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Differential DNA methylation reprogramming of various repetitive sequences in mouse preimplantation embryos

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Abstract

Genome-wide changes of DNA methylation by active and passive demethylation processes are typical features during preimplantation development. Here we provide an insight that epigenetic reprogramming of DNA methylation is regulated in a region-specific manner, not a genome-wide fashion. To address this hypothesis, methylation states of three repetitive genomic regions were monitored at various developmental stages in the mouse embryos. Active demethylation was not observed in the IAP sequences whereas methylation reprogramming of the satellite sequences was regulated only by the active mechanism. Etn elements were actively demethylated after fertilization, passively demethylated by the 8-cell stage, and de novo methylated at the morular and blastocyst stages, showing dynamic epigenetic changes. Thus, our findings suggest that the specific genomic regions or sequences may spatially/temporally have their unique characteristics in the reprogramming of the DNA methylation during preimplantation development.

Keywords: DNA methylation; Repetitive sequences; Epigenetic reprogramming; Preimplantation embryo; Mouse

DNA methylation at CpG dinucleotides in mammals plays important roles in a variety of biological processes during embryogenesis and in adult tissues such as tissue specific gene expression, cell differentiation, genomic imprinting, X-chromosome inactivation, carcinogenesis, and aging [1]. DNA methylation also functions to set up chromatin structure during mouse development [2]. Reprogramming of genome-wide DNA methylation takes place independently twice during preimplantation development and gametogenesis periods in mammals. Dynamic change of DNA methylation is observed during mouse preimplantation development. Reprogramming of DNA methylation occurs in early embryos by both active (DNA replication-independent) and passive (DNA replication-dependent) mechanisms [3,4]. The first genome-wide active demethylation is observed in the paternal genome during pronuclear formation of the zygote before the first cell division [5–7]. However, imprinted genes are not influenced by both active and passive demethylation mechanisms and maintain their methylation states throughout preimplantation development [3]. After the completion of the first cell cycle, DNA methylation of embryonic genomes is gradually erased during cleavage stage by a passive demethylation which is due to the lack of maintenance DNA methyltransferase (DNMT1) [8–11]. The oocyte form of DNMT1 (DNMTo) is predominantly localized in the cytoplasm of oocyte and then undergoes a transient nuclear localization at 8-cell stage [12–14]. In this process, DNMTo induces re-methylation with de novo methyltransferases (DNMT3a and 3b) and HDAC in the nucleus [15].

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The global methylation patterns have been mainly investigated in the preimplantation embryos by the indirect immunostaining method using the antibody against 5-methyl cytosine [5,7,16]. To test profiles of genome-wide methylation in early-stage embryos, alternatively methylation states of CpG islands in the target sequences could be determined by the bisulfite mutagenesis [17]. Non-coding DNA containing introns, repetitive elements, and potentially active transposable elements requires effective mechanisms for its long-term silencing [18]. However, little is known in the DNA methylation reprogramming of various repetitive genomic regions during early embryogenesis. Detailed analysis of genome-wide methylation profiles contributes to understanding DNA methylation reprogramming of early-stage embryos in the mammalian development. To address whether a genome-wide demethylation is the predominant mechanism for the methylation reprogramming during preimplantation development, we examined methylation profiles in various repetitive elements such as intracisternal A particle (IAP), early retrotransposons (Etn), and centromeric satellite of the mouse embryos by the bisulfite sequencing method. Our findings provide an insight essential to understanding the behaviors of specific genomic sequences regarding methylation reprogramming during preimplantation development.

