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Epigenetic changes associated with oocyte aging

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It is well established that the decline in female reproductive outcomes is related to postovulatory aging of oocytes and advanced maternal age. Poor oocyte quality is correlated with compromised genetic integrity and epigenetic changes during the oocyte aging process. Here, we review the epigenetic alterations, mainly focused on DNA methylation, histone acetylation and methylation associated with postovulatory oocyte aging as well as advanced maternal age. Furthermore, we address the underlying epigenetic mechanisms that contribute to the decline in oocyte quality during oocyte aging.

fertility, advanced maternal age, postovulatory oocyte aging, DNA methylation, histone modification

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Female germ cell meiosis is a long and extremely complex process, characterized by two consecutive cell divisions (meiosis I and II) and two cell cycle arrests: at the dictyate stage of prophase I (the germinal vesicle stage), and at metaphase II [1,2]. During the meiotic process, a series of changes of nuclear and cytoplasmic events take place to prepare the oocytes to obtain fertilization and subsequent developmental potentials.

Oocyte quality is essential for fertilization and subsequent embryo development [3]. Therefore, fertilization of freshly ovulated good quality oocytes typically produced by young females is important for normal embryo development into healthy offspring. It is well established that freshly ovulated MII stage oocytes have optimal fertilization potential when fertilization takes place during the time window between 6 and 24 h of ovulation, depending on species [4]. If fertilization does not occur within that time range, unfertilized oocytes remaining in the oviduct (*in vivo* aging) or culture (*in vitro* aging) will undergo a time-dependent deterioration in quality, a process called "oocyte aging". "Aged oocytes" are those oocytes in which aging already has occurred but not to the extent of degeneration [5,6]. On the other hand, with increasing age oocytes in the ovary suffer from progressive decline of quality and decrease in developmental potential. These oocytes are also being subjected to the aging process and become "aged oocytes" [7]. Here, to distinguish "aged oocytes" associated with postovulatory aging and advanced maternal age, we use for the former the term "aged oocytes" and for the latter the term "old oocytes", respectively. In contrast, we use the term "fresh oocytes" and "young oocytes", for their counterparts respectively.

DNA methylation, the most characterized epigenetic modification, is an epigenetic mechanism involved in transcriptional repression, global X chromosome inactivation, as well as genomic imprinting [8]. Genomic imprinting is a mechanism of transcriptional regulation that restricts expression of either the maternally or paternally inherited copy of the gene, whereas the opposite parental copy is repressed [9,10]. In general, genomic imprinting is character-

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ized by a multi-step process which involves erasure, establishment and maintenance of methylation mark [11,12]. In the female, genomic imprinting is established post-natally during oocyte growth, correlated with increasing oocyte diameter, as well as expression of Dnmts (DNA methyltransferases) [13–16]. Defects in imprints may likely occur during oogenesis or in early postfertilization development. Imprints are regulated by DNMTs, a family of *de novo* and maintenance methylating enzymes including five defined members in mammals: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L [17]. Appropriate expression of Dnmts is essential for regulation of imprints [17,18].

Except for methylation modification of DNA, there are diverse forms of modifications at the histone amino termini, including acetylation, phosphorylation, methylation, ADP-ribosylation, ubiquitination, sumoylation, biotinylation and proline isomerization [19–25]. It has been well documented that these histone modifications play important roles in cell cycle progression, DNA replication and repair, transcriptional activity and chromosome stability [26–28]. Among all forms of histone modifications mentioned above, some of them such as acetylation, phosphorylation and methylation have been shown to be essential for mammalian oocyte development as they display a meiosis stage-dependent nature [29]. Deregulation of these could disturb chromosome organization and segregation as well as delay progression of mammalian oocyte maturation. The dynamics, regulation mechanisms and functions of these three forms of histone modification during female meiosis have previously been reviewed in detail by us [29].

Previously, we and others have fully reviewed the cellular and molecular alterations as well as the mechanisms underlying oocyte aging [1,2,6,7]. The protein profiles during porcine oocyte aging have also been investigated by us [30]. In this review, we focus on epigenetic changes of oocyte aging related to postovulatory aging as well as advanced maternal age, with particular emphasis on the changes in DNA methylation and histone acetylation and methylation. Furthermore, the epigenetic mechanisms underlying oocyte aging are also addressed.

