—Mini Review—

Epigenetic Modification in Mouse Oocytes

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Abstract: Epigenetic modification is the main mechanism of transcriptional regulation that does not involve changes in DNA sequences, such as DNA methylation, acetylation and methylation of the N-terminal tail of histone. Recently, next-generation sequencing technology has provided detailed information about the DNA methylation status of the whole mouse genome in full-grown oocytes. However, it is still very hard to read histone codes in oocytes because a large number of cells (1×10^6 cells or more) are needed for such analyses. In addition, information that can be obtained from immunostaining analysis is limited to a global image of histone modification in oocytes. Consequently, a complete picture of individual epigenetic modifications in mouse oocytes has not yet been understood. In this paper, the DNA methylation required for functional oocytes is reviewed. The differences in DNA methylation between oocytes grown *in vivo* and *in vitro*, and the potential for manipulating epigenetic modifications in oocytes are also discussed.

Key words: oocyte, Genomic imprinting, DNA methylation, *in vitro* growth

DNA Methylation during Mouse Oogenesis

DNA methylation mainly occurs at the cytosine residues of CpG dinucleotides [1]. Non-CpG methylation is observed to a lesser extent, approximately 10% overall in germ cells [2, 3]. In general, DNA methylation in the promoter region suppresses gene transcription, whereas DNA methylation in the transcribed (gene body) regions positively correlates with gene expression [4–7]. DNA methylation is catalyzed by the DNA methyltransferases (DNMTs) DNMT1, DNMT3A, and DNMT3B, and the non-enzymatic cofactor DNMT3L [1]. DNMT1 is known as a maintenance methylase through which the methylation patterns of parent strands are clonally transmitted to daughter strands during DNA replication. DNMT3A and DNMT3B are *de novo* methylases that primarily determine the methylation patterns of naïve DNA strands. DNMT3L has no catalytic activity by itself but cooperates with other DNMT3 family members to carry out *de novo* methylation.

In non-growing oocytes, the global DNA methylation level is 2.3%, but this gradually increases as the oocytes grow [3, 8]. When an oocyte reaches the full-grown stage, genomic DNA is bisected into hypomethylated (less than 10% of CpG sites are methylated) and hypermethylated (more than 90% of CpG sites are methylated) regions, and the mean global DNA methylation level is approximately 40% [4]. Deletion of *Dnmt3a* or *Dnmt3l* but not *Dnmt1* and *Dnmt3b* during oogenesis leads to global hypomethylation in the full-grown oocytes. The DNA methylation level is decreased to 6.3% (*Dnmt3a* −/−) and to 3.2% (*Dnmt3l* −/−), which is similar to that of non-growing oocytes [3]. However, full-grown oocytes that lack DNMT3A and DNMT3L appear normal and can successfully undergo meiosis, early cleavage, and implantation [9–11]. Therefore, DNA methylation appears to be dispensable for oogenesis. On the other hand, during spermatogenesis, most of the CpG sites are hypermethylated and the mean global DNA methylation level in sperm is approximately 90%. Deletion of *Dnmt3a* or *Dnmt3l* during spermatogenesis results in azoospermia due to genome instability induced by the activation of retrotransposons [12]. Thus, the role of DNA methylation is quite different in oocytes and sperm.

The most significant role of DNA methylation during oogenesis is genomic imprinting. DNMT3A and DNMT3L are essential for *de novo* methylation at the imprinted regions in both the male and female germlines [9–11]. In mouse zygotes, the sperm–derived hypermethylated genome is actively demethylated [13]. The zygotic genome is passively demethylated through cell division, and consequently, the methylation level of the whole genome is decreased to the basal level at the blastocyst stage. Following genome-wide *de novo* methylation, the embryonic genome reacquires DNA methylation [14, 15]. However,

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imprinted regions are not affected by these methylation dynamics. The oocyte- or sperm-specific methylation status of the imprinted loci is protected against genome-wide demethylation at fertilization and against *de novo* methylation at gastrulation. The methylation status is maintained throughout ontogenesis except for germ cell lineage, resulting in parent-of-origin-specific gene expression [16].

