5'-acacactaccaggatcagac -3'
Evi1	5'-ctttgaatccaaggcagagc -3'	5'-gacagcatgtgcttctccaa -3'
Prdm4	5'-cgtgggaacagatgttgttg -3'	5'-tccaggacaccagtgtggta -3'
Suv39h1	5'-atggagtatgtgggagagat -3'	5'-caaaatgagagatgttgcca -3'
Suv39h2	5'-atgtaaatgtggagccgaga -3'	5'-ttaggggctttttcttctgtg -3'
G9a	5'-tacagcaaggaagaggatgg -3'	5'-atccacaccattgacacagg -3'
Eset	5'-gatgttcccctgtcctgtgt -3'	5'-acccatccttgcagtcacag -3'
GLP	5'-ggaacatcactcatttgcag -3'	5''-aagatcaagggtggttctgc -3'
Mll	5'-gttgtgtttctgattgagca -3'	5'-ttctcacgcttgtctgtctg -3'
Set7	5'-ggagtggtatcaagtggagct -3'	5'-tggttgtcccgttgtcagat -3'
Smyd3	5'-ctctttgtataatttcctcc -3'	5'-gaaatatcatgtacgccagt -3'
Prdm9	5'-catcccaaccactcagtcct -3'	5'-tcgtcctgtccatccacata -3'
0610039J04Rik	5'-aatcaactcttccagggtag -3'	5'-tcacagacacttagagacag -3'
2610014H22Rik	5'-tccatcagaatcaactccct-3'	5'-gttctcctctgctgcttcta -3'
Ash1l	5'-tgagattgtgggagaaacgg -3'	5'-tgtaaaaaaggtgtgctgac -3'
D12Ertd771e	5'-aaaaatcctgacctctctgt -3'	5'-cttcctcctccaactttctc -3'
Setmar	5'-attttgccaggagaagaact -3'	5'-attttctaaggggccatata -3'
Ezh1	5'-gatcaagagttccatgtcac -3'	5'-ccagcaacatcttcctctag -3'
Ezh2	5'-aaagacaccacctaaacgcc -3'	5'-gactctaaactcatacacct-3'
Nsd1	5'-gctggtgttttgtgtgttca -3'	5'-atgtttgtaaggtggcggct -3'
Prdm1	5'-agagagtacagcgtgaaaga -3'	5'-agtgagcattgtaagaagga -3'
Prdm2	5'-gaaagccagcatagggttct -3'	5'-aaggtgaggagactgtacga -3'
Prdm16	5'-aactttccccactccctcta -3'	5'-cttccgcttttctaccctgt -3'
Smyd1	5'-tttcaacaacttctgctcaa -3'	5'-ttcttttcttcttctgtgca-3'
Smyd2	5'-tttctttacctgtgagtgcc -3'	5'-tgttttccagacccatgtac-3'
Prdm5	5'-catctcaggagcggaagaac -3'	5'-ggcaaagtcctcttcacagc -3'
Prdm6	5'-tcccagagaacgccatattc -3'	5'-ttatctcgggctgattggac -3'
Dot1l	5'-caagaaaatgagtgctgcca -3'	5'-gagctgtgtgttcttctcct -3'
LSD1	5'-ctgagtggaaaacatctgca -3'	5'-gccacataagaataggagcc -3'
AOF1	5'-catctctctcccaacttacg-3'	5'-tcttcctaaacactaccaca -3'
Globin	5'-gcagccacggtggcgagtat -3'	5'-gtgggacaggagcttgaaat -3''

We obtained reproducible results in the expression patterns of 27 of the 31 genes by RT-PCR (Fig. 1). Expression patterns during preimplantation development could be divided into five types. The first group, which showed a constant expression pattern throughout preimplantation development, consisted of five genes: *Ash1l*, *EZh1*, *Nsd1*, *Prdm4* and *Suv39h*. The second group showed high levels of expression in oocytes and blastocysts; the level of expression decreased after fertilization and increased at the blastocyst stage. This group included ten genes: *Dot1l*, *Eset*, *GLP*, *G9a*, *Mll*, *Prdm1*, *Set7*, *Smyd2*, *Smyd3* and *Smyd4*. The genes in the third group were mainly expressed in oocytes; the expression level decreased after fertilization and remained low thereafter. Five genes, *2610014H22Rik*, *Ezh2*, *Prdm16*, *Setmar* and *Smyd1*, were included in this group. In the fourth group, which contained four genes, *D12Ertd771e*, *Prdm2*, *Suv420h1* and *Suv420h2*, the expression level was high only in blastocysts. Expression was not detected, or detected at very low levels, before the blastocyst stage. Finally, the fifth group comprised three genes, *Suv39h*, *Smyd* and *0610039J04Rik*, which showed expression patterns that could not be classified into any of the other four groups.

Histone demethylation is thought to be catalyzed by proteins with an AO domain. In our MGI search, we found two genes containing AO domains, *LSD1* and *AOF1*. Both were expressed at high levels in oocytes, and their expression levels decreased at the 2-cell stage (Fig. 2). The expression of *LSD1* then increased again at the blastocyst stage, placing it in group 2.

