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Gene-specific timing and epigenetic memory in oocyte imprinting

Diana Lucifero[1], Mellissa R.W. Mann[2], Marisa S. Bartolomei[2] and Jacquetta M. Trasler[1,*]

[1]McGill University, Montreal Children's Hospital Research Institute and Departments of Pediatrics, Human Genetics, and Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3H 1P3 and [2]Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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Imprinted genes are differentially marked during germ cell development to allow for their eventual parent-of-origin specific expression. A subset of imprinted genes becomes methylated during oocyte growth in both mouse and human. However the timing and mechanisms of methylation acquisition are unknown. Here, we examined the methylation of the *Snrpn*, *Igf2r*, *Peg1* and *Peg3* differentially methylated regions in postnatal growing mouse oocytes. Our findings indicate that methylation was acquired asynchronously at these different genes. Further analysis of *Snrpn* DMR1 revealed that parental alleles retain an epigenetic memory of their origin as the two alleles were recognized in a parental-specific manner in the absence of DNA methylation. In addition, we show that methylation acquisition was probably related to oocyte diameter and coincided with the accumulation of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* transcripts. Methylation of the repetitive retroviral-like intracisternal A particle also occurred during this same window of oocyte growth. These findings contribute to our understanding of the epigenetic mechanisms underlying imprint acquisition during female germ cell development and have implications for the practice of assisted reproductive technologies.

INTRODUCTION

DNA methylation is an epigenetic regulator of gene expression and acts as an important molecular mark underlying the parental-specific expression of genes subject to genomic imprinting (1). Imprinted genes account for the requirement of both maternal and paternal genomes in normal development and play significant roles in regulating embryo growth, placental function and neurobehavioral processes (2,3). Aberrant expression of several imprinted genes has been linked to the development of human diseases, including Beckwith–Wiedemann, Prader–Willi and Angelman syndromes (reviewed in 4).

To allow for the differential expression of imprinted genes, some epigenetic marking must distinguish the alleles inherited from the maternal versus the paternal genomes. To date DNA methylation remains the most widely investigated epigenetic modification associated with this differential marking of imprinted alleles. A number of genes regulated by imprinting contain differential methylation regions (DMRs) inherited from the gametes (5,6). In addition, DNA methylation is both a heritable and reversible epigenetic modification that is stably propagated after DNA replication and influences gene expression and chromatin condensation via the binding of factors such as methyl CpG binding proteins that subsequently recruit other gene silencing factors including histone deacetylases (reviewed in 7).

DNA methyltransferases (DNMTs) are a family of *de novo* and maintenance methylating enzymes responsible for the addition of a methyl group to the 5-position of cytosine within CpG dinucleotides (reviewed in 8). *Dnmt3L* mouse gene targeting studies demonstrate a critical role for this enzyme in the establishment of maternal methylation imprints in the female germ line (9,10). In addition, embryos derived via the transplantation of *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{+/-}$ ovaries have completely unmethylated *Igf2r*, *Peg1*, *Peg3* and *Snrpn* DMRs (10). Mice deficient for DNA methyltransferases *Dnmt1*, *Dnmt1o* or *Dnmt3L* display both loss of allele-specific methylation and expression of imprinted genes implying at the very least a role for methylation in imprint maintenance (9–12).

The erasure, establishment and maintenance of imprints are dynamic processes that must be correctly reprogrammed with every reproductive cycle. From mouse studies, erasure occurs around the time that primordial germ cells enter the

*To whom correspondence should be addressed at: McGill University, Montreal Children's Hospital Research Institute, 4060 Ste. Catherine Street West, Montreal, Quebec, Canada H3Z 2Z3. Tel: +1 5144124400, ext. 25235; Fax: +1 5144124331; Email: jacquetta.trasler@mcgill.ca

gonad (13–17). Imprint establishment occurs during gametogenesis, when maternal and paternal genomes are physically separated, and the timing of acquisition of genomic imprints is significantly different between the two germ lines. In the male germ line, methylation acquisition on *H19*, a maternally expressed gene, is initiated in prenatal prospermatogonia and complete postnatally by the pachytene stage of meiosis (18–20). Round spermatid microinjection experiments have confirmed that paternal imprints are complete by the haploid phase of spermatogenesis (21). In the female, elegant nuclear transplantation experiments in the mouse have suggested that imprint acquisition occurs in the postnatal growth phase of oogenesis (22,23). In complementary DNA methylation experiments, we have shown that the DMR1 methylation imprint on the paternally expressed bicistronic gene *Snurf–Snrpn* (24), hereinafter referred to as *Snrpn*, is acquired during oocyte growth and that establishment of the maternal methylation imprint on the gametic DMRs within *Igf2r* (25), *Peg1* (26) and *Peg3* (27), is complete in metaphase II (MII) oocytes (5). In agreement with our mouse data, a recent study on human oocytes indicated that *SNRPN* is methylated in late stage germinal vesicle (GV), metaphase I (MI) and MII oocytes (6). However, a thorough understanding of the timing and mechanisms underlying maternal methylation imprint establishment is lacking.

In this study, our first aim was to investigate and compare the methylation profiles of the DMRs of imprinted genes from different chromosomal regions (*Snrpn*, *Igf2r*, *Peg1* and *Peg3*) at five timepoints spanning the window of postnatal oocyte growth. Using bisulfite sequencing analysis, our findings suggest that, although each of these imprinted genes acquired methylation during postnatal oogenesis, methylation acquisition at these DMRs was asynchronous and may be governed by distinct mechanisms. Allele-specific methylation analysis of the *Snrpn* DMR revealed that, in the absence of DNA methylation, the parental identity of the *Snrpn* alleles was maintained as the maternally inherited allele acquired methylation prior to the paternally inherited allele and, thus, may be distinguishable via another epigenetic mechanism. This observed phenomenon of epigenetic memory was conserved when oocytes derived via the reciprocal cross were analyzed. We also show that methylation acquisition at the *Snrpn* DMR was related to oocyte diameter, and that the accumulation of *Dnmt3a*, *Dnmt3b* and *Dmnt3L* transcripts coincided with this increase in oocyte diameter and the timing of methylation acquisition on several imprinted genes. Analysis of the 5′ long terminal repeat (LTR) within non-imprinted intracisternal A particle (IAP) elements indicates that methylation of this repetitive retroviral-like sequence occurred during the same window of postnatal oocyte growth, and suggests a similarity between imprinted genes harboring maternal methylation and repeat elements, and their targeting by DNMTs.

RESULTS

Gene-specific establishment of DMR methylation during oocyte growth

In our first experiment, we assayed the timing of methylation acquisition for four imprinted genes harboring maternal methylation. In addition to being located on different chromosomes, each of the four genes has a well-defined DMR for which gamete-specific differences in methylation have been shown (5). The methylation status of a number of CpG sites within the *Snrpn*, *Igf2r*, *Peg1* and *Peg3* DMRs in 1, 5, 10, 15 and 25 dpp (days postpartum) oocytes was determined using bisulfite sequencing analysis (Fig. 1A–E and Supplementary Material Table 1). Non-growing oocytes from primordial follicles isolated at 1 (Fig. 1A) and 5 (Fig. 1B) dpp showed very few methylated CpG sites for all genes examined. In 10 dpp early-growing oocytes (Fig. 1C), which varied greatly in diameter (20–70 μm), we observed an increase in the number of methylated strands (percentage of strands hypermethylated defined as >50% of CpGs methylated on a given strand) for *Peg3* (38%), *Igf2r* (15%) and *Snrpn* (11%), but not for *Peg1* (0%). The majority of oocytes were probably isolated from preantral follicles.