1 Epigenetic changes of oocytes associated with advanced maternal age

1.1 DNA methylation

Although it has been well documented that decline of reproductive potentials is associated with increasing maternal age, the related mechanisms are still largely unclear. Genomic imprints play important roles in embryonic growth and development as well as placental functions. Defects in genomic imprinting likely take place during post-natal oocyte growth and/or early postfertilization development. If DNA methylation defects take place in oocytes, such defects should be observed in the subsequent progeny.

Very recently, through fluorescence staining and confocal microscopy, Yue *et al.* [31] examined global DNA methylation in oocytes and preimplantation embryos derived from young (6–8 weeks) and old (35–40 weeks) mice. The authors found that DNA methylation levels in the oocytes and preimplantation embryos (*in vivo* and *in vitro*) decreased significantly with the increasing maternal age. Moreover, the cleavage and blastocyst rates, as well as pregnancy rates of old female mice were lower when compared to those of young counterparts. This study indicated that decreased reproductive potential related to maternal aging might be due to defects in global DNA methylation during oogenesis and preimplantation embryo development [31].

An earlier investigation by Lopes *et al.* [32] investigated the relationships between reproductive and epigenetic outcomes associated with increasing maternal age. Blastocysts as well as mid-gestation embryos and placentas derived from young (age 8–10 weeks) and old (age 43–47 weeks) female C57BL/6 mice were examined. The authors found that the reproductive outcomes were impaired by maternal age, being consistent with earlier studies by Holinka *et al.* [33]. However, by epigenetic evaluation, the results showed that imprinted genes *H19* and *Snrpn* displayed normal monoallelic expression in 3.5 days post coitus (dpc) blastocysts derived from aged females. Moreover, the genes were also revealed to express monoallelically in 10.5 dpc embryos and their respective placentas. Furthermore, the authors demonstrated that methylation of the DMRs (differentially methylated regions) at imprinted genes *Snrpn*, *Kcnq1ot1*, *U2af1-rs1*, *Peg1*, *Igf2r* and *H19*, as well as genome-wide DNA methylation levels in embryos and placentas were unchanged by maternal aging. The data demonstrated that embryos capable of developing to mid-gestation appear to undergo normal acquisition and maintenance of DNA methylation patterning.

Taken together, maternal aging adversely affects global methylation during oogenesis and preimplantation development. By contrast, although reproductive outcomes are decreased during mid-gestation development in old females, the methylated imprints are normally maintained in the live embryos during this same period.

1.2 Histone methylation

The methylation profiles of various lysine residues at histone H3 and H4 have been characterized in oocytes of mice [34,35], porcine [36], sheep [37], bovine [38] and humans [39]. Compared to acetylation at histone lysine residues, methylation modification appears relatively stable during mammalian oocyte maturation [29]. For mice, H3K4me2, H3K9me2, H3K36me2, H3K79me2, H4K20me2, and H3K9me3 were methylated in GV oocytes, and such methylation patterns were maintained in MII oocytes [34,35].

However, methylation patterns were reported to be disrupted by increasing maternal age [34], as summarized in Table 1. Through evaluation of oocytes from young (2 months old) and old (10 months old) female mice, Manosalva and González [34] demonstrated that GV and MII oocytes derived from young females showed dimethylation of lysines 4, 9, 36 and 79 in histone 3 (H3K4me2, H3K9me2, H3K36me2, H3K79me2), lysine 20 in histone H4 (H4K20me2) and trimethylation of lysine 9 in histone 3 (H3K9me3). In contrast, a significant percentage of old GV and MII oocytes lacked methylation at H3K9me3, H3K36me2, H3K79me2 and H4K20me2. Interestingly, in this study, the authors demonstrated that there was a correlation of histone methylation defects in old GV and MII oocytes, as there was a similar percentage of methylated oocytes. Therefore, changes in methylation at histone lysine residues observed in old MII oocytes might have originated from old GV oocytes.

