# Analysis of DNA Methyltransferases (Dnmts) Expression during Early Development

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## ABSTRACT

There are replete numbers of reports which have apparently shown that established patterns of methylation are critical for normal mammalian development. Here, we report expression of the DNA methyltransferases (Dnmts) family during mouse early development. Transcription of Dnmt1o occurs in one-cell and morula stage embryos, whereas Dnmt1s transcripts were detectable in all cells and tissues examined during the study. Dnmt3a1 transcript was detected in all cells and Dnmt3a2 transcript was particularly detected in the oocyte and 1-cell stages. Low level Dnmt3b1 transcripts were expressed ubiquitously in oocyte, 1-cell, and preimplantation embryos except 2~4 cell stages. Dnmt3b3 transcripts were only detected in E7.5 embryo and ovary. Furthermore, Dnmt3l transcripts were detectable in all cells and tissues examined. Unlike Dnmt1, both Dnmt3a and Dnmt3b proteins existed in the nucleus of preimplantation embryos till the morula stage. These Results suggest that differences Dnmts expression level exist and genomic DNA methylation patterns may be determined partly through differential expression of Dnmts during early development.

(Key words : Methylation, Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l)

## INTRODUCTION

DNA methylation in vertebrates, mainly occurs at the 5'-position of cytosine in a CpG dinucleotide forming 5-methylcytosine (Gruenbaum et al., 1981). Formation of the cell type-specific DNA methylation pattern is one of the epigenetic events which goes hand in hand with the accompanying the production of diverse cell types in the body (Shiota et al., 2002). Generally, DNA methylation in gene-containing regions of the genome is inversely correlated with the transcriptional activity of associated genes (Kass et al., 1997; Bird et al., 1999) through direct (Tate et al., 1993) and/or indirect (Ng et al., 1999) mechanisms. Demethylation of male pronucleus in murine gets absolutely accomplished within 4 hr of fertilization (Santos et al., 2002) due to genomic DNA methylation (Monk et al., 1987) during preimplantation period in mammals.

In mammals, there are five members of DNA methyltransferase family; Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3l (Bestor et al., 1988; ; Okano et al., 1998a,b; Yoder and Bestor, 1998; Hata et al., 2002). Dnmt1 is considered as a maintenance methyltransferase based on the in vitro enzyme assay; in which it preferentially recognizes hemimethylated DNA (Pradhan et al., 1999), and localizes at replication foci in proliferating cells

(Bestor and Verdine, 1994). Inactivation of Dnmt1 in mice leads to global loss of methylation and biallelic expression or silencing of imprinted genes (Li et al., 1992, 1993; Caspary et al., 1998). Unlike Dnmt1, biological activity of Dnmt2 did not reveal methylase activity specific for CpGs (Yoder and Bestor, 1998), and knockout of this gene in the mouse showed no phenotypic effect (Okano et al., 1998b). Dnmt3a and Dnmt3b have de novo methyltransferase activity in vitro (Okano et al., 1998), and deletion of one of these genes causes extensive perturbations in DNA methylation patterns and embryonic lethality (Li et al., 1992; Okano et al., 1999).

Inappropriate genomic methylation is a likely cause of developmental abnormalities and epigenetic disease introduced by cloning technologies, assisted reproductive techniques and associated cell culture, and abnormal events during preimplantation development (Bourc'his et al., 2001; Dean et al., 2001; DeBaun et al., 2003). The observation of aberrant expression and localization of Dnmt1 isoforms in cloned mouse embryos (Chung et al., 2003) is of further concern particularly in terms of potential for inducing epigenetic abnormalities in ES cell. These above observations and findings indicate great deal of understanding in early epigenetic regulation is required as far as mouse reproduction is concerned. Therefore, study was designed to analyse the expression of the mRNAs and proteins of DNA methyltransferases during

mouse early development.