In mice, oocyte-specific methylation imprints are absent in non-growing oocytes but are gradually acquired during oocyte growth [17, 18]. Therefore, the imprinting status differs among the oocytes in the ovary. In contrast, all of the male germ cells in the testis possess integral sperm-specific methylation imprints since sperm-specific DNA methylation imprints are established in the prospermatogonia during the perinatal stage [19, 20]. Differentially methylated regions between the germ lines have been identified at imprinted loci: 19 of them are methylated in oogenesis, and 3 of them are methylated in spermatogenesis [4]. Loss of imprinted methylation leads to embryonic lethality, disorders, or phenotypic abnormalities [9–11, 21–23]. Thus, DNA methylation imprints are essential for producing functional germ cells in mammals.

Beckwith-Widemann syndrome, Silver-Russell syndrome, and transient neonatal diabetes mellitus 1 are the most well-known imprinting disorders. Some patients with such imprinting disorders exhibit loss of methylation imprints (LOM). The most common cause of LOM is the *ZFP57* mutation, although there are exceptions [24–26]. Recently, an association between epigenetic mutation and artificial reproductive technology (ART) has also been suggested, which will be discussed below [27].

Culture and Epigenetic Mutation

Methylation of DNA and histone requires S-adenosylmethionine (SAM) to act as a methyl donor. Methionine is an essential amino acid that cannot be synthesized *in vivo*. A mammal obtains methionine from food which is then converted to SAM by methionine adenosyltransferase [28]. In culture, cells uptake methionine from the medium and supplements such as fetal bovine serum (FBS) and bovine serum albumin. Epigenetic alterations caused by culture and manipulation of oocytes are thought to occur due to an excess of SAM, factors of the ectopic environment, or both. Epigenetic mutation possibly occurs during gametogenesis and embryogenesis; however, the specific cause is not known.

An earlier report focused on epigenetic mutation in bovine and ovine embryos produced *in vitro*. Bovine and ovine embryos derived from *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and culture of cleavage-stage embryos often exhibit large offspring syndrome [29]. This has been attributed to loss of methylation at the *IGF2R* locus and the consequent reduced expression of IGF2R in pre-implantation embryos in sheep [30]. Since the publication of this earlier study, the long-term effects of events occurring at the early developmental stages of embryos which are detrimental to late ontogeny have received substantial research attention. We also examined the long-term effect of *in vitro* growth (IVG) of mouse oocytes on their subsequent development [31]. IVG of oocytes may provide a new source of functional oocytes; however, it is difficult to produce offspring successfully from IVG oocytes because of their cytoplasmic deficiencies [31, 32]. Therefore, we used a nuclear transfer technique in which the nuclei of IVG oocytes were transferred into enucleated oocytes that were grown *in vivo*, and the developmental ability of these reconstituted oocytes was investigated following IVM, IVF, and embryo transfer (Fig. 1). In this strategy, the cytoplasmic deficiencies of IVG oocytes are negligible, and the influence of IVG on the oocyte genome can be evaluated. Full-grown, growing, and non-growing oocytes were cultured for 0, 11, and 21 days, respectively. Our results show that there were no significant differences in developmental ability among embryos reconstituted with nuclei of the oocytes cultured for 0, 11, or 21 days. More than 90% of the embryos developed to the blastocyst stage and approximately 30% of the embryos developed into pups in all experimental groups. The competency of the reconstituted oocytes was unaffected by the duration of culture. No obvious abnormalities were observed in any of the pups and placentae; however, the pups and placentae from the reconstituted eggs that contained the nuclei of oocytes cultured for 21 days were heavier than those obtained from non-manipulated eggs (IVF control). No significant differences were observed among the other experimental groups (Fig. 1) [31]. This suggests that the overgrowth phenotype arises from long-term culture and/or an ectopic environment during an earlier stage of oocyte growth. In addition, to analyze the DNA methylation status at imprinted loci in IVG oocytes, ovaries from newborn mice, which contain only non-growing oocytes, were cultured for 10 days and then the obtained IVG oocytes were compared with growing oocytes from 10-day-old mice (Fig. 2). The methylation levels of IVG oocytes with diameters of 45–50 μm and 50–55 μm were equal to those of size-matched groups of growing oocytes from 10-day-old mice [23]. Considering that oocyte-specific methylation imprints are established during oocyte growth, DNA methylation at imprinted loci might be susceptible to the