The majority of oocytes isolated at 15 dpp were roughly 70–80 μm in diameter; we suggest that these oocytes were derived from early antral follicles. A number of smaller oocytes probably from less mature preantral follicles were also collected at this stage. While regions within the DMRs of *Snrpn* (83%), *Igf2r* (63%) and *Peg3* (66%) continued to acquire methylation, *Peg1* remained hypomethylated (0%; Fig. 1D). Complete methylation of strands was observed for the first time at this stage in oogenesis with the exception of *Peg1*.

Germ cells isolated from 25 dpp follicles were composed of GV oocytes that were fully grown (70–80 μm) and meiotically competent. Strikingly, all *Peg1* strands sequenced were hypermethylated (Fig. 1E), suggesting that this gene underwent rapid *de novo* methylation late in oocyte development. *Snrpn* and *Igf2r* were 91 and 96% methylated, respectively, although a few strands remained hypomethylated at this late stage of oocyte growth. *Peg3*, which appeared to be one of the first genes to acquire methylation, was undermethylated compared to the other genes, although not significantly different from its methylation status at 15 dpp (Supplementary Material). *Snrpn*, *Igf2r* and *Peg3* continued to acquire methylation as these DMRs were densely methylated in MII oocytes (5). These results indicate that the establishment of methylation imprints proceeded in a gene-specific manner while oocytes were arrested at prophase I and transitioned from primordial to antral follicles.

Allele-specific methylation on *Snrpn*

To gain a more in-depth understanding of the timing and mechanisms underlying maternal methylation imprint establishment, *Snrpn* was subjected to further analysis. Allele-specific methylation dynamics of the *Snrpn* DMR1 were assayed in postnatal F_1 mouse oocytes by bisulfite sequencing, using strain-specific polymorphisms to differentiate the parental alleles (Fig. 2 and Supplementary Material Table 2). Methylation analysis of the *Snrpn* DMR1 in 1 dpp oocytes revealed both alleles to be essentially devoid of methylation, suggesting an equivalence of the parental alleles after methylation erasure (Supplementary Material Fig. 1). In 10 dpp oocytes (based on three independently collected sets of at least 500 oocytes each, from different litters), the maternal allele was substantially more methylated (67% strands hypermethylated) than the paternally derived allele (0%), suggesting an initial targeting of methylation to the maternally inherited *Snrpn* allele (Fig. 2A). Oocytes at 15 dpp (from two

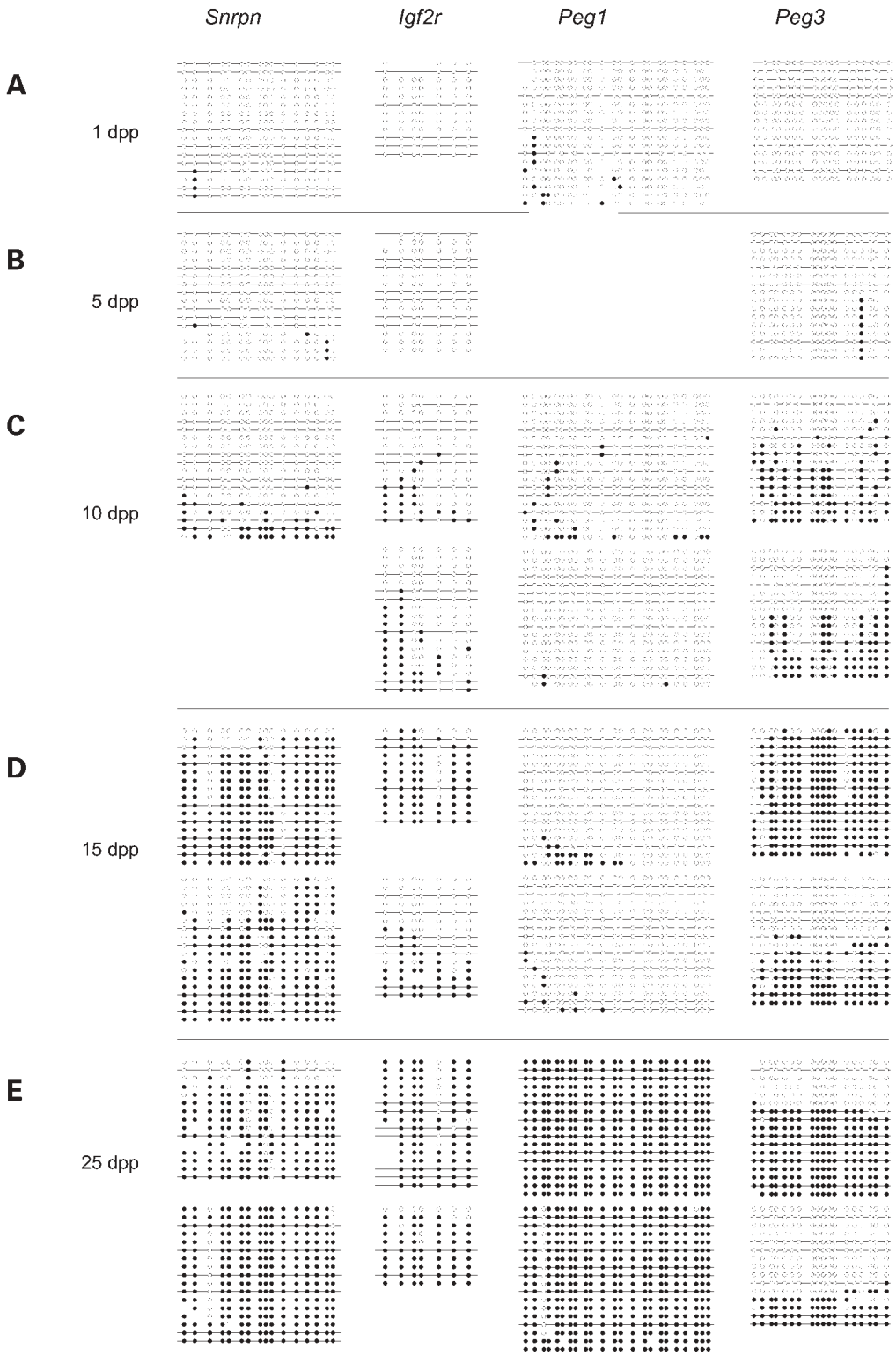

Figure 1. Gene-specific establishment of maternal methylation imprints for the *Snrpn*, *Igf2r*, *Peg1* and *Peg3* DMRs during postnatal oocyte growth. *Snrpn* resides within a well-characterized imprinting cluster on central chromosome 7, while *Igf2r*, *Peg1* and *Peg3* are located proximally on chromosomes 17, 6 and 7, respectively. Oocytes were analyzed at (**A**) 1, (**B**) 5, (**C**) 10, (**D**) 15 and (**E**) 25 dpp. Each line represents an individual clone. A solid circle indicates a methylated CpG site, an open circle denotes an unmethylated CpG and a missing circle represents a CpG site where the sequencing data were ambiguous. Space between strands of the same sample indicates that two sets of oocytes were independently bisulfite treated and analyzed. *H19* methylation served as control for somatic cell contamination (data not shown). For each sample, the bisulfite methylation data were analyzed by computing both the percentage of clones >50% methylated and the percentage methylated CpGs out of the total number of CpGs analyzed, and then subjected to statistical analysis using Fisher's exact test (Supplementary Material Table 1).