Materials and methods

Preparation of mouse embryos. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB). Preimplantation embryos were obtained as previously described [19]. Four-week-old female mice (C57BL/6J × CBA F1) were superovulated by the intraperitoneal injection of 5 IU Pregnant Mares' serum gonadotropin (PMSG), followed by an injection of 5 IU human chorionic gonadotropin (hCG) 48 h later. The female was mated with the stud male overnight. Zygotes were recovered from oviducts of the female at 20–22 h after the administration of hCG and the cumulus cells were detached from the embryos by the treatment with 1 mg/ml hyaluronidase (Sigma). Four-cell, 8-cell embryos, morulae, and blastocysts were picked up at each developmental stage during culturing the zygotes in M16 media at 37 °C, 5% CO_2 in air. To exclude the possibility of genomic contamination by attached cumulus cells, zona pellucidae of the embryos were removed by treatment with 0.5% pronase (Sigma) and then zona-free embryos were transferred into 1 μl lysis buffer (20 mM Tris, pH 8.0, 0.9% Tween 20, 0.9% Nonidet p40, and 0.4 mg/ml proteinase K). Sperm were collected from the cauda epididymis of 8-week-old males (C57BL/6J × CBA F1). Oocytes were recovered from females 16 h after hCG injection.

Genomic DNA isolation and bisulfite mutagenesis. All procedures were performed as described previously [20]. Embryos were pooled to collect approximately 200 diploid genomes. Zona-free embryos were transferred into 100 μl lysis buffer containing 5 μl proteinase K (20 mg/ml) and incubated at 55 °C overnight. Genomic DNA was recovered from the lysate by the ethanol precipitation after the addition of 5 μg tRNA as a carrier and dissolved in 10 μl DDW. The isolated genomic DNA was digested with *Bam*HI restriction enzyme in 20 μl of reaction buffer for 16 h and denatured with 0.3 N NaOH. Then, 235 μl of 5 M sodium bisulfite (pH 5) and 13.5 μl of 10 mM hydroquinone were added. The reaction mixture was overlaid with mineral oil and incubated at 55 °C for 16 h in the dark. The bisulfite-treated genomic DNA was purified by PCR purification kit (Quigen) according to the manufacturer's protocol, added with 40 μl of 90 °C distilled water and then eluted by centrifugation at 12,000*g*. The DNA sample was mixed with 4.4 μl of 3 N NaOH and incubated at 37 °C for 30 min. Following precipitation, the DNA was dissolved in 20 μl DDW.

PCR amplification and sequencing. IAP (GenBank Accession No. 1139546), Etn (GenBank Accession No. 6625641), and centromeric satellite sequences (GenBank Accession No. 10046896) were amplified using the following PCR conditions; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s in the first PCR and then another 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s in the nested PCR. Primers used for amplifying IAP element were 5′-ACCTCTATCTCC ACTCTCATTA-3′ and 5′-TTTTGATTGGTTGTAGTTTATGGT-3′ for the first PCR, and 5′-ATTAATATAAATAACCTATTTACTCT TA-3′ and 5′-TTTGATTGGTTGTAGTTTATGGT-3′ for hemi-nested PCR. Primers for ETn elements were 5′-CTTAACTACATTTC TTCTTTTACC-3′ and 5′-AGTTAGYGTTAGTATGTGTATTTGT-3′ for the first PCR, and 5′-TCTAAATTCCTCTCTTACAACT-3′ and 5′-AGTTAGYGTTAGTATGTGTATTTGT-3′ for the hemi-nested PCR. For the amplification of the centromeric satellite sequences, two sets of primers, 5′-TACACACTAAAAAAACT AAA TTATAA-3′ and 5′-TTYGTTATATTTTAGGTTTTTTAGA-3′ for the first PCR, and 5′-ATACACACTTTAAAACATAAAATAT AA-3′ and 5′-TTYGTTATATTTTAGGTTTTTTAGA-3′ for the hemi-nested PCR were used. The resulting PCR products were cloned into the pGEM T-easy vector (Promega) and then sequenced using ABI PRISM-377.

Results and discussion

To trace DNA methylation patterns during early embryogenesis, DNA samples were isolated from mature oocytes, sperm, preimplantation embryos, and cumulus cells as the control of differentiated cells. Primers for the IAP elements, which are present approximately 1000 copies in the mouse genome [21], were designed to amplify 212-bp fragments containing 9CpG dinucleotides and the resulting PCR products were individually cloned and sequenced. As shown in Fig. 1, the IAP element was heavily methylated in sperm (79%, 95/120 CpG sites) and oocyte genomes (66%, 67/101 CpG sites), which was similar to methylation states of cumulus cells (73%, 85/117 CpG sites). This high methylation level was maintained throughout 1-cell to 8-cell stages. Reduction of DNA methylation in the IAP element was detected in morular-stage embryos and further reduction in blastocysts. IAP sequences are largely resistant to demethylation during preimplantation development [22] and moderately demethylated in blastocysts [23]. Specific sites in imprinted genes are also resistant to active demethylation and maintain a methylated state during early embryogenesis [3]. The results indicate that the active demethylation does not occur in the IAP sequences and the passive demethylation appears to be begun at the morular stage.