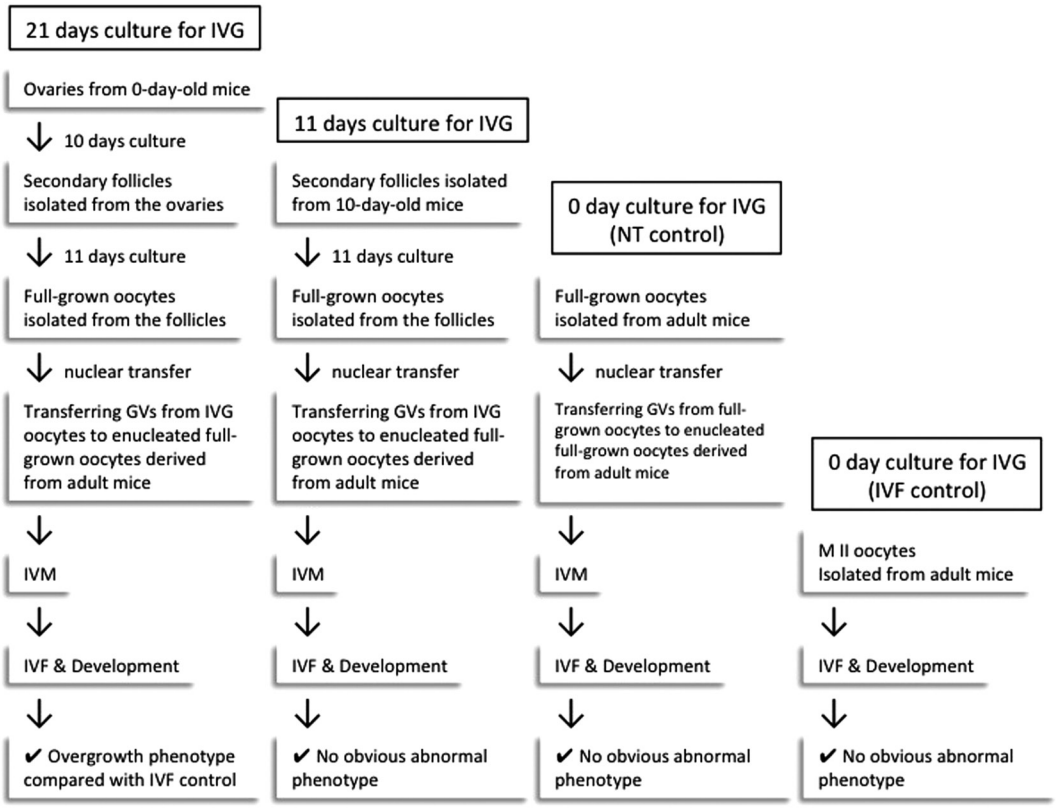

Fig. 1. Schematic representation of the experimental design used to examine the long-term effect of the *in vitro* growth (IVG) of mouse oocytes on their ontogeny.
Ovaries isolated from 0-day-old mice were cultured for 10 days on Transwell membranes in minimal essential medium (MEM)-alpha supplemented with 10% fetal bovine serum (FBS). On day 10, secondary follicles were isolated from ovaries and subjected to follicular culture. The secondary follicles were isolated from *in vitro*-derived ovaries and ovaries from 10-day-old mice. These follicles were cultured for 11 days in MEM-alpha supplemented with 5% FBS, 0.1 IU/ml follicle-stimulating hormone, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium. Full-grown oocytes at the germinal vesicle (GV) stage were isolated from the resultant follicles and Graafian follicles of adult mice. The GVs of IVG oocytes were transferred to the cytoplasm of GV oocytes that were isolated from adult mice. As a control, GVs were transferred between GV oocytes from adult mice (NT control). After *in vitro* maturation, chromosomes at the metaphase in the second meiosis (MII) were transferred from the reconstituted oocytes to the ovulated and enucleated MII-stage oocytes to yield cytoplasmic competency. As another control, MII oocytes were subjected to *in vitro* fertilization (IVF control). This scheme is a simplified and modified version of the original experimental design [31].

IVG. However, we were not able to obtain evidence to directly support this hypothesis, which is consistent with the results reported by Smitz *et al*. [33]. In contrast, some studies have shown that superovulation and/or culture of embryos affect the imprinted expression and methylation status in oocytes, embryos, and/or placentae [34–36]. Currently, there is not enough evidence to conclude that epigenetic mutation is due to ART. To understand this relationship better, epigenetic information of the whole genome in a large population of experimental animals produced by several kinds of ART is required.

Potential for Artificial Control of Epigenetic Modification

As described above, oocyte-specific methylation imprints are established during oocyte growth, which require DNMT3A and DNMT3L [9–11]. Oocyte-specific methylation imprints are absent in non-growing oocytes that lack DNMT3A and DNMT3L expression [37]. The expression levels of DNMT3A and DNMT3L gradually increase as the oocytes grow (Fig. 3). These facts led us to hypothesize that one reason for the lack of oocyte-specific methylation imprints in non-growing oocytes might