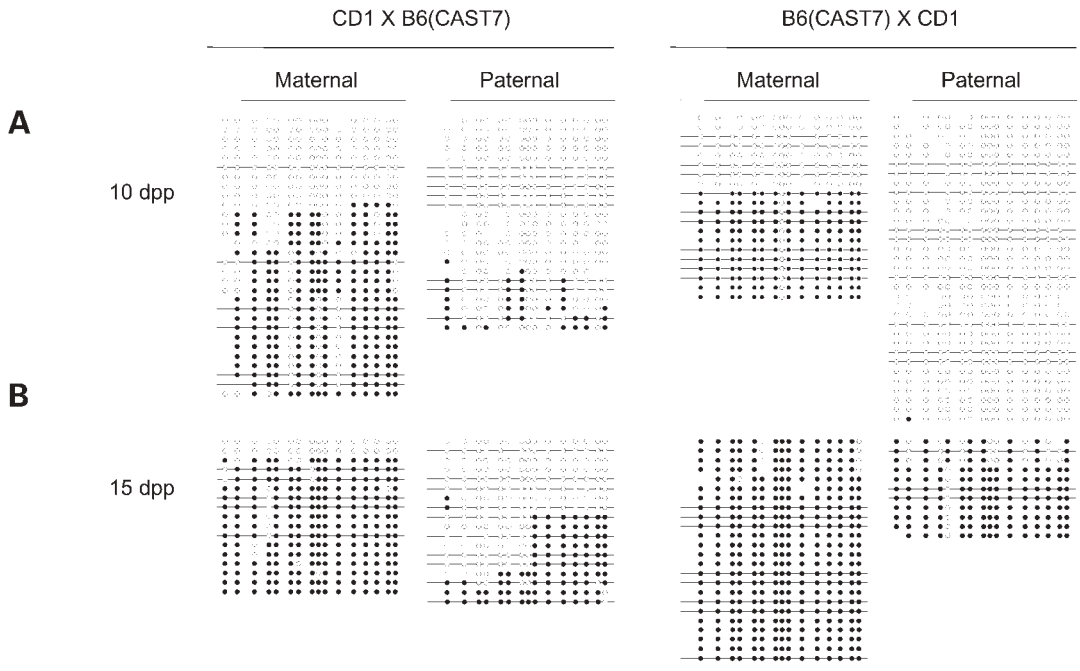

Figure 2. Allele-specific methylation status of *Snrpn* DMR1 in growing oocytes that were isolated at (**A**) 10 dpp, and (**B**) 15 dpp from CD1 × B6(CAST7) (left) and B6(CAST7) × CD1 (right) F_1 females. DNA isolation and bisulfite sequencing analysis were performed on four 10 dpp and three 15 dpp sets of F_1 oocytes (roughly 500 each). Details as described in Figure 1. The maternally inherited allele appeared to acquire methylation prior to the paternal allele, suggesting that another epigenetic mark continues to distinguish *Snrpn* parental alleles in the absence of methylation in growing oocytes. See Supplementary Material Figure 1 for allele-specific methylation profile of *Snrpn* in 1 dpp oocytes and Supplementary Material Table 2 for statistical analysis of 10 and 15 dpp data.

independently collected sets of at least 500 oocytes each, from different litters) continued to acquire methylation allele-specifically with the *Snrpn* maternal allele substantially more methylated (88%) than the paternal allele (22%; Fig. 2B). As observed in the non-allele-specific methylation analysis of *Snrpn* 15 dpp oocytes (Fig. 1), 15 dpp was the first timepoint where all 16 CpGs analyzed were methylated within a given strand. In these F_1 samples, complete methylation occurred exclusively on the maternally inherited allele (Fig. 2B). Although some paternal strands were highly methylated, complete methylation of paternally inherited strands probably occurred later in oocyte growth.

To differentiate between strain-specific effects versus parental allele directed acquisition, DNA methylation was assayed in 10 and 15 dpp oocytes isolated from F_1 pups derived via the reciprocal cross. A similar pattern of methylation establishment was observed with the maternal allele hypermethylated compared with the paternal allele (Fig. 2A and B, right). In 10 dpp oocytes, maternal and paternal strands exhibited 60 and 0% methylation, respectively. In 15 dpp oocytes, the maternal allele was 100% methylated while the paternal allele was 82% methylated. In contrast to expectation from the analysis of 1 dpp oocytes (Supplementary Material Fig. 1), the parental *Snrpn* alleles were not equivalent but retained their identity in the absence of a DNA methylation mark within the area of DMR1 examined.

Imprint establishment is related to oocyte diameter

Having established that maternal methylation imprints were conferred in a gene- and allele-specific manner, we next assessed whether the acquisition of *Snrpn* methylation was related to oocyte diameter. We isolated oocytes from 15 dpp females and grouped them according to diameter, with one pool containing oocytes that ranged between 20 and 50 μm and a second pool containing oocytes that were 60–80 μm. Bisulfite sequencing analysis of *Snrpn* showed that 15 dpp oocytes of small diameter were devoid of methylation (0%) while strands from larger diameter 15 dpp oocytes were hypermethylated (94%; Fig. 3A and Supplementary Material Table 3). Our observations that maternal methylation imprint establishment was related to oocyte diameter suggests that the accumulation of some enzyme(s) or factor(s) may be necessary for methylation imprint establishment.

Dnmt3 enzyme profiles during oocyte growth

As a first step in determining which DNMT is mediating the methylation of these imprinted DMRs during oogenesis, we assayed expression of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* during postnatal oocyte growth using RT–PCR. Our results indicate that all three *Dnmts* were expressed during oocyte growth (Fig. 4). *Dnmt3L* transcripts appeared to be particularly abundant in growing oocytes and were differentially expressed in 20–50 versus 60–80 μm 15 dpp oocytes (Fig. 4, bottom right panel). The *de novo* methyltransferases, *Dnmt3a* and *Dnmt3b*, were expressed at lower levels, but with a similar expression profile to that seen for *Dnmt3L* where peak expression occurred in 15 dpp oocytes.

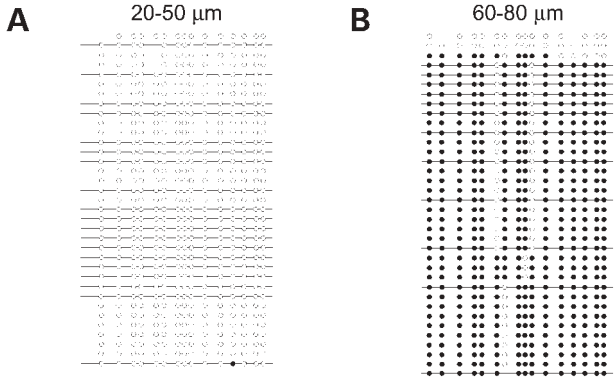

Figure 3. *Snrpn* DMR1 methylation profile of 15 dpp oocytes of different diameters. Methylation status of *Snrpn* DMR1 in (**A**) 20–50 μm and (**B**) 60–80 μm 15 dpp oocytes. *Snrpn* methylation acquisition appeared to be related to oocyte diameter. Details as described in Figure 1. See Supplementary Material Table 3 for statistical analysis.