## MATERIALS AND METHODS

### Reagents, Collection of Oocyte and Embryos

C57BL/6Ncrj (B6) mice were obtained from Wako Pure Chemicals Ind. (Osaka, Japan). Metaphase II (MII) oocytes were collected from the oviducts of 7 weeks old superovulated B6 female mice 20 hr after the injection of human chorionic gonadotropin (hCG; Teikoku Zouki, Japan). Collected MII oocytes were incubated in 1 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA)/PBS for 5 min at room temperature to remove cumulus cells, then the oocytes were washed over 5 times in PBS containing 1% BSA (Sigma). Superovulated B6 females were individually caged with B6 stud male overnight to obtain preimplantation embryos, and examined for the presence of a vaginal plug (VP) on the next morning. Noon of the day on which VP was observed was designated as embryonic day 0.5 (E0.5).

Embryos were collected from oviducts at E0.5 (1-cell stage) after hCG injection. Two-cell embryos were obtained from oviducts of superovulated females killed 1.5 days after hCG administration. Four- and eight-cell stage embryos were obtained from oviducts of superovulated females killed 60 and 72 hrs after hCG administration, respectively. Some of the eight-cell stage embryos were cultured in 50 μl microdrops of potassium-enriched synthetic oviductal medium (KSOM) under mineral oil at 37°C in an atmosphere of 5% $CO_2$ in air to obtain morula and blastocyst stage embryos. Postimplantation embryos were dissected out of uterine horns at E7.5. For RNA preparation, only the morphologically normal oocytes and embryos were pooled and stored in 5 μl of diethyl pyrocarbonate-treated phosphate-buffered saline at −80°C until use. Approximately 5 oocytes and 1~3 preimplantation embryos were used for RNA preparation. Kidney, liver and ovary were collected from 60 days old B6 mice.

### Culture and Isolation of Embryonic Stem (ES) Cells

The ES cell line MS12, derived from B6 mouse embryo, was a kind gift from Dr. H. Suemori (Kyoto University, Kyoto, Japan). ES cell was cultured on mitomycin C (Sigma-Aldrich)-treated STO cells in the presence of 1000 units/ml leukemia inhibitory factor (LIF, ESGRO®; Chemicon, Temecula, CA, USA) under standard conditions. Before preparation of RNA from ES cells, mitomycin C treated STO cells were removed by taking advantage of their higher adhesiveness to the surface of tissue culture dish compared with ES cells. In brief, cells were harvested by incubation in 0.05% trypsin/ 1 mM EDTA and re-plated on fresh tissue culture dishes. After 30 min incubation at 37°C, non-attached cells were collected and added to fresh tissue culture dishes again. After further 30 min incubation at 37°C, non-attached ES cells were recovered and stored at −80°C until use.

### RNA Isolation and RT-PCR

Into the microfuge tube containing the collected oocytes or embryos in 5 μl of DEPC-treated PBS, 100 μl of Trizol reagent (Invitrogen, Carlsbad, CA) and 2 μl of 20 mg/ml glycogen (MBI, Fermentas) were added. Subsequently, RNA was isolated following manufacturer's instructions and finally dissolved in 10 μl of DEPC-treated water. RNA preparation from adult tissues was also performed with the Trizol reagent. Synthesis of cDNA was carried out using random hexamers and SuperScript II first-strand synthesis system (Invitrogen) according to manufacturer's instructions. PCR amplifications were carried out with 1 μl aliquots of cDNA in a total reaction volume of 20 μl by using r-*Taq* polymerase (Toyobo, Tokyo, Japan). The primer sequences, annealing temperature and their approximate sizes are listed in Table 1. PCR reactions were performed under the following conditions: 95°C, 1min; 35 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 1min; final extension 72°C, 10 min. β -actin was used in all experiments as a positive control to ensure the quality and mRNA of quantity. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

### Immunocytochemistry

Anti-rat Dnmt1 antiserum has been generated against the amino-terminal polypeptide (amino acids 108-318) of Dnmt1 protein (Kimura *et al.*, 1998) that is a common region between Dnmt1o and Dnmt1s. Anti-Dnmt3a and anti-Dnmt3b antibodies (Aoki *et al*, 2001) were kindly provided by Dr. H. Sasaki (National Institute of Genetics, Japan).