To determine methylation states of another transposable element, Etn, 241-bp fragments containing 15 CpG dinucleotides were analyzed in the mouse embryos

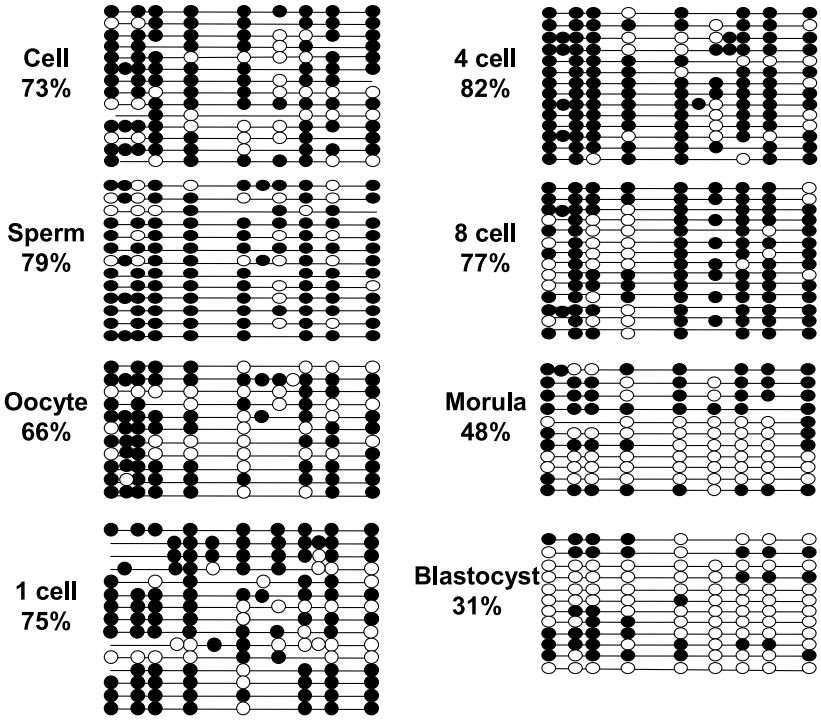

Fig. 1. Methylation profiles of the IAP transposable element sequences. Blank and filled circles indicate unmethylated and methylated CpGs, respectively. Percent methylation is the proportion of methylated CpG sites relative to the whole CpG sites examined.

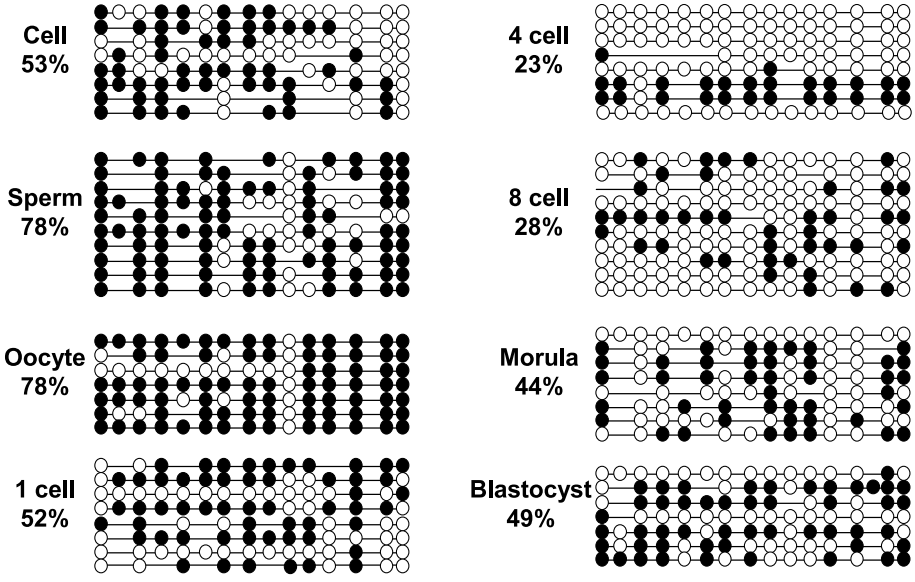

Fig. 2. Methylation profiles of the Etn transposable element sequences.