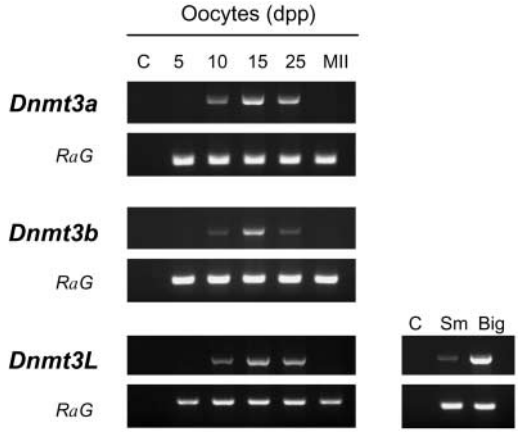

Figure 4. Expression analysis of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* during postnatal oogenesis. Oocytes were isolated at 5, 10, 15 and 25 dpp and from ovulated MII oocytes (C, minus reverse transcriptase control lane). Amplification of rabbit α-globin (*RαG*) was used as an internal control for RNA extraction and amplification efficiency. For *Dnmt3a* and *Dnmt3b* (top and middle panels) 150 oocytes were used per lane. For *Dnmt3L*, 25 oocytes were used per lane. *Dnmt3a*, *Dnmt3b* and *Dnmt3L* appeared to be expressed in growing oocytes as methylation was being acquired at maternally methylated DMRs. *Dnmt3L* transcripts appeared to be particularly abundant in growing oocytes. Bottom right panel, expression of *Dnmt3L* in 15 dpp oocytes ($n = 25$) of different diameters. Oocytes were isolated at 15 dpp and grouped into two pools according to size: 20–50 μm (Sm for small) and 60–80 μm (Big). *Dnmt3L* expression was up-regulated as oocyte diameter increased.

IAP LTR methylation and expression in oocytes

To address the idea that methylation acquisition occurs postnatally in growing oocytes on a more global level, we assessed the methylation profile of non-imprinted IAPs during this same developmental window. IAP sequences are retrovirus-like transposable elements found in ~1000 copies in the mouse genome. Methylation of the 5′-LTR of IAPs is important for keeping these elements transcriptionally silent and inactive (28). Our results show that IAPs LTR methylation was also acquired during oocyte growth (Fig. 5A and Supplementary Material Table 4) with the percentage of hypermethylated strands in 1, 5, 10 and 15 dpp and MII oocytes at 12, 33, 82, 82 and 66%, respectively (15 dpp and MII methylation profiles not significantly different by Fisher's exact analysis, see Supplementary Material).

Using RT–PCR, we next determined whether IAP methylation during oocyte growth correlates with transcriptional silencing of IAP elements. IAP transcripts are divided into two subfamilies, type I and type II, which account for roughly 60 and 40% all IAPs, respectively (29). Our analysis, which investigated the expression profile of the more abundant subfamily, indicated that type I IAPs were present in 10, 15 and 25 dpp oocytes and were down-regulated in MII oocytes (Fig. 5B). Although this result may suggest that methylation resulted in IAP transcriptional down-regulation, it may also reflect the general phenomenon of decreased RNA synthesis in MII oocytes (30).

DISCUSSION

Methylation of imprinted genes occurred during oocyte growth

In this study, we show that a number of imprinted genes acquired their methylation imprint during postnatal oocyte growth and that, while some genes were methylated early in oocyte development, others were methylated in more mature fully grown oocytes. In our earlier study, we had examined *Snrpn* methylation at three timepoints during oocyte growth (1 and 10 dpp and in MII oocytes) (5). Here, by expanding our analysis to include additional timepoints as well as multiple DMRs within imprinted genes, we show that the acquisition of methylation imprints during oocyte development was a more generalized phenomenon. These findings are important in light of recent results indicating that human *SNRPN* is also methylated in GV oocytes (6). Several studies have suggested that there is an increased incidence of human imprinting disorders associated with alterations in the methylation of imprinted genes in children conceived by assisted reproductive technologies (ARTs) (31–35). We and others (36–39) have argued that it is thus critical to achieve a better understanding of the timing and mechanisms underlying imprint establishment and maintenance, as well as the conditions that may adversely affect imprints, such as ovulation protocols and culture conditions. The fact that maternal methylation imprints appear to be established during the later stages of oocyte development in human, as they are in mouse, provides support for the continued use of the mouse model in studying imprinting mechanisms.

Gene-specific establishment of maternal methylation imprints

We first investigated the timing of methylation acquisition of four imprinted genes with a maternal methylation imprint, *Snrpn*, *Igf2r*, *Peg1* and *Peg3*, in postnatal growing oocytes. Our use of bisulfite sequencing analysis, a very sensitive and powerful assay for investigating the methylation status of

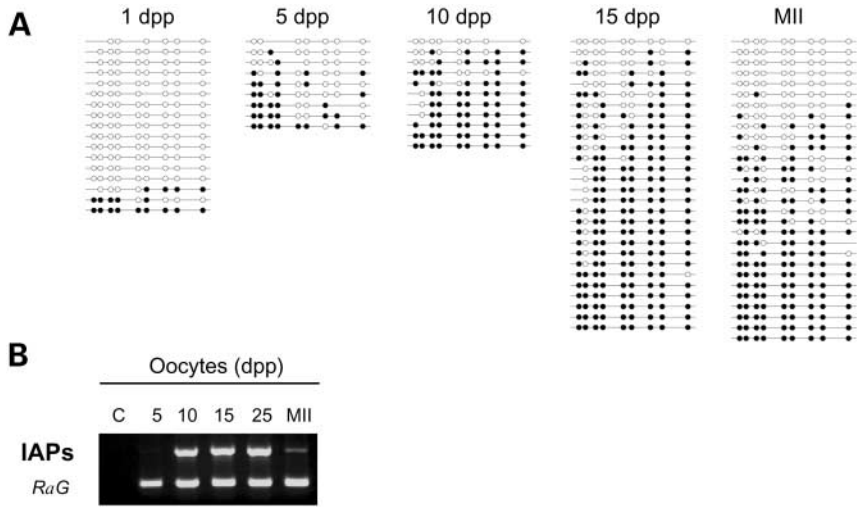

Figure 5. IAP methylation and expression during postnatal oocyte growth. (**A**) Methylation status of the IAPs 5′-LTR in 1, 5, 10 and 15 dpp, and MII oocytes. Bisulfite results for 15 dpp and MII were obtained from two sets of oocytes. Details as described in Figure 1. See Supplementary Material Table 4 for statistical analysis. (**B**) Expression analysis of IAP type I transcripts in 5, 10, 15 and 25 dpp and MII oocytes (25 oocytes were used per lane) (C, control lane). IAP type I transcripts were expressed in 10, 15 and 25 dpp growing oocytes and appeared to be down-regulated in MII oocytes.

multiple CpGs within a region of interest, enabled five imprinted genes to be examined simultaneously in the same sets of oocytes, permitting the methylation of each gene to be analyzed in relation to the others. In each set of oocytes, *H19* DMR methylation was examined first and the lack of methylated strands verified that there was no somatic contamination in the oocyte preparations (data not shown). Our findings indicate that methylation on the *Snrpn*, *Igf2r*, *Peg1* and *Peg3* DMRs was acquired asynchronously in a gene-specific manner while oocytes were arrested at prophase I and transitioned from primordial to antral follicles (summarized in Fig. 6).

