Dynamic CpG and Non-CpG Methylation of the Peg1/Mest Gene in the Mouse Oocyte and Preimplantation Embryo*

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In somatic tissues, the CpG island of the imprinted Peg1/Mest gene is methylated on the maternal allele. We have examined the methylation of CpG and non-CpG sites of this differentially methylated CpG island in freshly ovulated oocytes, in vitro aged oocytes, and preimplantation embryos. The CpG methylation pattern was heterogeneous in freshly ovulated oocytes, despite the fact that they all were arrested in metaphase II. After short in vitro culture, Peg1/Mest became hypermethylated, whereas prolonged in vitro culture resulted in demethylation in a fraction of oocytes. Non-CpG methylation also occurred in a stage-specific manner. On alleles that were fully methylated at CpG sites, this modification was found, and it became reduced in two-cell stage embryos and blastocysts. These observations suggest that the process of establishment of the methylation imprint at this locus is more dynamic than previously thought.

Establishment of the mature epigenetic configuration of the genome is part of the maturation process of the gametes and is essential for normal development after fertilization. DNA methylation of CpG sites is one of the epigenetic modifications that regulates gene expression (for review, see (1)). The genome undergoes widespread changes in CpG methylation during germ cell maturation. Imprinted genes are of particular interest, because they are frequently associated with CpG-rich regions that are methylated differentially on the paternal and maternal chromosomes (for a review on imprinting, see Ref. 2). These differentially methylated regions (DMRs)¹ are believed to play an important role in the parental origin-dependent regulation of individual imprinted genes and of entire genomic regions during embryonic development. The methylation profile typical for the paternal or maternal chromosome is believed to be established during maturation of the male and female germ cells, but the exact kinetics of this process is still unclear. Some observations have even suggested that some imprinted genes reach their mature methylation profile only after fertilization (3).

Peg1/Mest is a typical imprinted gene that is predominantly expressed from the paternal allele in the mesoderm and its derivatives (4). The methylation analysis of Peg1/Mest revealed that the CpG island in the promoter region was completely methylated on the maternal and unmethylated on the paternal chromosomes (5). It has been shown that the human PEG1/MEST is already unmethylated in spermatogonia (6). In the maternal germ line, Peg1/Mest is fully methylated in ovulated oocytes that are arrested in metaphase of the second meiotic division (MII) (7).

In a previous study (8), we have detected methylation heterogeneity in growing oocytes at several imprinted loci, including Peg1/Mest. The heterogeneity was substantially increased in the oocytes matured in vitro, suggesting that the methylation imprint, at this stage, is unstable and can be influenced by the cellular environment. In the present study, we investigated whether changes in the methylation imprint occurred at the Peg1/Mest locus during the last stages of oocyte maturation. Although DNA methylation has been found predominantly on cytosines that are part of CpG dyads, it has been shown that cytosines in non-CpG sites can also be modified (9–12). Non-CpG methylation was detected in mouse oocytes and two-cell embryos on several sites of the maternal allele of the non-imprinted neurofibromatosis type 1 and the adenosine deaminase deamidase genes (10). Non-CpG methylation has also been observed in mammalian cell lines (11, 12). Although its biological significance is unclear, non-CpG methylation was shown to occur in the CpG-methylated alleles of the rat Sphk1 through a non-coding RNA-dependent mechanism (12). Here, we describe an unexpected heterogeneity of both CpG and non-CpG methylation of the 5'-DMR of Peg1/Mest that is subject to dynamic changes during the aging of oocytes and the early preimplantation development of fertilized embryos.