Embryos were freed of zona pellucida by a brief exposure to acidified Tyrode's solution (pH 2.5) and fixed for 15 min at room temperature in a freshly prepared 4% formaldehyde/ PBS, and then the fixed specimens were incubated in a blocking buffer (3% BSA/ 0.5% Triton X-100) for at least 1 hr. For immunofluorescence detection of Dnmt1o, Dnmt3a and Dnmt3b, the anti-Dnmt1 antisera, the anti-Dnmt3a antibody and the anti-Dnmt3b antibody were used at a dilution of 1:50, 1:100 and 1: 1000, respectively, in the blocking buffer. Bound antibodies were detected by FITC-conjugated goat anti-rabbit IgG (JIR) at 1:500 dilution in the blocking buffer. Immunostained embryos were finally counterstained in 10 μg/ml DAPI (Roche Diagnostics)in the blocking buffer for 30 min at room temperature. For microscopic examination, immunostained embryos were transferred into a drop of the mounting medium (Biomeda, Foster, CA) placed on a glass microscope slide. A coverslip was gently

Table 1. The sequences of PCR Primers used for RT-PCR

| Genes | Primer sequence | Annealing temperature(°C) | Base pairs |
|---|---|---|---|
| Dnmt1o | Foward 5'-GGTTGATTGAGGGTCATT-3'<br>Reverse 5'-GCAGGAATTCATGCAGTAAG-3' | 55 | 235 |
| Dnmt1s | Foward 5'-GGGTCTCGTTCAGAGCTG-3'<br>Reverse 5'-GCAGGAATTCATGCAGTAAG-3' | 55 | 201 |
| Dnmt3a1 | Foward 5'-CGAGGGCTTGACATCAGGGTC-3'<br>Reverse 5'-CACTCCGCTTCTCCAAGTCTCC-3' | 62 | 414 |
| Dnmt3a2 | Foward 5'-AGGGGCTGCACCTGGCCTT-3'<br>Reverse 5'-TCCCCCACACCAGCTCTCC-3' | 61 | 279 |
| Dnmt3b | Foward 5'-GTAGCGCAGCGATCGGCGCCGG-3'<br>Reverse 5'-CCCGCTGGCACCCTCTTCTTC-3'. | 65 | 279 |
| Dnmt3b isoforms<br>(exons9-13) | Foward 5'-TGGGATCGAGGGCCTCAAAC-3'<br>Reverse 5'-TTCCACAGGACAAACAGCGG-3' | 60 | 284 or 224 |
| Dnmt3b isoforms<br>(exons19-23) | Foward 5'-GCGACAACCGTCCATTCTTC-3'<br>Reverse 5'-CTCTGGGCACTGGCTCTGACC-3' | 58 | 549 or 361 |
| Dnmt3l | Foward 5'-GTTCTGACGACCCTGCTGTC-3'<br>Reverse 5'-TCAATGCTCCGTCGGTTCAC-3' | 60 | 317 |
| β-actin | Foward 5'-GTGGGCCGCTCTAGGCACCAA-3'<br>Reverse 5'-CTCTTTGATGTCACGCACGATTTC-3' | 65 | 531 |

placed on the drop containing embryos to spread the mounting medium and slightly squash the specimen, and was subsequently sealed with clear nail polish. The embryos were then examined using an Axioskop microscope (Zeiss) equipped for epifluorescence with fluorescein and UV filter sets. Obtained images were processed by deconvolution software IPLab program (M&S, Tokyo, Japan).