An independent study inferred that maternal imprint acquisition occurred asynchronously based on expression of imprinted genes in parthenogenetic embryos derived from manipulated primordial to fully grown oocytes (23). However, these studies did not determine the mechanistic basis (i.e. DNA methylation or chromatin structure) of the asynchronous imprinting. Imprint establishment was suggested by Obata and Kono (23) to occur in primordial to primary follicle stages oocytes (5–15 dpp) for *Snrpn*, *Znf127* and *Ndn*, in secondary follicle stage oocytes (10–20 dpp) for *Peg3*, *Igf2r* and $p57^{kip2}$, in tertiary to early antral follicle stage oocytes (15–20 dpp) for *Peg1* and in antral follicle stage oocytes for *Impact* (23). This suggested sequence of imprint establishment (23) closely resembled our results here, where *Snrpn* methylation was initiated in 10 dpp oocytes, *Peg3* and *Igf2r* had similar methylation imprint profiles and *Peg1* acquired methylation at a late stage in oocyte growth.

Each of the genes we investigated is found in different chromosomal regions: *Snrpn* is found within a well-characterized imprinting cluster that spans a large region of central chromosome 7, while *Igf2r*, *Peg1* and *Peg3* are located proximally on chromosomes 17, 6 and 7, respectively (www.mgu.har.mrc.ac.uk/imprinting/all_impmaps.html). We suggest that the gene-specific establishment of imprints may be explained by the fact that these genes are either present in distinct chromosomal regions or are within different surrounding chromatin environments.

Epigenetic memory in oocyte imprinting

Our non-allele-specific methylation analysis of the DMRs of each imprinted gene in 1 dpp oocytes, where the strands sequenced were devoid of methylation, suggests an equivalence of the parental alleles at this time. Further analysis of *Snrpn* revealed that parental alleles retained an epigenetic memory of their parental origin as the two alleles continued to be recognized in a parental-specific manner (summarized in Fig. 6). Our allele-specific analysis of imprint acquisition on *Snrpn* in oocytes indicates that the methylation imprint was initially established in preantral early growing oocytes on the maternally inherited allele. In contrast, the paternally inherited allele becomes methylated in more mature oocytes derived from antral follicles. Thus, our finding that the maternal and paternal alleles of *Snrpn* are differentially marked during oocyte growth indicates that the parental alleles were not equivalent, and retained their identity in the absence of DMR1 methylation.

Our findings for *Snrpn* are reminiscent of the sequence of methylation acquisition on the *H19* DMR during spermatogenesis. Both parental alleles of *H19* are unmethylated in 13.5 dpc (days postcoitum) prospermatogonia. Methylation is initiated and complete on the paternally inherited allele in 15.5 dpc prospermatogonia while methylation on the maternal allele is postponed until 18.5 dpc and is not completed until the end of meiosis I (19). Similar to *Snrpn* in the female germ line, this differential pattern of allele-specific methylation establishment on *H19* indicates that some epigenetic modification in the male germ line distinguishes the parental alleles in the absence of DNA methylation.

Strain-specific differences in mouse oocytes can affect epigenetic inheritance (40,41) and could explain the parental allele-specific methylation differences reported for *H19* (19). Here, we analyzed *Snrpn* DMR1 methylation in reciprocal cross F_1 oocytes to determine whether our allele-specific

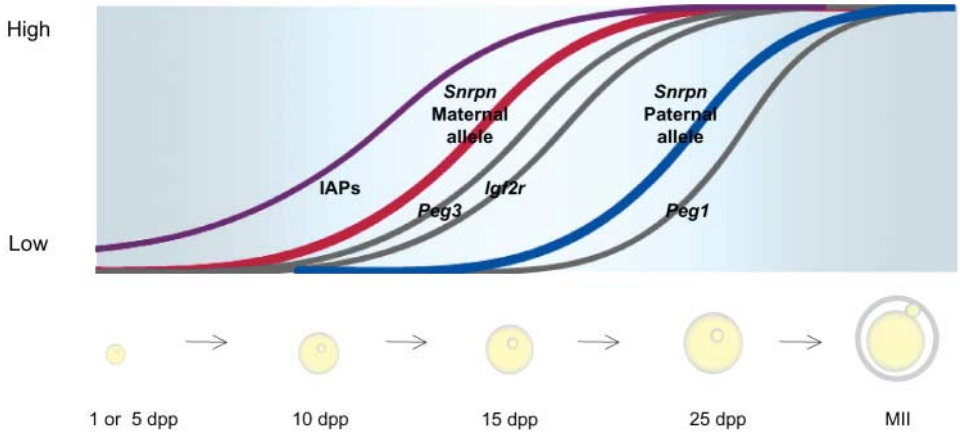

Figure 6. Schematic depicting the gene- and parental allele-specific dynamics underlying methylation acquisition during postnatal oogenesis. The top panel roughly depicts the methylation profiles of IAPs, *Peg3*, *Igf2r*, *Snrpn* and *Peg1* in growing oocytes. Our findings indicate that non-imprinted IAPs (purple line) began acquiring methylation in non-growing and early-growing oocytes. Methylation was initiated on the *Peg3*, *Igf2r* (gray lines) and *Snrpn* (red and blue lines) DMRs in early-and mid growing oocytes (in descending order according to their percentage strands with >50% mCpGs), while *Peg1* (gray line) remained hypomethylated in comparison to the other genes and acquired methylation late in oocyte development. Our results for *Snrpn* suggest a non-equivalence of the parental alleles in the absence of DNA methylation in growing oocytes, as the maternally inherited allele (red line) acquired methylation prior to the paternally derived allele (blue line). The bottom panel depicts the various stages of oocytes that were investigated.

observations were dependent on the direction of the cross used to generate our oocytes. Our findings that the maternal allele was also hypermethylated when compared with the paternal allele in reciprocal cross oocytes imply that this differential establishment of maternal imprints was not strain-specific. However, it should be noted that, although the order of methylation was the same, the degree to which alleles were methylated appeared to be significantly different between the crosses. While these observations may reflect the fact that the number of oocytes collected with larger diameters may have varied between pools, they also suggest possible strain-specific differences in the DNMT content and activity of oocyte cytoplasms.

Together our results implicate the existence of another epigenetic mark in the two germ lines. We propose that differential chromatin structure directs the establishment of gametic imprints in a gene-specific manner and marks the parental alleles of imprinted genes during germ cell development. While methylation imprints are erased in PGCs, other epigenetic modifications may not be removed. The remaining epigenetic modifications may direct DNA methylation exclusively to the *Snrpn* maternal DMR1 in oocytes before 10 dpp and to the paternal *H19* DMR in prospermatogonia prior to 18.5 dpc.

Differential modifications on the histone tails of imprinted parental alleles have been described. Allele-specific acetylation and methylation profiles of specific histone residues have been reported for *Igf2*, *H19*, *Igf2r*, *Snrpn* and *U2af1-rs1* (42–45). We propose that chromatin modifications may mark the alleles of imprinted genes during early germ cell development and initially target DNA methylation to one parental allele. Collecting the number of germ cells needed to carry out the allele-specific chromatin immunoprecipitation assays or downscaling and adapting such assays to allow analysis of chromatin modifications in germ cells remains a significant challenge to the testing of this hypothesis.

Methylation acquisition at *Snrpn* DMR1 correlated with an increase in oocyte diameter and the accumulation of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* transcripts

During postnatal female germ cell development, the oocyte grows greatly in volume and accumulates a variety of factors necessary for both meiotic maturation and early embryo development (30). We concluded from our results on oocytes from 1, 5, 10, 15 and 25 dpp mice that there is a progressive stage-related increase in methylation of the imprinted genes examined. Since the oocytes were selected at different ages, it is also possible that there is an age effect. Experiments to test an age effect would be challenging and require the isolation of oocytes of different sizes from the adult ovary. However, our results on *Snrpn* methylation in oocytes of different sizes isolated from the ovaries of 15 dpp mice support our conclusion of stage-specific methylation. Our observations that maternal methylation imprint establishment was related to oocyte diameter suggests that the accumulation of some enzyme(s) or regulatory molecule(s) necessary for imprint establishment is the limiting factor in methylation imprint establishment. We therefore examined the expression of the most recently characterized DNMT enzymes, DNMT3a, DNMT3b and DNMT3L, in growing oocytes.