EXPERIMENTAL PROCEDURES

Preparation of Oocytes and Embryos—Fully grown germinal vesicle-stage oocytes were obtained by scratching follicles from the ovaries of 6–8-week-old F1 female mice (C57BL/6 × CBA) 48 h after the intra-peritoneal injection of 5 IU of pregnant mare serum gonadotrophin (Folligon®, Intervet, Angers, France). MII oocytes were obtained from the oviducts of females superovulated by intraperitoneal injection of 5 IU of PMSG followed 48 h later by injection of 5 IU of hCG (Chorulon®, Intervet, Angers, France). Oocytes were recovered 14 h after the hCG injection into M2 medium (Sigma), and cumulus cells were removed with 0.03% hyaluronidase (Sigma), followed by either direct methylation analysis or culture in M16 medium (Sigma) at 37 °C under 5% CO2/air followed by methylation analysis. Fertilized embryos were obtained from superovulated F1 females mated to F1 males.
For DNA methylation analysis, oocytes or embryos (two-cell stage and blastocysts) were treated with 50 mg/ml Pronase (Roche Applied Science) to remove the zona pellucida with the remaining attached cumulus cells. Three times with 500 ml of M2 medium, and immobilized in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 1.5% NuSieve® GTG® agarose droplets (FMC, Rockland, MA). Replicate agarose blocks were prepared from each cell type studied and were analyzed in parallel.

**DNA Methylation Analysis—** The methylation profile of Peg1/Mest was determined by a slightly modified technique of genomic sequencing after bisulfite treatment (6, 13). Briefly, 5–10 oocytes or embryos, embedded in a small agarose block, were incubated overnight in 200 ml of lysis solution (0.5 mM EDTA, pH 8, containing 2 mg/ml proteinase K) at 50 °C. The agarose/cell drops were then either used immediately or stored at 4 °C for up to 2 weeks. The DNA was denatured with 300 ml of freshly prepared 0.3 M NaOH for 30 min at 50 °C. Aliquots (900 ml) of bisulfite solution (4 M sodium bisulfite (Sigma) containing 125 mM hydroquinone (Sigma), pH 5-0) were added to each reaction tube. The reaction mixture was incubated at 50 °C in the dark for 5–18 h, as previously described. The beads were then heated to 80 °C for 10 min to melt the agarose, and then modified DNA was purified using the Wizard DNA Clean-Up System (Promega) according to the manufacturer’s instructions. Methylation was completed by the addition of a 0.3 M NaOH solution at 37 °C, followed by neutralization with an equal volume of 6 M ammonium acetate, pH 7.0. The bisulfite-treated DNA was ethanol-precipitated, resuspended in water (10 ml), and either used immediately for PCR or stored at −20 °C.

We amplified a region encompassing 22 CpGs in the differentially methylated promoter region of Peg1/Mest (GenBank accession number AF017994; nucleotides 2220–2556) by semi-nested PCR: forward external primer, 5′-ATCTTACGGTTTGGAAATCTGTTGTTTCT-3′; forward internal primer, 5′-AGAGGATTAGTAGGAAGGGGTGTTAG-3′; reverse internal primer, 5′-ACACAAAAACACACACACACACT-3′; reverse primer, 5′-ACGCGCTGACCGATCTGTCG-3′. The products of the PCR reaction were subcloned into pCR4-TOPO (Invitrogen) and sequenced.

PCR amplification is a potential source of sampling bias because of the small number of DNA molecules available for amplification in a single reaction. Under these conditions, amplification of the entire target pattern is random and does not depend on the initial frequency of the pattern in the population of template DNA molecules. This makes it impossible to differentiate whether two identical patterns are derived from two different original template molecules with identical methylation patterns or whether they both result from the amplification of the same DNA fragment. Unless the amplification is done on a batch of several thousands of oocytes, the statistical validity of the result obtained from a single batch with a single amplification is uncertain. The only way to overcome the problem is to increase the number of independent amplifications on independent batches. Obviously, when different patterns are obtained in the same experiment or identical patterns are obtained in different experiments, there can be no doubt about their origin from an independent template. Therefore, we sequenced only 2–4 clones from each agarose block and performed several independent sets of DNA modification, amplification, cloning, and sequencing. The sequences obtained were classified into two categories on the basis of their methylated CpG content. The “methylated” and “unmethylated” class contained all the sequences that were fully or almost fully methylated or fully or almost fully unmethylated, respectively. The frequency of the two methylation profiles found in different cell populations was compared using a χ2 test with the correction of Yates for small samples (25).