during preimplantation development (Fig. 2). The Etn element was heavily methylated in both sperm (78%, 94/119 CpG sites) and oocytes (78%, 80/102 CpG sites). Cumulus cells showed a lower methylation level (53%, 54/101 CpG sites) than germ cells. The methylation level of the Etn element was reduced to 52% (55/106 CpG sites) in 1-cell embryos and completely demethylated in 8-cell embryos (0%, 0/92 CpG sites). Interestingly, de novo methylation was observed in compact morulae and its methylation level was maintained until blastocyst stage. Similarly, the active demethylation occurs in the paternal genome, the passive demethylation is observed during cleavage up to the 8-cell stage and the de novo methylation was detected at the 16-cell stage in bovine embryos [16]. These findings suggest that Etn elements in the mouse embryos undergo dynamic

epigenetic changes, such as DNA demethylation and de novo methylation, during early embryogenesis.

In order to examine the methylation states of centromeric satellite sequences which are part of heterochromatin sequences, 210 bp DNA fragments containing 7CpG dinucleotides were amplified from bisulfite-treated genomic DNA by PCR (Fig. 3). The centromeric satellite sequence was heavily methylated in cumulus cells (65%, 83/127 CpG sites). The sperm genome (47.5%, 56/118 CpG sites) was more heavily methylated than the oocyte genome (21%, 24/115 CpG sites) although both germ cells showed a low methylation level compared to cumulus cells. Thus, a dynamic demethylation of the centromeric satellite region took place in 1-cell embryos (16%, 17/109 CpG sites) after fertilization and its methylation level was maintained that way until the blastocyst stage. The epigenetic reprogramming of this sequence seems to be regulated only by the active mechanism, not by the passive demethylation process. Single-copy genes are also undermethylated by active mechanism and keep their low methylation states until the blastocyst stage in the mouse [24]. In bovine embryos, promoter regions of single-copy genes appear to be resistant to active demethylation and gradually demethylated during preimplantation development [25,26]. The results demonstrate that methylation reprogramming of the satellite sequences is regulated only by the active mechanism during preimplantation development.

Active demethylation in the genome of sperm has been observed by immunostaining using the antibody against 5-methyl cytosine [5,7,16] and by bisulfite sequencing for line 1 elements [22]. It is suggested that the active methylation is achieved by remodelling of sperm chromatins through the replacement of protamines to histones in the egg cytoplasm, which is accomplished before the first cell division. Genome-wide reprogramming is conserved in several mammalian species and the active demethylation, taking place in the absence of DNA replication, is detected in bovine, rat, and pig zygotes [16]. It is proposed that CpG sites of single-copy genes carrying heavily methylated states in sperm, not in oocytes, are rapidly demethylated in the zygote after fertilization, whereas those methylated in both oocytes and sperm show a delayed demethylation [24,27]. In this study, centomeric satellite sequences were actively demethylated after fertilization, but methylation reprogramming of IAP and Etn elements, which are heavily methylated in both sperm and oocytes, did not exhibit any delayed demethylation. Thus, the repetitive sequences do not appear to be reprogrammed in preimplantation embryos by the germ cell-dependent demethylation mechanism.