DNMT3a and DNMT3b are *de novo* methyltransferases (46), and their involvement in establishing methylation imprints has been suggested by the observation that *Igf2r*, *Peg1*, *Peg3* and *Snrpn* DMRs are unmethylated in embryos derived via the transplantation of *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{+/-}$ ovaries (10). *Dnmt3L* mouse knockout studies demonstrate an essential role for this enzyme in the establishment of maternal methylation imprints in the female germ line (9,10). As *Dnmt3L* does not share any of the conserved DNMT catalytic motifs responsible for enzymatic activity, it is postulated to be a regulator of maternal imprint establishment. Our results indicate that all three enzymes are expressed during oocyte growth with similar

expression profiles (Fig. 4). *Dnmt3L* transcripts appear to be particularly abundant in growing oocytes, further supporting an important role for this DNMT in the establishment of methylation imprints. Expression of *Dnmt3L* in growing oocytes, but not primary oocytes, was also reported by Bourc'his *et al.* (9). It has been shown that DNMT3L stimulates methylation by DNMT3a (but not DNMT3b) at the *Snrpn* and *Igf2r* DMRs *in vitro* (47). In growing oocytes, DNMT3L may serve to recruit *de novo* methyltransferases such as DNMT3a to the DMRs of imprinted genes and direct methylation at these sites. While a number of candidate factors have been postulated to play a role in maternal imprint establishment, there remains the possibility that other proteins such as methyl-binding and chromatin remodeling proteins or some yet to be characterized DNMT may also be involved.

IAP methylation and expression in oocytes and implications for the repeat-like nature of imprinted gene DMRs

Having shown that DNA methylation is established at the DMRs of several imprinted genes in growing oocytes, we were interested in determining whether a non-imprinted class of sequences that is known to be methylated acquires methylation within the same developmental window. The great majority of CpGs present in the mammalian genome are contained within repetitive DNA elements. We chose to analyze the methylation status of the 5′-LTR of IAP retroviral repeat sequences. Several studies have investigated the methylation status of IAP elements in germ cells and embryos as a means of gaining insights into the methylation reprogramming events that occur during development. Previous studies have suggested that IAPs are hypomethylated in non-growing primary oocytes (48,49) and hypermethylated in fully grown oocytes (48,50) and, like imprinted genes, appear to resist demethylation in the zygote and preimplantation embryo (50). We now show using bisulfite sequencing that multiple CpG sites within the 5′-LTR of IAP elements were relatively undermethylated in 1 dpp oocytes, and acquired methylation during postnatal oocyte growth in a manner comparable to imprinted genes.

The similarity in *de novo* methylation acquisition suggests a likeness in the nature and regulation of IAPs and imprinted genes, as has been suggested by others (50). It has been shown that the short tandem repeat elements within the DMRs of some imprinted genes are important for imprinting (51). DNMTs and other components of the complex that drive methylation imprints may recognize these repeats as the signal directing methylation. From our results, methylation appears to be targeted to IAPs earlier during oocyte growth than the imprinted genes we examined. We speculate that the degree to which imprinted genes are repeat-like may be one of the factors involved in determining the timing of methylation imprint acquisition.

DNA methylation has been proposed as an IAP transcriptional silencing mechanism (48). Embryos completely deficient in the major maintenance methyltransferase *Dnmt1* show a 50- to 100-fold increase in the expression of all classes of IAP transcripts (48). Because our IAP expression analysis was limited to type I transcripts whereas our bisulfite methylation analysis detected the majority of IAPs with LTRs, a direct correlation between IAP methylation and expression levels may not be appropriate. However, data from our analyses of growing oocytes, showing active transcription of type I IAP elements that were probably methylated, suggest that methylation may not be sufficient to repress IAP transcription and that other epigenetic mechanisms are probably required for silencing.

Significance

Maternal imprinting defects have been reported for a number of clinical syndromes. In one syndrome, thought to represent a global disorder of imprinting in the female germ line, biparental hydatidiform moles develop as a result of a maternal imprinting establishment defect (52,53). Moreover, several reports have linked ARTs to an increased incidence of rare imprinting diseases (reviewed in 36,37). Methylation analysis of affected ART-conceived children has revealed a consistent defect in the methylation status of the maternally inherited allele at the *SNRPN* (Angelman syndrome) and *KCNQ1OT1* (Beckwith–Wiedemann syndrome) DMRs (31–35). It has been postulated that genetic defects (52,53) and techniques used in human ARTs (36,37) may perturb maternal imprint establishment and/or maintenance. Our data and those of others (23) indicate that some maternal imprints are established relatively late in oogenesis. Since imprint acquisition during oocyte growth may be vulnerable to ARTs, such as *in vitro* maturation of oocytes and ovarian hyperstimulation, epigenetic evaluation of oocyte manipulations should be included in prospective studies on ARTs and imprinting disorders.

MATERIALS AND METHODS

Oocyte collections and mice

Oocytes were isolated from dissociated ovaries at 1, 5, 10, 15 and 25 dpp while MII oocytes were collected from 7- to 8-week-old females as described (5,54) and were washed free of somatic cells and stored at −80°C until ready for use. Briefly, ovaries of 1, 5, 10 and 15 dpp mice were dissected in PBS, pH 7.2, and transferred to conical tubes containing 2 ml of 3 mg/ml polyvinylpyrrolidone (Sigma) prepared in PBS, 2 mg/ml collagenase (Sigma), 0.025% trypsin (Gibco BRL) and 0.02 mg/ml DNase (Sigma). The contents were shaken at 250 rpm in a 37°C incubator for 3–10 min depending on the age of the ovary and subsequently diluted by half with Hepes-buffered MEM, pH 7.2 (Gibco BRL), modified as described (55). The dissociation of oocyte–cumulus cell complexes was carried out by vigorously drawing the MEM-H/PBS solution in and out of a micropipette. GV oocytes were isolated by puncturing ovarian follicles of 25 dpp mice using a needle. MEM-H used for the isolation of 25 dpp oocytes was supplemented with 50 µg/ml dibutyryl cyclic AMP to prevent GV breakdown. MII oocytes were collected from 7- to 8-week-old females that were superovulated by injection of 7.5 IU of pregnant mares' serum gonadotropin (Sigma), followed 44–48 h later by 5 IU of human chorionic gonadotropin (Sigma). Twenty hours post-hCG, MII oocytes were recovered from the oviducts and the cumulus cells were dispersed with 1 mg/ml hyaluronidase (Roche Diagnostics). For all stages collected, oocytes were picked up using a mouth-controlled drawn-out glass pipette and washed free of somatic cells by transfer through

three dishes of MEM-H. Only cumulus-free, non-fragmented and 'healthy' looking oocytes were chosen for analysis. Somatic cell contamination was eliminated as a source of methylated strands, as the *H19* DMR, that is paternally methylated, was completely unmethylated for each sample (data not shown).