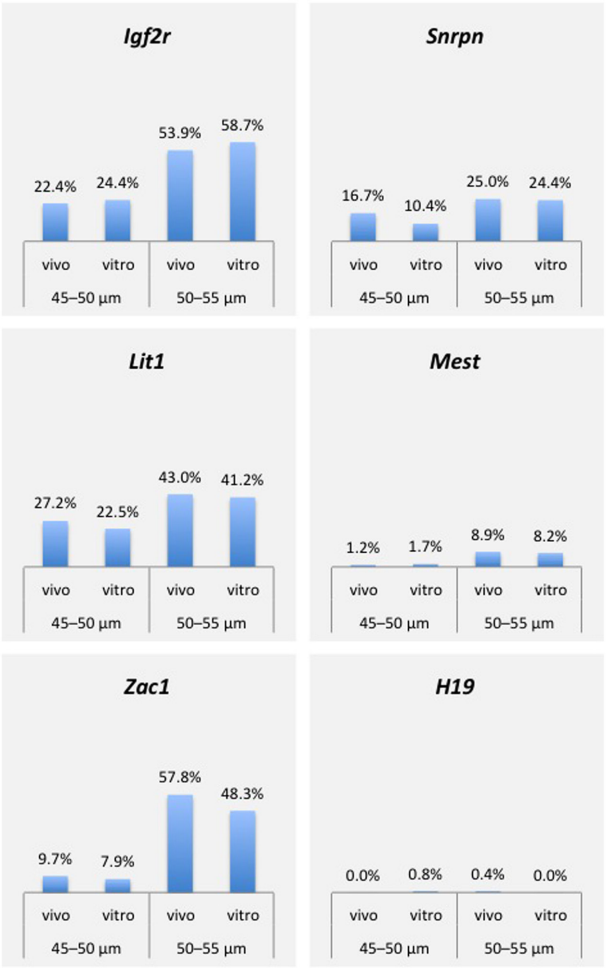

Fig. 2. DNA methylation levels at imprinted loci in growing oocytes derived from 10-day-old mice (*in vivo*) and ovaries after 10 days of culture (*in vitro*).
Methylation imprints of *Igf2r*, *Lit1*, *Zac1*, *Snrpn*, and *Mest* are established during oogenesis, whereas the methylation imprint of *H19* is established during spermatogenesis. *Igf2r*, *Lit1*, *Zac1*, *Snrpn*, and *Mest* are fully methylated (approximately 100%), whereas *H19* is not methylated in full-grown oocytes of adult mice. No significant alteration of DNA methylation levels was observed at any of the imprinted loci.

be the absence of DNMT3A and DNMT3L expression. To test this hypothesis, we produced transgenic (Tg) mice to induce overexpression of DNMT3A and DNMT3L prematurely in oogenesis [37]. Western blot analysis showed that DNMT3A and DNMT3L expression was successfully induced in non-growing oocytes derived from Tg mice as well as in full-grown oocytes. However, oocyte-specific methylation imprints were still lacking in the non-growing oocytes of Tg mice. This indicates that the presence of DNMT3A and DNMT3L is necessary but not sufficient for the establishment of oocyte-specific methylation imprints. One possible explanation for the inability of DNMT3A to catalyze *de novo* methylation at imprinted loci is a resistant state at the imprinted loci which prevents the actions of DNMT3A and DNMT3L. Although co-expression of DNMT3A and DNMT3L is restricted to germ cells, their presence alone is not sufficient to control imprint acquisition in oocytes (Figs. 3 and 5) [37].