Table 1 Changes in histone methylation modification at diverse histone lysine residues of mouse oocytes with advanced maternal age [34][a)]

Residues	GV		MII	
	Young	Old	Young	Old
H3K4me2	+	Un	+	↓
H3K9me2	+	Un	+	↓
H3K36me2	+	↓	+	↓
H3K79me2	+	↓	+	↓
H4K20me2	+	↓	+	↓
H3K9me3	+	↓	+	↓

a) Intense and decreased signals are denoted by + and ↓, respectively; Un indicates unchanged.

1.3 Histone acetylation

Acetylation modification taking place on histone H3 and H4 during mammalian oocyte maturation has been well documented in various species. There are at least four highly conserved lysines on H4 (K5, K8, K12 and K16) and two on H3 (K9 and K14) that are subjected to acetylation modification. Histone acetylation during mammalian oocyte maturation is a dynamic process and is species-specific. For mice, the above lysine residues on H3 and H4 are acetylated in GV oocytes. However, with the resumption of meiosis, deacetylation was observed in MII oocytes, with the exception of H4K8ac which was reported present or absent regarding acetylation [40,41]. For humans, H4K5, H4K8, H4K12 and H4K16 were acetylated in GV oocytes, while partial deacetylation in MII oocytes was shown [42]. Histone acetylation patterns in oocytes were affected by increasing maternal age; these changes are summarized in Table 2.

The effects of increasing maternal aging on histone acetylation of oocytes were firstly reported by Akiyama *et al.* [40], who compared the profiles of acetylation at various lysine residues on H4 using mouse oocytes from young (age 3 weeks) and old (age 10 months) BDF1 females. The authors revealed that H4K12 remained acetylated in 40% of the MII-stage oocytes of older mice, whereas histone deacetylation was complete in the oocytes of young mice. In addition, comparison of acetylated H4K8 in MII oocytes of young and old mice showed that the signal in old mice was higher, although two other lysines, H3K14 and K4K16, showed complete deacetylation in MII oocytes regardless of maternal age. Moreover, this study also suggested that the ability of deacetylating histone decreased, but did not disappear in MII oocytes with increasing maternal age. A subsequent observation by Manosalva and Gonzalez [41] further confirmed that the deacetylation level of H4K8 in mouse oocytes was affected by maternal age. Moreover, the authors compared the acetylation of H4K12 in GV and MII oocytes from different advanced maternal ages, and found that the acetylation of H4K12 decreased with age in GV oocytes at 11, 13, and 15 months (70%, 55%, 20%), and increased relative to age in old MII oocytes (57%, 60%, 80%). The abnormal acetylation of H4K12 in GV oocytes and deacetylation in MII oocytes were correlated, indicating such errors likely origin from GV oocytes.

Aside from the mouse model, histone acetylation changes in oocytes associated with increasing maternal age was also observed in humans. By using immunofluorescence coupled with confocal microscopy, Berg *et al.* [42] found that similar to the mouse, human GV oocytes showed in-

Table 2 Changes in histone acetylation modification at various histone lysine residues with advanced maternal age[a)]

Species		Age		GV		MII		
		Young	old	Young	Old	Young	Old	
Mouse	H4K5	8 w	40–44 w	100%	Un	0%	Un	[41]
	H4K8	8 w	40–44 w	100%	Un	0%	Un	[41]
		3 w	40 w			0%	↑	[40]
	H4K12	8 w	40–44 w	100%	↓ (83%)	0%	↑ (19%)	[41]
		3 w	40 w			0%	↑ (40%)	[40]
		8 w	35–40 w				↑	[65]
	H4K16	8 w	40–44 w	100%	↓ (60%)	0%	Un	[41]
Human	H4K12	av 26.9 y	av 38.3 y			32%	↑ (56%)	[42]

a) Decreased and increased signals are denoted by ↓ and ↑, respectively; Un indicates unchanged; w and y indicate week and year, respectively.

tense staining signals at H4K5, H4K8, H4K12 and H4K16. Although gradual deacetylation took place during maturation, a substantial percentage of the oocytes had residual acetylation of the above lysines in MII oocytes. The authors further compared the acetylation profiles of H4K12 in oocytes of different female ages (young maternal age: 24–30 years; middle maternal age: 31–35 years; advanced maternal age: 36–42 years). They demonstrated that increasing amounts of acetylated oocytes were found with advanced aging, indicating that advanced maternal aging gradually affected the deacetylation ability of H4K12 during the final stages of human oocyte maturation.