To confirm the non-CpG methylation occurring at the Peg1/Mest promoter region, we used a methylation-sensitive enzyme. Oocytes and blastocysts embedded individually in an agarose block were incubated overnight in 200 ml of lysis solution (0.5 mM EDTA, pH 8, containing 2 mg/ml proteinase K) at 50 °C. The agarose drops were then washed several times in a large volume of TE buffer (100 mM Tris-CH, pH 7.3, 5 mM EDTA). Genomic DNA was digested with 10 units of PstI (Invitrogen) at 37 °C for 16 h within the agarose blocks. The part of the promoter region was subsequently amplified by PCR using the following primers: forward primer, 5′-ATCTTATAGAAAGAAGGGGAGG- TAGGG-3′ (nucleotides 2242–2266); reverse primer, 5′-AACAGC GGACGGACTTAAAAAGCC-3′ (reverse complement to nucleotides 2395–2419).

**RESULTS**

**CpG Methylation of Peg1/Mest in MII Oocytes—** To investigate the ontogeny of the methylation imprint during the last stages of female germ cell maturation, we examined the DNA methylation pattern of the Peg1/Mest DMR in the promoter region (Fig. 1). This region has been reported differentially methylated on the parental chromosomes of somatic cells with the maternal copy being fully methylated (5). We used the bisulfite sequencing method adapted for the study of very small samples, as previously described (6). First, we examined the Peg1/Mest methylation in fully grown germinal vesicle-stage oocytes (Fig. 2A). In agreement with our previous results (8), we found both fully methylated and unmethylated patterns. As a next step, we studied the DNA methylation pattern of Peg1/Mest in freshly ovulated MII oocytes (14 h after hCG treatment). Because the maternal allele of this gene is methylated in somatic cells and MII oocytes represent the last cellular stage before fertilization, we expected to find the analyzed region hypermethylated. However, as shown in Fig. 2B, fully methylated, mixed, and unmethylated patterns were observed. The 38 fragments derived from bisulfite-modified DNA obtained in 16 independent experiments showed 15 different CpG and non-CpG methylation/unmethylation patterns. Some patterns were found several times in independent experiments. The possibility that somatic cell contamination could be the cause of the observed heterogeneous methylation was ruled out (see “Experimental Procedures”). Only fully methylated copies of region 2 of the Igf2r gene were found in our oocyte preparations (not shown). These unexpected results indicate a high heterogeneity of Peg1/Mest methylation in MII oocytes, suggesting that the methylation imprint of this gene is not established in all oocytes before fertilization.

To determine whether the maternal CpG methylation pattern could become established between ovulation and fertilization, we cultured MII oocytes in vitro and performed methylation analysis. After culture in M16 medium for 8 h (corresponding to 22 h after hCG treatment), all 29 Peg1/Mest clones obtained from eight independent PCR reactions were found hypermethylated (Fig. 2C) with only four CpG and non-CpG methylation patterns. We did not find any unmethylated alleles. The difference in the frequency of unmethylated/hypermethylated copies in the in vitro cultured oocytes compared with the freshly ovulated MII oocytes is, statistically, highly significant (p < 0.001). This observation suggests the existence of de novo methylation activity acting in unfertilized oocytes.
after ovulation but before fertilization. Interestingly, unmethylated alleles were again observed in aged oocytes obtained after 28 h of in vitro culture (i.e. 42 h after hCG treatment) (Fig. 2D). Four of sixteen clones analyzed in four independent experiments were unmethylated.

We also analyzed the methylation patterns in two-cell embryos, blastocysts, and somatic cells (Fig. 3), and as expected, both hypermethylated and hypomethylated alleles were seen, with similar proportions in each case. These profiles were reminiscent of the expected imprinted pattern in which the maternal allele is hypermethylated and the paternal allele is unmethylated.