On the other hand, excess of DNMT3A and DNMT3L accelerates the establishment of methylation imprints during oocyte growth. Four of six analyzed imprinted regions were hypermethylated in the growing oocytes derived from Tg mice at a much earlier stage than those derived from wild-type mice. To determine whether these accelerated methylation imprints in the growing oocytes of Tg mice were functional genomic imprinting after fertilization, we produced fertilized embryos containing nuclei from growing oocytes of Tg mice, and examined allele-specific DNA methylation states and expression of imprinted genes in the embryos at the mid-gestational stage [37]. Our results showed that maternal-specific hypermethylation patterns derived from Tg growing oocytes were maintained in the embryos at the *Igf2r* locus. Activation of maternal expression of *Igf2r* was also observed in the embryos containing the genome from Tg growing oocytes, but not in the embryos containing the genome from growing oocytes of wild-type mice. In contrast, loss of methylation at the *Lit1*, *Zac1,* and *Impact* loci was observed in the embryos containing the genome from Tg growing oocytes as well as in those containing the genome from growing oocytes of wild-type mice (Fig. 4). Allele-specific expression was uncontrolled at the *Lit1*, *Zac1* and *Impact* loci. Moreover, DNA methylation mosaicism at maternal alleles was observed in embryos, indicating that accelerated acquisition of methylation imprints are passively erased after fertilization. Therefore, DNA methylation at imprinted loci acquired during oocyte growth is insufficient, suggesting that maintenance mechanisms for oocyte-specific DNA methylation imprints, presumably additional epigenetic modifications, are required for functional genomic imprinting (Fig. 5) [37].

Epigenetic Modification other than DNA Methylation

KDM1B, a histone H3 lysine4 (H3K4) demethylase has been found to be essential for DNA methylation imprints at *Grb10*, *Mest*, *Zac1*, and *Impact*, but not at *Igf2r*, *Lit1*, and *Snrpn* [38]. This is consistent with the fact that DNMT3L interacts specifically with unmethylated histone