2 Epigenetic changes of oocytes associated with postovulatory oocyte aging

2.1 DNA methylation

It has been well shown that sex-specific DNA methylation patterns of imprinted genes are established during gametogenesis. In mouse MII stage oocytes, the maternally imprinted genes *Peg1*, *Peg3* and *Snrpn* are hypermethylated, while paternally imprinted gene *H19* is hypomethylated [13,43,44]. These methylation patterns in gametes must be maintained during early development after fertilization.

We previously examined methylation patterns of imprinted genes in mouse oocytes during the postovulatory aging process [45]. In our study, methylation of two maternally imprinted genes, *Snrpn* and *Peg1*, were examined in *in vivo* and *in vitro* oocytes at 13, 21 and 29 h post hCG injection, respectively. By using BS (bisulfite and sequencing) and COBRA (combined bisulfite restriction analysis), the results showed that *Snrpn* maintained the complete methylation pattern in oocytes at 13 and 21 h, whereas lost methylation was observed in some oocytes at 29 h of hCG injection. Similar to a previous report [43], *Peg1* displayed a hypermethylated pattern in fresh oocytes. The hypermethylated pattern was maintained when time progressed to 21 and 29 h post hCG injection. By contrast, Imamura *et al.* [46] reported that *Peg1* lost methylation in oocytes during postovulatory aging. For *Peg1*, our result differed from that of Imamura *et al.* [46]. Such discrepancy may be due to different oocyte aging times, or different domains of the same gene examined. Altogether the data indicate that the defects of DNA methylation induced by oocyte aging are time-dependent.

Previous studies have shown that postovulatory oocyte aging gives rise to offspring suffering from a series of defects including an increased number of growth-retarded pups, delayed development of the righting reflex, and higher spontaneous motor activity and emotionality, as well as decreased reproductive fitness and longevity of offspring [47,48]. To study whether these phenomena are associated with abnormal DNA methylation in offspring, we assayed DNA methylation patterns of imprinted genes in oocytes from offspring that had been derived from postovulatory aged oocytes [49]. We found that postovulatory oocyte aging caused a decline in reproductive outcomes, being consistent with previous reports [47,48]. Nevertheless, our results showed that methylation patterns at DMRs of *Peg3*, *Snrpn*, *Peg1* and *H19* in oocytes from aged-oocyte offspring were basically normal, with only a small number of oocytes showing aberrant methylation in DMR of *Peg3*. Altogether, postovulatory oocyte aging causes a decline in reproductive outcomes but does not evidently disrupt the acquisition of methylated imprints in oocytes from viable offspring.

2.2 Histone acetylation

As discussed above, histone acetylation modification at lysine residues in oocytes is influenced by maternal aging. Similarly, they are also disrupted during postovulatory oocyte aging. We examined the acetylation levels of various lysine residues on histones H3 and H4 in *in vivo* and *in vitro* postovulatory aged mouse oocytes at 14, 19, and 24 h of hCG injection [50]. By using fluorescence staining with specific antibodies, we found that AcH3K9, AcH3K14, AcH4K5, AcH4K8, AcH4K12, and AcH4K16, with the exception of a weak signal for AcH4K8, did not display signals in oocytes at 14 h post-hCG. Interestingly, with the progression of postovulatory time to 19 h of hCG injection, fluorescence signals for AcH4K8 and AcH4K12 intensified and increased gradually. When the time of aging progressed to 24 h of hCG injection, AcH3K14 was also detected and displayed an increase of fluorescence signals in the oocytes. Our observation was further confirmed by Liu *et al.* [51] who found that AcH3K14 and AcH4K12 showed an increase in acetylation in mouse oocytes during postovulatory aging. Although unlike mouse oocytes, AcH4K12 was acetylated in mature porcine oocytes, its acetylation level increased during postovulatory aging [52]. These data indicate that changes in oocyte histone acetylation with postovulatory aging occur regardless of the *in vivo* or *in vitro* environment. Furthermore, the number of changed histone acetylation modification should be increased with progression of oocyte aging.