Detection of Non-CpG Methylation at the Peg1/Mest Locus—
We frequently found clones in which some of the non-CpG cytosines were not converted to uracil by the bisulfite reaction. Such partial conversion potentially reflects non-CpG methylation. Interestingly, the incidence of non-conversion differed depending on the post-ovulatory age of oocytes. In the fully grown germinal vesicle stage, this type of non-conversion was distributed randomly over the entire sequence (Fig. 2E). In the oocytes, 14 h after hCG treatment, two preferential sites showing potential non-CpG methylation were located between CpG sites 12 and 13, although some other sites of non-conversion were also detected (Fig. 2F). In contrast, 22 h after hCG treatment, the observed patterns were less heterogeneous. For example, the two sites between CpG sites 12 and 13 were found resistant to bisulfite modification in all clones with 100% CpG methylation (Fig. 2G). In addition to these two sites, we found four other cytosines that were frequently insensitive to bisulfite modification. When compared with the CpG methylation in Peg1/Mest, the incidence of the non-conversion at non-CpG sites seemed to be well correlated with the methylation of CpG sites, whereas the vast majority of the unmethylated sequences did not have resistant non-CpG cytosines. In in vitro aged oocytes (after 28 h of culture), two-cell-, and blastocyst-stage embryos, the incidence of the non-conversion became lower and was restricted only to some non-CpG sites, even in hypermethylated sequences (Figs. 2H and 3). It is also to be noted that somatic cells did not show any sign of non-CpG methylation, even in hypermethylated conditions (Figs. 2H and 3). However, their conversion was complete after a prolonged treatment. Contrary to the TH gene, the sites in the Peg1/Mest DMR were resistant to conversion even after prolonged bisulfite reaction (not shown). These observations strongly suggested that the non-conversion from cytosine to uracil did not result from incomplete bisulfite modification but reflects the methylation of these non-CpG sites. To verify this possibility, we applied an alternative approach based on restriction enzyme digestion and subsequent amplification. We

![Fig. 2. Cytosine methylation pattern of Peg1/Mest DMR in fully grown (A and E), freshly ovulated (B and F), and oocytes cultured in vitro for 22 (C and G) and 42 h (D and H). The methylation patterns of CpG sites are shown on the left side. Each row of circles represents a single cloned allele, with open circles for non-methylated CpG site and filled circles for methylated cytosines. The CpG sites are numbered from 1 to 22 as on Fig. 1. Methylation of the non-CpG sites is shown on the right side. Each row of squares represents the same clone as on the left. Only non-CpG sites in which we observed at least once the non-conversion of cytosine to uracil are shown. The relative positions of the non-CpG sites are indicated relative to the CpG sites numbered from 1 to 22. The number of independent experiments is indicated (n = ) for each stage analyzed.](http://www.jbc.org/doi/abs/10.1074/jbc.M108.005703)

![Fig. 3. DNA methylation of the Peg1/Mest DMR at CpG and non-CpG sites after fertilization. A, two-cell embryos. B, blastocysts. C, somatic cells. The symbols are the same as on Fig. 2. Note that no non-CpG methylation was observed in somatic cells.](http://www.jbc.org/doi/abs/10.1074/jbc.M108.005703)
digested the genomic DNA of oocytes and blastocysts with a methylation-sensitive enzyme, PstI (15). A recognition site for this enzyme is present in the sequence analyzed with a bisulfite-resistant cytosome (Fig. 1, underlined). We performed PCR amplification of the region encompassing the PstI site. An amplification product is expected only if the site is methylated, thus resistant to PstI digestion. As shown in Fig. 4, an amplification product is always obtained when the oocyte or blastocyst DNA is not digested by the restriction enzyme. Two of three independent amplifications using PstI-digested genomic DNA from MII oocytes (20 h after hCG treatment) showed positive bands, indicating the existence of non-CpG methylation. In contrast, we could not see any amplification products in PstI-digested blastocyst DNA derived from hundreds of the somatic cells, indicating that the digestion was complete and that Peg1/Mest sequences were unmethylated in the context of non-CpG sites. This is in accordance with the bisulfite sequencing that did not detect non-CpG methylation at this stage. Therefore, it is likely that the results obtained by bisulfite sequencing reflect non-CpG methylation of the DNA.