## RESULTS

### Dnmt1 RNA and Protein Expression during Preimplantation Development

Dnmt1o and Dnmt1s transcripts were detected by RT-PCR in the oocytes, early embryonic stages before and after implantation, ovary and ES cell (Fig. 1A). Dnmt1o transcript was detected in the oocyte, 1- and 2-cell stage embryos, morula and ovary, while the transcript was not detectable in the 4- and 8-cell stages, blastocyst, ES cells, and in the E7.5 embryos. Similarly, no Dnmt1o transcript was detected in the kidney and liver of the adult mice (data not shown). In contrast, Dnmt1s transcript was detectable in all cells and tissues examined; amount of Dnmt1s transcript was high in 7.5dpc embryo, ovary and ES cells, while it was low in other tissues and cells including the oocytes and preimplantation embryos. Thus, expression of Dnmt1o is not allowed in the restricted embryonic stages including 4- and 8-cells, blastocysts and embryonic stages after implanta-

tion. In this data, the expression of Dnmt1o is more severely controlled compared with Dnmt1s. Interestingly, the 1-cell stage embryos contained higher amount of Dnmt1o mRNA than oocytes. Considering that the amount of B-actin mRNA was similar between the oocyte and 1-cell stage, the increase of Dnmt1o transcript seems to depend on the de novo synthesis at the stage. Regarding the Dnmt1o, the transcription occurs in oocytes and in 1-cell and morula stage embryos, while it is suppressed in 4- and 8-cell, blastocyst stages and in post-implantation embryos. The amount and localization of Dnmt1 were observed in very high levels in cytoplasm of preimplantation embryos (Fig. 1B).

### Expression of Isoform of Dnmt3a and Dnmt3b during Preimplantation Development

To determine the Dnmts responsible for methylation during early development, we investigated isoforms of Dnmt3a and Dnmt3b by RT-PCR in the oocytes, early embryonic stages before and after implantation, ovary and ES cell (Fig. 2). Dnmt3a1 transcript was detected in all cells examined including the oocytes, 1- to 8-cell stage embryos, morulae and blastocysts. In contrast Dnmt3a2 transcript was specifically detected in the oocyte and 1-cell stages, but not detectable in the 2-cell to blastocyst stages.

Regarding the Dnmt3b, based on presence or absence of exon 10 and/or exon21/22, there are many variants (Fig. 2A). Although Dnmt3b2 and Dnmt3b6 transcripts may be detected in preimplantation embryos, low level Dnmt3b1 transcripts were expressed ubiquitously in oo-

cyte, 1-cell and preimplantation stage embryos except 2- to 4-cell stages (Fig. 2B). In contrast, clear band of Dnmt3b1 transcripts were detected in D7.5 embryo, ovary and ES cells. Dnmt3b3 transcripts were only detected in E7.5 embryo and ovary. In addition, Dnmt3l transcripts were detectable in all cells and tissues examined; Dnmt3l transcripts were high in oocyte, 1-cell and ES cell, while it was low in E7.5 embryo and ovary including the preimplantation embryos (2- to 8-cells, morula and blastocyst).