3 Epigenetic changes: underlying mechanisms of oocyte aging

As discussed above, there are a series of epigenetic changes related to oocyte aging. Together with the meiosis-specific roles of epigenetic modifications during mammalian oocyte growth and maturation, it is suggested that epigenetic changes may contribute to oocyte aging.

Acetylation levels at some histone lysine residues in mouse and porcine oocytes were clearly increased during *in vivo* and *in vitro* postovulatory aging (Table 3). To further

Table 3 Profiles of acetylation modification at diverse histone lysine residues in oocytes during postovulatory aging[a)]

Species		Aging time (h)	Fresh	Aged	Refs.
Mouse	H3K9	10	–	Un	[50]
	H3K14	10–20	–	↑	[50,51,66]
	H4K5	10	–	Un	[50]
	H4K8	10	–	↑	[50]
	H4K12	10–12	–	↑	[50,51]
	H4K16	10	–	Un	[50]
		20		↑	[66]
Pig	H4K12	24		↑	[52]

a) Weak/absent and increased signals are denoted by – and ↑, respectively; Un indicates unchanged.

determine the correlation between oocyte aging and histone hyperacetylation, histone acetylation levels were examined in oocytes through artificial delay or acceleration of the aging progression. Caffeine can delay the progress of aging in mouse and porcine oocytes [5,30,50]. Interestingly, the delay of aging by caffeine is correlated with hypoacetylation at H3K4 and H4K12. By contrast, TSA (Trichostatin A), an inhibitor of HDACs (histone deacetylases) can artificially interfere with the status of histone acetylation [53]. We found that mouse oocytes cultured in M16 medium with 100 nmol L^{-1} TSA for 5 h were acetylated in H3K14 and H4K12 but not in H3K9 and H4K5 when these four sites were examined [50]. Moreover, the higher activation rate in the TSA group compared to that of the control group indicated that mouse oocyte aging was accelerated. Thus, artificially accelerated oocyte aging correlated to hyperacetylation at lysine residues. Taken together, these data clearly demonstrate that a decrease and increase of the acetylation level at some histone lysine residues is correlated to delayed and accelerated oocyte aging, respectively. These evidences further confirm that epigenetic changes of oocytes may be an underlying mechanism of oocyte aging.

It has been well established that a decline in female fertility with increasing maternal age is due to high frequency of errors in meiotic chromosome segregation during female meiosis [1,2]. Studies suggested that defects in deacetylation at some histone lysine residues in old oocyte may be involved. Histone acetylation has been shown to be related to chromosome segregation [29]. On one hand, the presence of TSA in the medium during mammalian oocyte *in vitro* maturation resulted in hyperacetylation at some lysine residues and a high frequency of spindle defects and chromosome missegregation in mouse and porcine oocytes [40,54,55]. Inadequate histone deacetylation during mouse oocyte meiosis may cause aneuploidy and embryo death. On the other hand, old mouse oocytes have higher levels of acetylation at H4K8 and H4K12 when compared to that of young oocytes [40]. Similar to old mouse oocytes, it has been reported that defective deacetylation of H4K12 in human oocytes is associated with advanced maternal age as well as chromosome misalignment [42]. In summary, inadequate histone deacetylation during mammalian oocyte meiosis leads to aneuploidy. Old female oocytes display defects in deacetylation, accompanied with high frequency of aneuploidy. Thus, inadequate histone deacetylation might be the underlying mechanism of oocyte quality decline with increased maternal age.