For the *Snrpn* diameter specific methylation analysis, 15 dpp were collected as described above, measured and pooled into the 20–50 and 60–80 μm size groups using a stage micrometer. For the other methylation and expression experiments, all 1–5 dpp and 25 dpp oocytes collected were 10–20 and 70–80 μm in diameter, respectively, whereas our 10 and 15 dpp oocyte pools were more heterogeneous. At 10 dpp, the oocytes collected ranged from 20 to 70 μm in diameter with greater than 50% of oocytes being larger than 50 μm. At 15 dpp, roughly 90% of oocytes were greater than 60 μm and ranged in diameter from 20 to 80 μm.

Oocytes used in the non-allele specific experiments were obtained from CD-1 mice (Charles River Canada, St Constant, QC, Canada). C57BL/6J and *Mus musculus castaneus* were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To facilitate the isolation of F_1 hybrid mice for the allele-specific bisulfite analysis of *Snrpn* in oocytes, a strain of mice that served as the source of *M. m. castaneus* allele was used (56). Natural matings between CD1 and C57BL/6J(CAST-7) mice were used to produce females for the isolation of oocytes. Parental origin of the sequenced strands was determined using strain-specific single nucleotide polymorphisms for *Snrpn* and *H19* (56). Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care.

DNA isolation and bisulfite sequencing

DNA was isolated from 450 to 500 oocytes at 1, 5, 10, 15 and 25 dpp, digested using *Hind*III (Gibco BRL) and bisulfite treatment was carried out as previously described (5,57). At least two independent sets of oocytes from mice of different litters were collected and bisulfite treated separately; all six genes were amplified from each set of oocytes. Nested PCR amplification for *Snrpn*, *Igf2r*, *Peg1*, *Peg3*, *H19* and non-nested PCR amplification for IAPs were carried out on each set of isolated oocytes as previously described (5,12). Clones containing the appropriate inserts were sequenced using an ABI 310 sequencer. Only sequences with >95% bisulfite conversion efficiency were used for analysis. Sequence differences between clones with similar CpG methylation profiles were verified to ensure unique clones were represented.

Primers

Primers specific for bisulfite-converted DNA for *Snrpn*, *Igf2r*, *Peg1*, *Peg3*, *H19* and IAPs were as previously described (5,12). The regions analyzed for each of these genes are within CpG islands. We examined a total of 16 CpG sites in a 419 bp fragment of *Snrpn* (2151–2570 bp, AF081460), seven CpG sites in a 205 bp fragment of *Igf2r* (796–1001 bp, L06446), 23 CpG sites in a 562 bp fragment of *Peg1* (1089–1651 bp, AF017994), 18 CpG sites in a 286 bp fragment of *Peg3* (2770–3056 bp, AF105262), 16 CpG sites in a 422 bp fragment of *H19* (1304–1726 bp, U19619) and nine CpG sites in a 212 bp fragment of IAPs (100–312 bp, M17551).

RT–PCR

Total RNA was extracted from roughly 500 each 5, 10, 15 and 25 dpp and MII oocytes using Trizol reagent (Invitrogen) supplemented with 10 μg of mussel glycogen (Boehringer Ingelheim) (58). Prior to RNA extraction 0.125 pg of rabbit globin mRNA (Sigma) was added per oocyte for each sample. Amplification of rabbit α-globin was used as an internal control for RNA extraction and amplification efficiency (59). Oocyte RNA pellets were dissolved in 50 μl of DEPC-treated water and 25 or 150 oocytes were used in the SuperScript One-Step RT–PCR System (Invitrogen) to analyze *Dnmt3a*, *Dnmt3b*, *Dnmt3L* and IAP (type I) expression levels. RT–PCR products were electrophoresed through 2% agarose gels. The amplification product sizes were as follows: *Dnmt3a*, 601 bp (671–1272 bp, NM_007872); *Dnmt3b*, 506 bp (556–1062 bp, AF068628); *Dnmt3L*, 533 bp (632–1165 bp, NM_019448); IAPs, 418 bp [1651–2069 bp (29), M17551]. See Supplementary Material for primer sequences. Primers were designed to span introns for the transcripts investigated. RT–PCR experiments were repeated on at least two samples of RNA from independently collected and extracted oocytes at each timepoint.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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REFERENCES

1. Reik, W. and Walter, J. (2001) Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet.*, **2**, 21–32.
2. McGrath, J. and Solter, D. (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, **37**, 179–183.
3. Surani, M.A., Barton, S.C. and Norris, M.L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, **308**, 548–550.
4. Paulsen, M. and Ferguson-Smith, A.C. (2001) DNA methylation in genomic imprinting, development, and disease. *J. Pathol.*, **195**, 97–110.
5. Lucifero, D., Mertineit, C., Clarke, H.J., Bestor, T.H. and Trasler, J.M. (2002) Methylation dynamics of imprinted genes in mouse germ cells. *Genomics*, **79**, 530–538.
6. Geuns, E., De Rycke, M., Van Steirteghem, A. and Liebaers, I. (2003) Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Hum. Mol. Genet.*, **12**, 2873–2879.
7. Li, E. (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.*, **3**, 662–673.
8. Bestor, T.H. (2000) The DNA methyltransferases of mammals. *Hum. Mol. Genet.*, **9**, 2395–2402.
9. Bourc'his, D., Xu, G.L., Lin, C.S., Bollman, B. and Bestor, T.H. (2001) Dnmt3L and the establishment of maternal genomic imprints. *Science*, **294**, 2536–2539.