## Expression and Localization of Dnmt3a and Dnmt3b during Preimplantation Development

Immunocytochemistry revealed that Dnmt3a mainly localized in the nucleus and/or perinuclear region in the 1-, 2-, and 4-cell stage embryos (Fig. 3). In 8-cell, morula
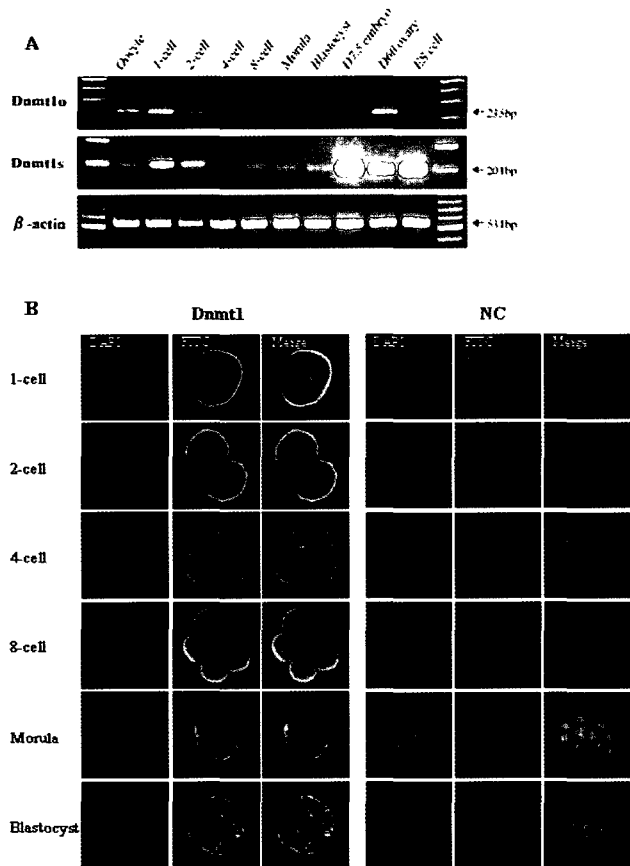
**Fig. 1. Expression and subcellular localization of Dnmt1 during early development.** (A) RT-PCR analysis of the expressions of Dnmt1o and Dnmt1s. Predicted sizes of the PCR products are shown on the right of each panel. Expression in embryos at 7.5 days of development (D7.5 Embryo), adult ovary (D60 ovary) and ES cell are also shown. The experiment was repeated three times. (B) immunostaining of Dnmt1 in preimplantation embryos. 4,6-Di-amidinophenylindole counterstain is false-colored red (DAPI) and antibody staining is falsecolored green (FITC). Treatment with primary antibodies was omitted from the procedure in the negative controls (NC).
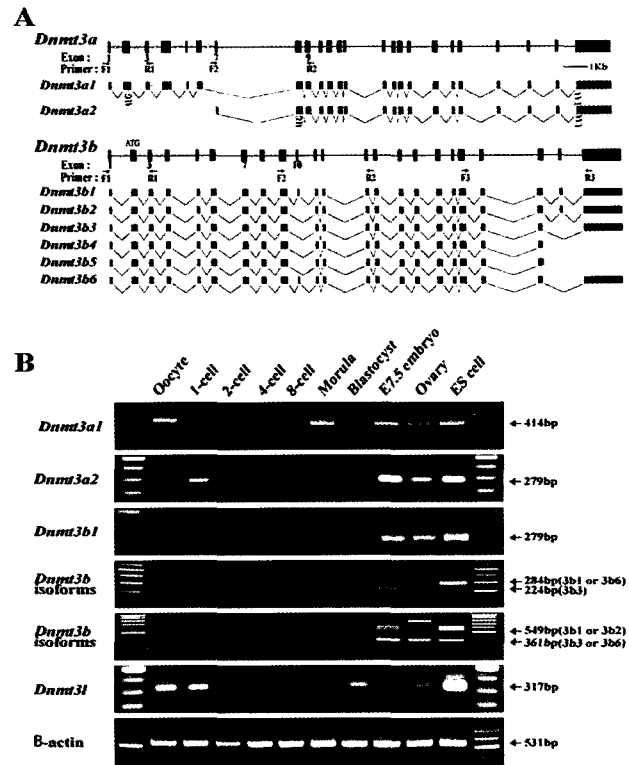
**Fig. 2. Expression of Dnmt3a, 3b isoforms and Dnmt3l during preimplantation period and development.** (A) The structure of Dnmt3a and Dnmt3b gene and mRNAs. Exons are shown as black bars. The primers used for RT-PCR are shown under the corresponding exons (F, forward R, reverse). (B) The expression of Dnmt 3a1 and Dnmt3a2 was determined by RT-PCR using primers specific to Dnmt3a1 or Dnmt3a2. The same RNA samples were analyzed by RT-PCR using Dnmt3b-specific primers exons1-3, exons 9-13 and exons 19-23. The experiment was repeated three times.
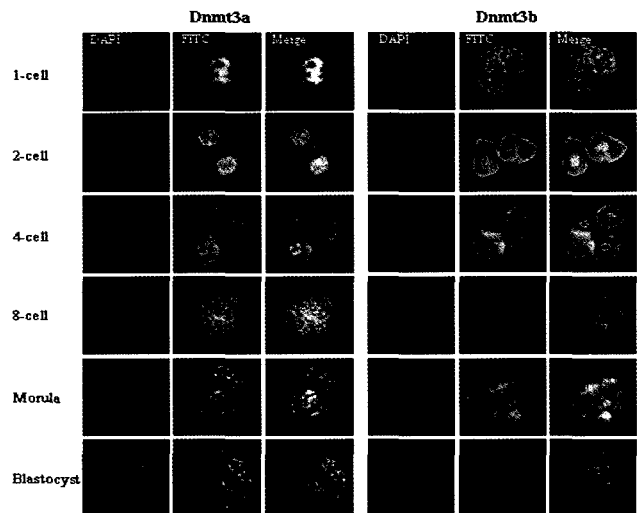
**Fig. 3. Subcellular localization of Dnmt3a and Dnmt3b during early development.** Immunostaining of Dnmt3a and Dnmt3b in preimplantation embryos. DAPI counter stain is false-colored red (DAPI) and antibody staining is false-colored green (FITC).