4 Disruption of epigenetic regulation in oocytes of advanced maternal age

Transcript profiles of mouse old oocytes have been determined on a global scale by using microarray techniques [56,57]. Hamatani *et al.* [57] compared the mRNA expression profiles of MII oocytes from young (5–6 weeks old) C57BL/6 females with those of aged (42–45 weeks old) C57BL/6 females. Based on microarray analysis, the authors found that of approximately 11000 genes whose transcripts were detected in MII oocytes about 5% (530) were affected by maternal aging. Interestingly, among the disrupted gene transcripts some were involved in epigenetic modification. For example, both oocyte-specific and somatic forms of maintenance DNA methyltransferase (Dnmt1o and Dnmt1s, respectively) [58] were downregulated in old oocytes, while this gene is essential for the maintenance of DNA methylation imprints during preimplantation development [59,60]. Dnmt-associated protein-1 (Dmap1) was also downregulated. This gene mediates distinct preimplantation epigenetic reprogramming processes [61]. Dnmt3L was downregulated in old oocytes whereas the level of *de novo* methyltransferase Dnmt3b transcript was upregulated. A different strain of mice (B6D2F1) at an older age (66 weeks) was used in another microarray-based gene expression study, and many of the same changes were detected, including Dnmt3a and Dmap1 [56]. Thus, it is suggested that DNA methylation might be potentially influenced in old oocytes and/or during postfertilization development.

Besides on mRNA levels, expression profiles of various Dnmt proteins have also been investigated in old oocytes. Most recently, the protein expression levels of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L in old mouse oocytes were shown to be significantly lower than in young oocytes [31]. In addition, through immunostaining coupled with confocal microscopy, Zhang *et al.* [62] examined the effects of maternal age on the expression profiles of various Dnmt proteins during meiosis by using mouse oocytes from young (7–8 weeks) and old (40–47 weeks) Kunming females. The authors observed that maternal aging did not affect localization of Dnmt1 in oocyts, and this protein was observed in the cytoplasm during maturation regardless of maternal age. However, Dnmt3a and Dnmt3b were detected only in the cytoplasm and both were absent in the cytoplasm around chromosome-cytoplasm trafficking in old oocytes. Moreover, Dnmt3L was also detected in abnormal localization in old oocytes [62]. Control of subcellular protein localization is a common theme for DNMT3a and DNMT3b, as proteins

were seen in the nucleus only when methylation was occurring [63]. It is concluded that maternal aging affects the cytoplasm-to-nuclear trafficking of DNA methyltransferases and thus protein methylation in mouse oocytes. Therefore, DNA methylation might be altered in old oocytes, and maternal age affects Dnmts protein expression and localization.

Collectively, not only the expression on mRNA and protein levels of Dnmts was adversely affected by maternal aging, but also the localization was adversely affected in oocytes. These changes may lead to dysfunction of Dnmts, which catalyze DNA methylation. Thus DNA methylation may be susceptible to disruption by maternal aging. As discussed above, acquisition and maintenance of DNA methylation are adversely affected in embryos during the preimplantation period other than in embryos which are capable of developing to mid-gestation. It should be noted that (i) only global methylation profiles have been determined during preimplantation development, while DNA methylation patterns of individual genes are unclear; (ii) the number of examined imprinted genes is very limited among nearly one hundred known imprinted genes [64]; (iii) only live embryos were examined at mid-gestation. Finally, it is also possible that although defects in expression and localization of Dnmts in old oocyte are observed, such aberration is still not enough to disturb acquisition and maintenance of DNA methylation.

5 Concluding remarks

Oocyte quality declines during the aging process relative to postovulatory aging as well as advanced maternal age. During oocyte aging a series of epigenetic changes take place. The defects of epigenetic modifications in aged and old oocytes suggest that epigenetic mechanism may contribute to oocyte aging.

Although oocyte aging related to postovulatory aging and advanced maternal age are two different physiological processes, they both share many similar epigenetic changes, especially alterations of acetylation at histone lysine residues.

The acetylation levels of some histone lysine residues are clearly changed when the aging process is artificially delayed or accelerated, further confirming that the oocyte aging process is correlated to changes in histone acetylation at lysine residues. Therefore, this information offers the possibility to develop strategies to delay oocyte aging process through adjusting the histone acetylation levels.

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