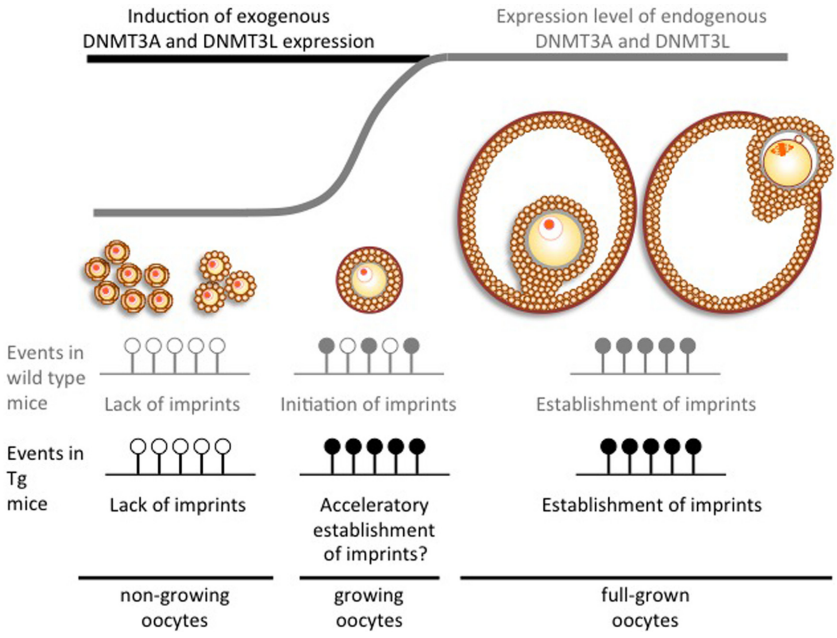

Fig. 3. Expression of DNA methyltransferases and the establishment of DNA methylation imprints during oocyte growth.
The bold line indicates expression levels of DNMT3A and DNMT3L. Open circles represent unmethylated CpG sites at certain imprinted loci. Filled circles represent methylated CpG sites at certain imprinted loci.

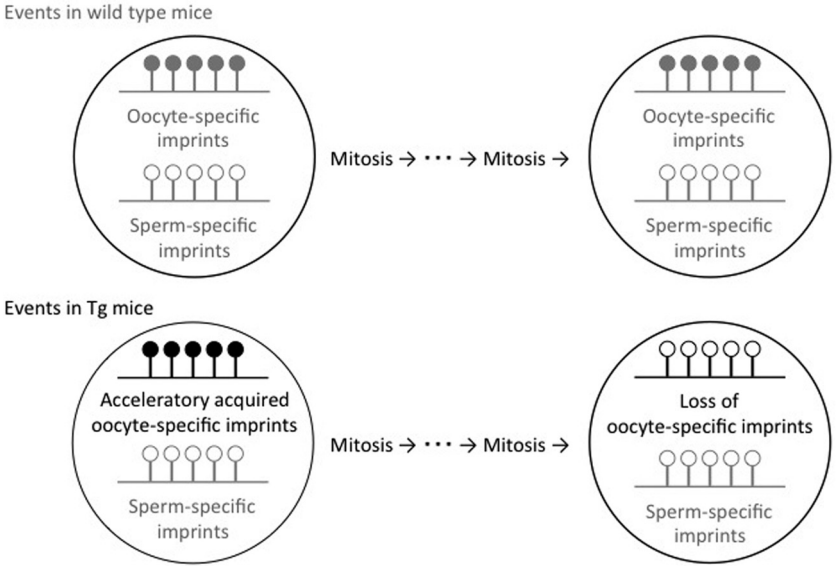

Fig. 4. Function of DNA methylation imprints during embryogenesis.
Functional methylation imprints from full-grown oocytes were maintained after fertilization and cell division (top); however, embryos containing the genome of growing oocytes derived from Tg mice showed loss of imprints sometime during embryogenesis, in spite of the acquisition of oocyte-specific DNA methylation imprints (bottom).