and blastocyst stage embryos, Dnmt3a was mainly detected in the cytoplasm, but a weak signal still remained in the nucleus. Thus, Dnmt3a protein reside at the all-preimplantation cleavage stages. Dnmt3b mRNA variants were not clearly detected throughout the preimplantation stages, and was detectable high in the ovary, E7.5 embryos and ES cells (Fig. 2). Dnmt3b protein was detected by immunocytochemistry in the preimplantation embryos (Fig. 3). Dnmt3b was mainly localized in the cytoplasm and plasma membrane cortex in the 1-, 2-, and 4-cell stage embryos, as well as located extensively in cytoplasmic foci in 1- and 8-cell stages and morula stage embryos (Fig. 3). In the blastocyst, both Dnmt-3a and Dnmt3b are found in the cytoplasm. However, unlike Dnmt1, Dnmt3b did not appear to be excluded from the nucleus during preimplantation development. Thus, both Dnmt3a and Dnmt3b proteins existed in the nucleus of preimplantation stage embryos till the morula stage. Thus, Dnmt3a and Dnmt3b proteins exist in the preimplantation embryo with different subcellular localization.

## DISCUSSION

We have analysed the expression of the mRNAs and proteins of DNA methyltransferase, which regulate or recognize CpG methylation status in order to elucidate which factors may be relevant to the regulation of DNA methylation information during mouse preimplantation embryonic development. We observed differential expression patterns and transcript usage throughout the mouse developmental stages tested. Expression of Dnmt1 mRNA was detected in one-cell and morula stage embryos, whereas Dnmt1s transcripts were detectable in all cells and tissues examined. Thus, expression of Dnmt1o is not allowed in the restricted embryonic stages including 4-, 8-, blastocyst and embryonic stages after implantation. In the current report, the expression of Dnmt1o is more severely controlled compared with Dnmt1s.

It is obscured and not determined when actually the transcription of Dnmt1o and Dnmt1s begins. Various mRNA species transcribed in oocytes are carried over into the preimplantation embryo and transcription was is limited at early embryonic stages (Aoki et al., 2003). Dnmt1o protein has been reported to be excluded from the nucleus during preimplantation development except for 8-cell stage (Mertineit et al., 1998), making it an unlikely candidate for regulating formation of the methylation pattern at its own gene. Dnmt1o detection at morula stage may be explainable by transcription at this stage rather than the carrying over from previous stages, because the transcript was undetectable at 4- and 8-cell stages. As shown in Fig. 1B, amount and localization of Dnmt1 were observed in very high levels in cyto-

plasm of preimplantation embryos. In our data, nucleic Dnmt1 protein localization in eight cell embryo is different from the pattern of cytoplasmic localization seen in the previous reports (Howell et al., 2001; Ratnam et al., 2002). The difference in intracytoplasmic distribution of Dnmt1 is likely to be due to differences of used antibody.