**DISCUSSION**

In the present study, we showed that the hypermethylated pattern characteristic for the maternal allele of imprinted Peg1/Mest in somatic cells is not yet established in all oocytes at the moment of ovulation. A significant subset of oocytes carries unmethylated alleles despite the fact that all of the oocytes are arrested in metaphase of the second meiotic division (Fig. 2). Reminiscent of our observations, it has been reported that the methylation imprint at the SNRPN locus is not established in ovulated human oocytes (3). Another study found that the methylation of this gene was already completed in the ovulated oocytes (16). These contradictory data on SNRPN and the observations on Peg1/Mest in this study suggest that the heterogeneity and irregularity of methylation imprint establishment in morphologically and physiologically homogenous oocyte populations might reflect the inherent physiological differences between the cells. After *in vitro* culture for 8 h, all oocytes contained a fully methylated Peg1/Mest DMR (Fig. 2), indicating that *de novo* methylation occurred after ovulation to reach the mature imprint pattern. Recently, Lucifero et al. (7) have reported that the Peg1/Mest DMR was fully methylated in oocytes obtained 20 h after hCG injection, which contributes to 6 h after ovulation. The concordance of these findings supports the notion that the mature maternal methylation imprint is established after ovulation. In oocytes cultured *in vitro* for 28 h after ovulation, unmethylated sequences were again detected, further emphasizing the highly dynamic nature of DNA methylation of the Peg1/Mest locus. It is known that the oocytes gradually lose the capacity to undergo normal development and the fetsuses derived from the fertilization of aged oocytes frequently display developmental defects (17). The reduced developmental competence can attribute to the consequence of accumulated epigenetic alterations at imprinted loci.

It is unclear how the observed *de novo* methylation and active demethylation activities can act on condensed chromosomes. The large amounts of various methyltransferases present in oocytes may be responsible for progressive methylation of the Peg1/Mest DMR (18). Oocytes contain an, as yet, unidentified demethylating activity that has been shown to act after fertilization, preferentially on the paternal genome (19, 20). Our data are the first to show that this activity can demethylate DNA even before fertilization.

The bisulfite sequencing method also allowed us to detect methylation of cytosines in a non-CpG context (Fig. 2). We have shown, by an independent method, that the non-CpG methylation is not an artifact resulting from incomplete bisulfite modification (Fig. 4). Earlier studies demonstrate the existence of non-CpG methylation in a wide range of organisms, from plants to mammals (for review, see Ref. 21). In mammals, non-CpG methylation has been detected in several gene loci and repeated sequences (22). In the Peg1/Mest, non-CpG methylation found in oocytes occurred only in sequences that had all or almost all the CpG sites methylated (Fig. 2). In two-cell- and blastocyst-stage embryos, non-CpG methylation became reduced, and it was absent in somatic cells (Fig. 3), suggesting that this modification occurs transiently on the CpG-methylated allele. Similarly to our observations, maternal allele-specific non-CpG methylation of the neurofibromatosis type 1 and adenosine deaminase genes was observed in oocytes and two-cell-stage embryos simultaneously with CpG methylation but did not persist beyond the two-cell stage (10). The observed decrease of the non-CpG methylation after fertilization is compatible with both the existence of an active demethylating mechanism and passive dilution that would result in 50% reduction of the non-CpG-methylated alleles, although no definitive conclusion can be drawn in the absence of compelling data.

Our observations suggest that establishment of the methylation imprint in the oocyte may be a more dynamic process than previously thought. The actual methylation pattern of a given sequence may be determined by the equilibrium of methylation and demethylation activities that depend on other ongoing cellular processes. This conclusion is supported by the observation that environmental stress, such as *in vitro* culture, can affect the methylation of imprinted genes in preimplantation embryos (23), follicular oocytes (8), or embryonic stem cells (24). Our results may have practical implications for the use of oocytes obtained after hormonal stimulation in human medicine.

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**Fig. 4. Variable methylation of the non-CpG site in Peg1/Mest in oocytes detected by PCR following PstI digestion.** The genomic DNA from three oocytes (obtained 20 h after the hCG injection) and three blastocysts was digested with the methylation-sensitive enzyme, PstI. Undigested material (+) and controls (−) were also included. The region encompassing the PstI site was subsequently amplified (see Fig. 1). An amplification product is expected only if the site is methylated and resistant to PstI cleavage.
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