Epigenetic reprogramming such as DNA methylation is spatially/temporally regulated in mammalian development. In mouse primordial germ cells (PGC), de novo methylation is acquired during early development and migration into the genital ridge. After their entry into the genital ridge, this DNA methylation is rapidly erased in the regions within imprinted and non-imprinted loci [28]. Our results also provide an additional insight or

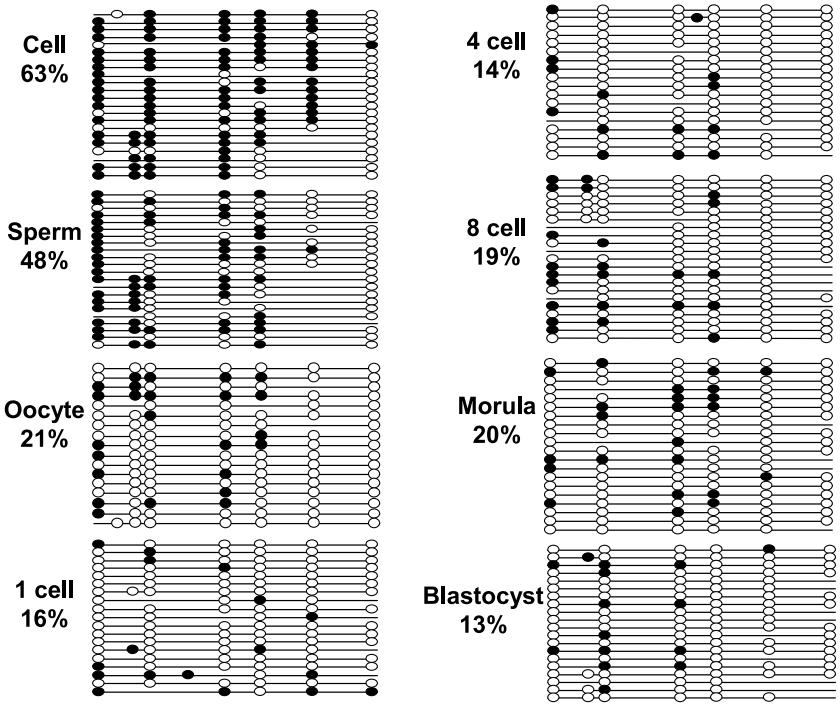

Fig. 3. Methylation profiles of the centromeric satellite transposable element sequences.

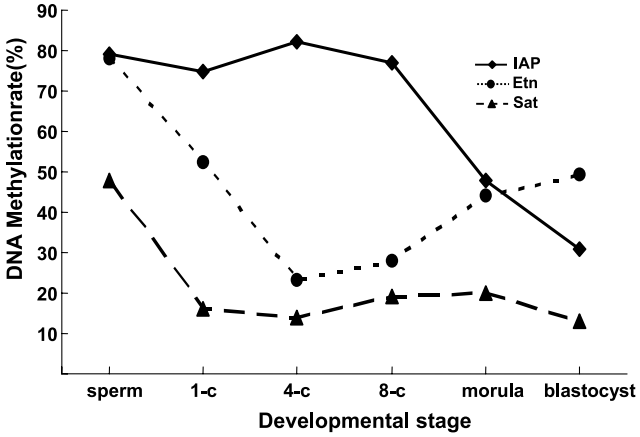

Fig. 4. Various patterns of the DNA methylation reprogramming during preimplantation development. Each of three repetitive sequences shows independent reprogramming patterns of the DNA methylation in early mouse embryos.

knowledge that the features of the DNA methylation changes may be dependent on various genomic regions in early-stage embryos. The mouse major and minor satellite sequences are undermethylated in both sperm and oocytes as compared to somatic cells [29]. In this study, the methylation levels of sperm and oocyte genomes were different although DNA methylation state of both germ cells was lower than that of somatic cells (Fig. 3).

A typical DNA methylation reprogramming is an essential process that early embryos should be undergone to get developmental competence; the paternal genome is demethylated by an active mechanism immediately after fertilization, the maternal genome is demethylated by a passive mechanism that depends on DNA replication during preimplantation development [16]. In this study, we have demonstrated that the reprogramming of DNA methylation in the various repetitive sequences including IAP sequences, retrotransposon elements, and centromeric satellite sequences works independently in a region-specific manner during early embryonic development in the mouse (Fig. 4). It is concluded that the specific genomic regions or sequences have spatially/temporally their unique characteristics in the reprogramming of the DNA methylation during preimplantation development, suggesting that the epigenetic reprogramming occurs independently on the distinct genomic regions by different mechanisms. Understanding the detailed mechanisms of DNA methylation reprogramming in early development will be important for animal cloning and embryonic stem cell research.

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