DNA methylation patterns of genomic imprinting in oocyte and preimplantation embryos occur in settings of dramatic changes in the intracellular composition and localization of forms of Dnmt1 methyltransferase (Mertineit et al., 1998). In MII oocytes and in all preimplantation embryos, there are Dnmt1o protein, approximately 50,000-fold higher on a per-nucleus basis in the oocyte as compared to amount of Dnmt1s protein present in a cycling somatic cell (Carlson et al., 1992). Dnmt1o's only known function during the period spanning oocyte maturation and preimplantation development is to maintain methylation patterns on imprinted genes during a single S phases of preimplantation development (Howell et al., 2001). Expression of Dnmt1 were observed in very high levels in cytoplasm of preimplantation embryos. Results of the experiments presented here revealed is that methylation patterns for all S phases are not maintained by the Dnmt1s. Therefore, DNA cytosine methyltransferases other than Dnmt1s are required for maintaining imprintings during preimplantation development.

Transcripts of the Dnmt3 family methyltransferase were detected in mouse mature oocyte, 1-cell (Dnmt 3a1, Dnmt 3a2, Dnmt3b1, Dnmt3l), and preimplantation embryos (Dnmt3a1, Dnmt3l). Our expression data indicate that the Dnmt3a1 and Dnmt3l are candidate regulators of methyl-CpG based epigenetic information during oocyte and preimplantation embryos. The present results, along with the dynamic changes during preimplantation stage, suggested the coordinate function of Dnmt3a, Dnmt3a2, Dnmt-3b1 and Dnmt3l for methylation information of several regions during early development.

The expression of the Dnmt3a2 transcript was tested due to the association of expression of this transcript with the cells and tissues in which de novo methylation occurs, including testis, ovary, and ES cells (Chen et al., 2002; Weisenberger et al., 2002). In the data presented, expression of the Dnmt3a2 transcript was detected in matured oocyte, 1-cell, ovary and ES cell. However, we have identified Dnmt3a2 as a candidate regulator of the de novo methylation during oogenesis and early embryogenesis. The murine Dnmt3l gene is expressed in the oocyte, of both newborn and adult mice (Bourc'his et al., 2001; Hata et al., 2002). Alternatively spliced variants of Dnmt3b methyltransferase have been identified in mouse (Yin et al., 1999; Chen et al., 2002). We showed that different Dnmt3b variants exhibit different tissue distribution. Dnmt3b1 and Dnmt3b6 are the predominant forms in ES cell, whereas Dnmt3b3 are ex-

pressed at relatively high levels in ovary and D7.5 embryo. Dnmt3a and Dnmt3b variants show different biochemical properties and expression patterns (Chen *et al.*, 2002), and they may have distinct function in early development.

We have previously proposed in our previous report that the maintainence of the complete DNA methylation pattern of Dnmt1o T-DMR, all of Dnmt1, Dnmt3a and Dnmt3b were required in the ES cells (Ko *et al.*, 2005). Co-operation between Dnmt3a family methyltransferases has been implicated in the establishment of maternal methylation imprints (Bourc'his *et al.*, 2001; Aapola *et al.*, 2002; Chedin *et al.*, 2002; Hata *et al.*, 2002). Furthermore, both Dnmt1 and Dnmt3 were required for stable maintenance of global methylation in the mouse ES cells (Chen *et al.*, 2003). Presence of Dnmt3a and Dnmt3b in the preimplantation embryos also supports their contribution in the methylation of preimplantation embryos.

It is desirable to further characterize expression of the Dnmt3 family methyltransferases during mammalian preimplantation stage. The expression data described here provides further evidence of the developmental regulation of expression of methyltransferases and indicates that DNA methylation is presumed likely to be highly regulated in mouse oocyte and preimplantation embryo.

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