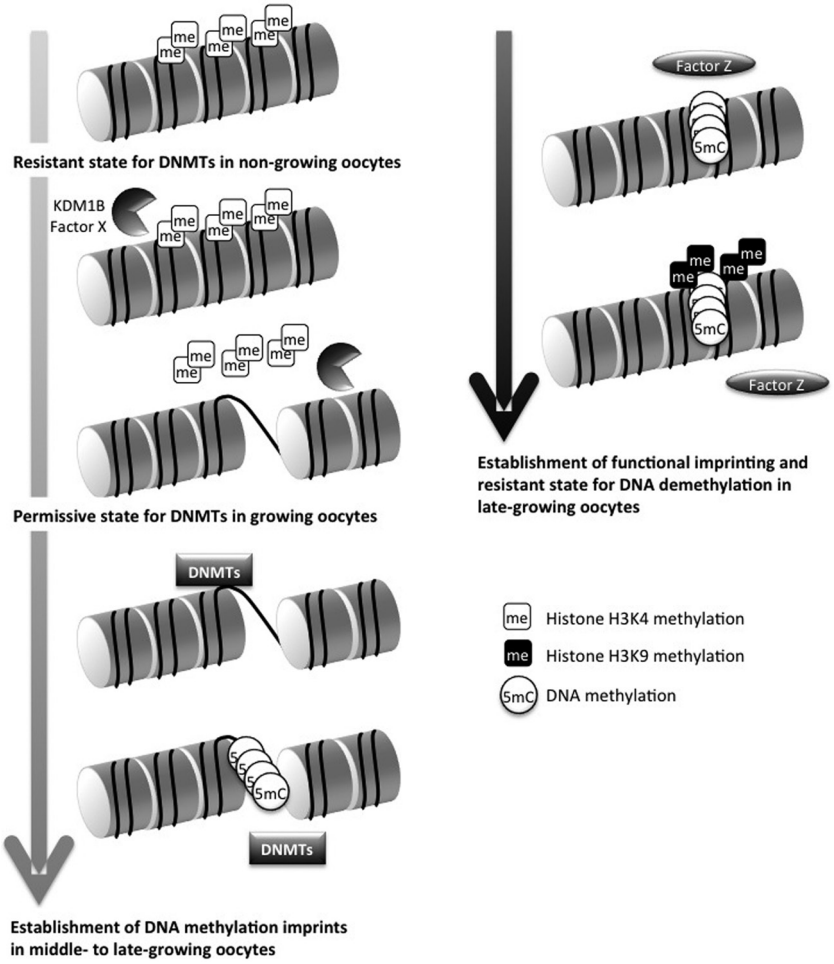

Fig. 5. Putative mechanisms behind the establishment of functional imprinting during oocyte growth. Acquisition of DNA methylation imprints is modulated by mechanisms that control the shift from the resistant to the permissive chromatin state at imprinted loci. The transition to a permissive state occurs in the early to middle oocyte growth phase. Unknown factor(s), X, in addition to KDM1B regulate extension of the permissive state for the DNA methyltransferases DNMT3A and DNMT3L (DNMTs). Methylation imprints may recruit additional epigenetic modifications, such as H3K9 dimethylation, in the late oocyte growth phase. Finally, oocytes establish functional imprinting until reaching the full-grown stage, and methylation imprints protect against DNA demethylation after fertilization.

H3K4 in *in vitro* interaction assays [39, 40]. Furthermore, protection against DNA demethylation at imprinted loci after fertilization was achieved by PGC7, H3K9 dimethylation, and ZFP57 [24–26, 41, 42]. Thus, mechanisms for the establishment of functional imprinting are complex and not yet fully elucidated.

Recently, histone deacetylase inhibitors have been evaluated as potential therapeutic drugs for cancer. An oral histone deacetylase inhibitor drug has been approved by United States Food and Drug Administration for use in cancer therapy. This is a challenge to the control of epigenetic modifications. Further studies are needed to obtain a better understanding of the epigenetic modifications occurring during oogenesis and their potential contributions to the development of reproductive medicine.

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