10. Hata, K., Okano, M., Lei, H. and Li, E. (2002) Dnmt3L cooperates with the Dnmt3 family of *de novo* DNA methyltransferases to establish maternal imprints in mice. *Development*, **129**, 1983–1993.
11. Li, E., Bestor, T.H. and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
12. Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasler, J.M. and Chaillet, J.R. (2001) Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. *Cell*, **104**, 829–838.
13. Szabo, P.E. and Mann, J.R. (1995) Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. *Genes Dev.*, **9**, 1857–1868.
14. Kato, Y., Rideout, W.M., III, Hilton, K., Barton, S.C., Tsunoda, Y. and Surani, M.A. (1999) Developmental potential of mouse primordial germ cells. *Development*, **126**, 1823–1832.
15. Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El Maarri, O., Reik, W., Walter, J. and Surani, M.A. (2002) Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.*, **117**, 15–23.
16. Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A. and Ishino, F. (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development*, **129**, 1807–1817.
17. Szabo, P.E., Hubner, K., Scholer, H. and Mann, J.R. (2002) Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. *Mech. Dev.*, **115**, 157–160.
18. Davis, T.L., Trasler, J.M., Moss, S.B., Yang, G.J. and Bartolomei, M.S. (1999) Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics*, **58**, 18–28.
19. Davis, T.L., Yang, G.J., McCarrey, J.R. and Bartolomei, M.S. (2000) The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum. Mol. Genet.*, **9**, 2885–2894.
20. Ueda, T., Abe, K., Miura, A., Yuzuriha, M., Zubair, M., Noguchi, M., Niwa, K., Kawase, Y., Kono, T., Matsuda, Y. *et al.* (2000) The paternal methylation imprint of the mouse H19 locus is acquired in the gonocyte stage during foetal testis development. *Genes Cells*, **5**, 649–659.
21. Shamanski, F.L., Kimura, Y., Lavoir, M.C., Pedersen, R.A. and Yanagimachi, R. (1999) Status of genomic imprinting in mouse spermatids. *Hum. Reprod.*, **14**, 1050–1056.
22. Bao, S., Obata, Y., Carroll, J., Domeki, I. and Kono, T. (2000) Epigenetic modifications necessary for normal development are established during oocyte growth in mice. *Biol. Reprod.*, **62**, 616–621.
23. Obata, Y. and Kono, T. (2002) Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J. Biol. Chem.*, **277**, 5285–5289.
24. Shemer, R., Birger, Y., Riggs, A.D. and Razin, A. (1997) Structure of the imprinted mouse Snrpn gene and establishment of its parental-specific methylation pattern. *Proc. Natl Acad. Sci. USA*, **94**, 10267–10272.
25. Stoger, R., Kubicka, P., Liu, C.G., Kafri, T., Razin, A., Cedar, H. and Barlow, D.P. (1993) Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell*, **73**, 61–71.
26. Lefebvre, L., Viville, S., Barton, S.C., Ishino, F. and Surani, M.A. (1997) Genomic structure and parent-of-origin-specific methylation of Peg1. *Hum. Mol. Genet.*, **6**, 1907–1915.
27. Li, L.L., Szeto, I.Y., Cattanach, B.M., Ishino, F. and Surani, M.A. (2000) Organization and parent-of-origin-specific methylation of imprinted Peg3 gene on mouse proximal chromosome 7. *Genomics*, **63**, 333–340.
28. Walsh, C.P. and Bestor, T.H. (1999) Cytosine methylation and mammalian development. *Genes Dev.*, **13**, 26–34.
29. Poznanski, A.A. and Calarco, P.G. (1991) The expression of intracisternal A particle genes in the preimplantation mouse embryo. *Dev. Biol.*, **143**, 271–281.
30. Picton, H., Briggs, D. and Gosden, R. (1998) The molecular basis of oocyte growth and development. *Mol. Cell Endocrinol.*, **145**, 27–37.
31. Cox, G.F., Burger, J., Lip, V., Mau, U.A., Sperling, K., Wu, B.L. and Horsthemke, B. (2002) Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am. J. Hum. Genet.*, **71**, 162–164.
32. DeBaun, M.R., Niemitz, E.L. and Feinberg, A.P. (2003) Association of *in vitro* fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am. J. Hum. Genet.*, **72**, 156–160.
33. Gicquel, C., Gaston, V., Mandelbaum, J., Siffroi, J.P., Flahault, A. and Le Bouc, Y. (2003) *In vitro* fertilization may increase the risk of Beckwith–Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am. J. Hum. Genet.*, **72**, 1338–1341.
34. Maher, E.R., Brueton, L.A., Bowdin, S.C., Luharia, A., Cooper, W., Cole, T.R., Macdonald, F., Sampson, J.R., Barratt, C.L., Reik, W. and Hawkins, M.M. (2003) Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *J. Med. Genet.*, **40**, 62–64.
35. Orstavik, K.H., Eiklid, K., van der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O. and Buiting, K. (2003) Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am. J. Hum. Genet.*, **72**, 218–219.
36. Lucifero, D., Chaillet, J.R. and Trasler, J.M. (2004) Potential significance of genomic imprinting defects for reproduction and assisted reproductive technology. *Hum. Reprod. Update*, **10**, 3–18**.**
37. Gosden, R., Trasler, J., Lucifero, D. and Faddy, M. (2003) Rare congenital disorders, imprinted genes, and assisted reproductive technology. *Lancet*, **361**, 1975–1977.
38. Schultz, R.M. and Williams, C.J. (2002) The science of ART. *Science*, **296**, 2188–2190.
39. Winston, R.M. and Hardy, K. (2002) Are we ignoring potential dangers of *in vitro* fertilization and related treatments? *Nat. Cell Biol.*, **4** (suppl.), s14–s18.
40. Latham, K.E. (1994) Strain-specific differences in mouse oocytes and their contributions to epigenetic inheritance. *Development*, **120**, 3419–3426.
41. Sapienza, C., Paquette, J., Tran, T.H. and Peterson, A. (1989) Epigenetic and genetic factors affect transgene methylation imprinting. *Development*, **107**, 165–168.
42. Fournier, C., Goto, Y., Ballestar, E., Delaval, K., Hever, A.M., Esteller, M. and Feil, R. (2002) Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. *EMBO J.*, **21**, 6560–6570.
43. Grandjean, V., O'Neill, L., Sado, T., Turner, B. and Ferguson-Smith, A. (2001) Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. *FEBS Lett.*, **488**, 165–169.
44. Hu, J.F., Pham, J., Dey, I., Li, T., Vu, T.H. and Hoffman, A.R. (2000) Allele-specific histone acetylation accompanies genomic imprinting of the insulin-like growth factor II receptor gene. *Endocrinology*, **141**, 4428–4435.
45. Gregory, R.I., Randall, T.E., Johnson, C.A., Khosla, S., Hatada, I., O'Neill, L.P., Turner, B.M. and Feil, R. (2001) DNA methylation is linked to deacetylation of histone H3, but not H4, on the imprinted genes Snrpn and U2af1-rs1. *Mol. Cell. Biol.*, **21**, 5426–5436.
46. Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell*, **99**, 247–257.
47. Chedin, F., Lieber, M.R. and Hsieh, C.L. (2002) The DNA methyltransferase-like protein DNMT3L stimulates *de novo* methylation by Dnmt3a. *Proc. Natl Acad. Sci. USA*, **99**, 16916–16921.
48. Walsh, C.P., Chaillet, J.R. and Bestor, T.H. (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat. Genet.*, **20**, 116–117.
49. Lees-Murdock, D.J., De Felici, M. and Walsh, C.P. (2003) Methylation dynamics of repetitive DNA elements in the mouse germ cell lineage. *Genomics*, **82**, 230–237.
50. Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J. and Reik, W. (2003) Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. *Genesis*, **35**, 88–93.
51. Reinhart, B., Eljanne, M. and Chaillet, J.R. (2002) Shared role for differentially methylated domains of imprinted genes. *Mol. Cell. Biol.*, **22**, 2089–2098.
52. Judson, H., Hayward, B.E., Sheridan, E. and Bonthron, D.T. (2002) A global disorder of imprinting in the human female germ line. *Nature*, **416**, 539–542.
53. El Maarri, O., Seoud, M., Coullin, P., Herbiniaux, U., Oldenburg, J., Rouleau, G. and Slim, R. (2003) Maternal alleles acquiring paternal methylation patterns in biparental complete hydatidiform moles. *Hum. Mol. Genet.*, **12**, 1405–1413.
54. Eppig, J.J. and Telfer, E.E. (1993) Isolation and culture of oocytes. *Meth. Enzymol.*, **225**, 77–84.

55. Clarke, H.J., Rossant, J. and Masui, Y. (1988) Suppression of chromosome condensation during meiotic maturation induces parthenogenetic development of mouse oocytes. *Development*, **104**, 97–103.
56. Mann, M.R., Chung, Y.G., Nolen, L.D., Verona, R.I., Latham, K.E. and Bartolomei, M.S. (2003) Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol. Reprod.*, **69**, 902–914.
57. Warnecke, P.M., Mann, J.R., Frommer, M. and Clark, S.J. (1998) Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. *Genomics*, **51**, 182–190.
58. Ratnam, S., Mertineit, C., Ding, F., Howell, C.Y., Clarke, H.J., Bestor, T.H., Chaillet, J.R. and Trasler, J.M. (2002) Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. *Dev. Biol.*, **245**, 304–314.
59. Temeles, G.L., Ram, P.T., Rothstein, J.L. and Schultz, R.M. (1994) Expression patterns of novel genes during mouse preimplantation embryogenesis. *Mol. Reprod. Dev.*, **37**, 121–129.