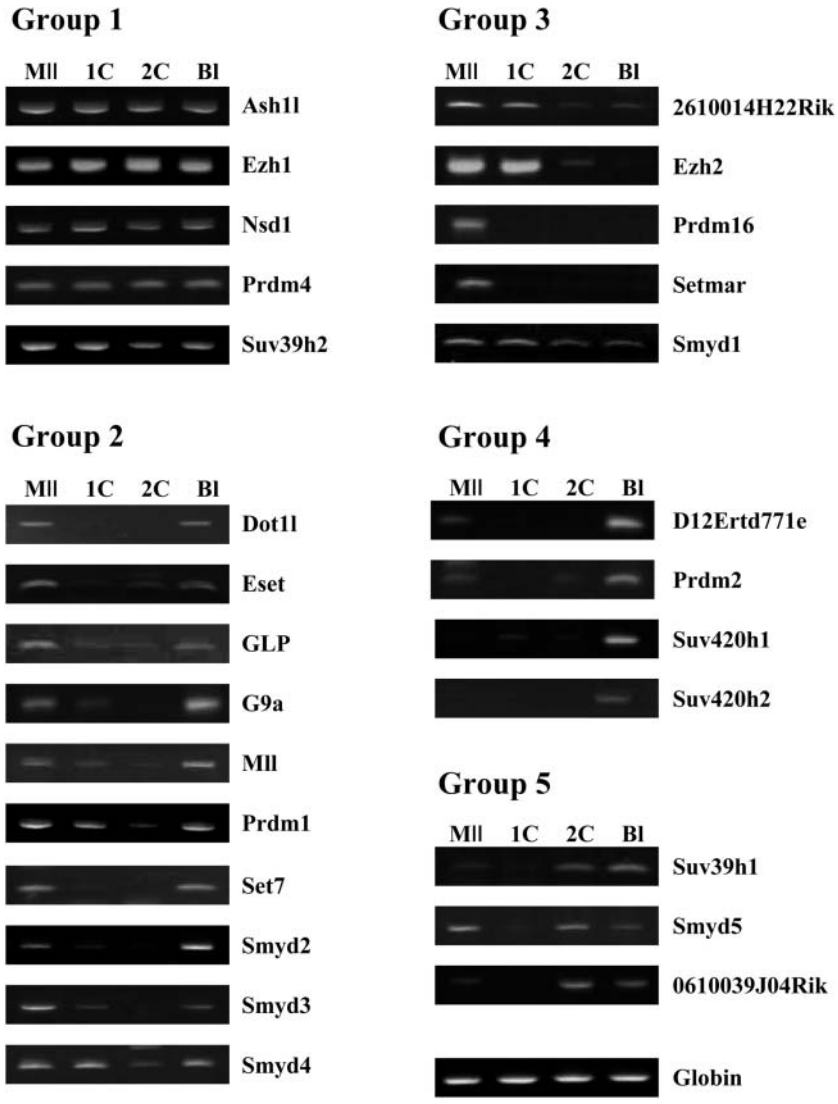

Fig. 1. Expression of histone methylases (HMTs) in mouse preimplantation embryos. Expression of HMTs was examined by RT-PCR in MII stage oocytes (MII) and in embryos at the 1-cell (1C), 2-cell (2C), and blastocyst (Bl) stages. Rabbit α-globin mRNA was added to the total RNA samples as an external control. The experiments were conducted three times, and similar results were obtained in each case.

AOF1 expression remained at a low level in blastocysts, placing it in group 3. Thus, several HMTs and HDMs genes were classified in groups 2, 3, and 4 (more than 70%; 21 of 29), in which the expression level was high in MII oocytes and/or blastocyst stage embryos.

Discussion

We investigated the expression of various HMTs and HDMs in MII stage oocytes and preimplantation embryos and found that their expression levels changed dynamically during preimplantation development. Their expression patterns were classified into five groups, and many of them (more than 70%) were classified into groups 2, 3 and 4, in which the expression level was high in MII stage oocytes and/or blastocyst stage embryos. Cells in both of these stages are differentiated. Rewriting epigenetic markers is considered to be important both in MII stage oocytes, in which the differentiated genome in gametes and

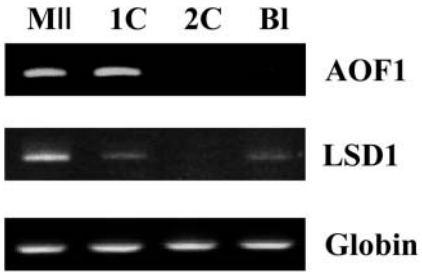

Fig. 2. Expression of histone demethylases (HDMs) in mouse preimplantation embryos. Expression of HDMs was examined by RT-PCR in MII stage oocytes (MII) and in embryos at the 1-cell (1C), 2-cell (2C), and blastocyst (Bl) stages. Rabbit α-globin RNA was added to the total RNA samples as an external control. The experiments were conducted three times, and similar results were obtained in each case.

transferred somatic nucleus can be reprogrammed, and in blastocyst stage embryos, in which totipotent zygotes begin to differentiate. At these stages, cell characteristics and gene expression patterns are dynamically changed following alteration of epigenetic markers. Among several epigenetic markers, histone methylation is considered to be a stable modification in somatic cells. To rewrite this stable marker, a high expression level of HMTs and HDMs may be necessary.

Although several studies have recently reported on the functions of various HMTs, for example, *Suv39h*, *G9a*, *GLP* and *Eset* [26–29], many of the genes described in this study have not been well investigated. Little is known regarding their expression patterns, and many of their targets have yet to be identified. We found that all of the HMT candidates were expressed in oocytes and/or preimplantation embryos. Among them, *Smyd2*, *Smyd4* and *Setmar* were abundantly expressed in oocytes, while the expression levels of *2610014H22Rik* and *D12Ertd771e* increased after fertilization. In conclusion, various HMTs dynamically alter their expression levels in different ways during preimplantation development. These results suggest that dynamic alterations of various histone methylations are involved in regulating changes in gene expression patterns during preimplantation development.

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