



# Epigenetic modifications and reprogramming in paternal pronucleus: sperm, preimplantation embryo, and beyond

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**Abstract** Pronuclear/zygotic stage is the very first stage of life. In this period, paternal pronucleus undergoes massive chromatin remodeling called “paternal reprogramming” including protamine-histone replacement and subsequent acquisition of epigenetic modifications. Although these consecutive events are required for the initiation of maternal-zygotic transition, the precise role of paternal reprogramming and its effect on subsequent embryonic development has been largely unknown to date. Recently, various new techniques, especially next-generation sequencing (NGS) and RNAi microinjection contribute to unveil the epigenetic transition from both paternal and maternal to early preimplantation embryos, suggesting not only the simple transcriptional regulation by transcription factors but also dynamic structural alteration of chromatin to initiate the wave of zygotic gene transcription. This review summarizes such recent progress for understanding the epigenetic transition in sperm and preimplantation embryos, and further argue about its transgenerational effect.

**Keywords** Protamine · Histone · Epigenetic modification · Fertilization · Pronucleus · Preimplantation development · Zygotic gene activation · Transgenerational epigenetic inheritance

## Abbreviations

ATAC-seq	Assay for transposase-accessible chromatin with high throughput sequencing
CHX	Cycloheximide
ChIP-seq	Chromatin immunoprecipitation sequencing
DHS	DNase I hypersensitivity site
dKO	Double knock-out
DRB	5,6-dichlorobenzimidazole riboside
FISH	Fluorescent in situ hybridization
FRAP	Fluorescent recovery after photobleaching
GFP	Green fluorescent protein
ICR	Imprinting control region
iPSC	Induced pluripotent stem cell
MERVL	Murine endogenous retrovirus-L
MNase	Micrococcal nuclease
NGS	Next-generation sequencing
NPM	Nucleoplasm
PN	Pronuclear stage
RNA-seq	RNA sequencing
ROSI	Round spermatid injection
SCNT	Somatic cell nuclear transfer
ZGA	Zygotic gene activation

## Introduction

The fertilized egg provides a unique environment where two nuclei, derived from either an oocyte or a sperm, exist independently in the same cytoplasm. Although these two nuclei both contain parental haploid genomes, their chromatin states are markedly different mainly because the paternal chromatin undergoes massive epigenetic reprogramming by protamine-histone exchange. Histones newly incorporated into the paternal chromatin are predominantly supplied from maternal stock, and they eventually acquire

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new modifications such as methylation. Most of the modifications are asymmetric between paternal and maternal pronuclei. Due to the asymmetric chromatin states, several nuclear events in each pronucleus occurs separately until the first fusion. This review first summarizes these asymmetric epigenetic events occurring in mouse zygotes, and later argues their physiological significance such as transcriptional regulation. In addition, we will also describe sperm-mediated epigenetic transgenerational inheritance, which has recently been attracting attention in the field.

## The regulation of minor zygotic gene regulation

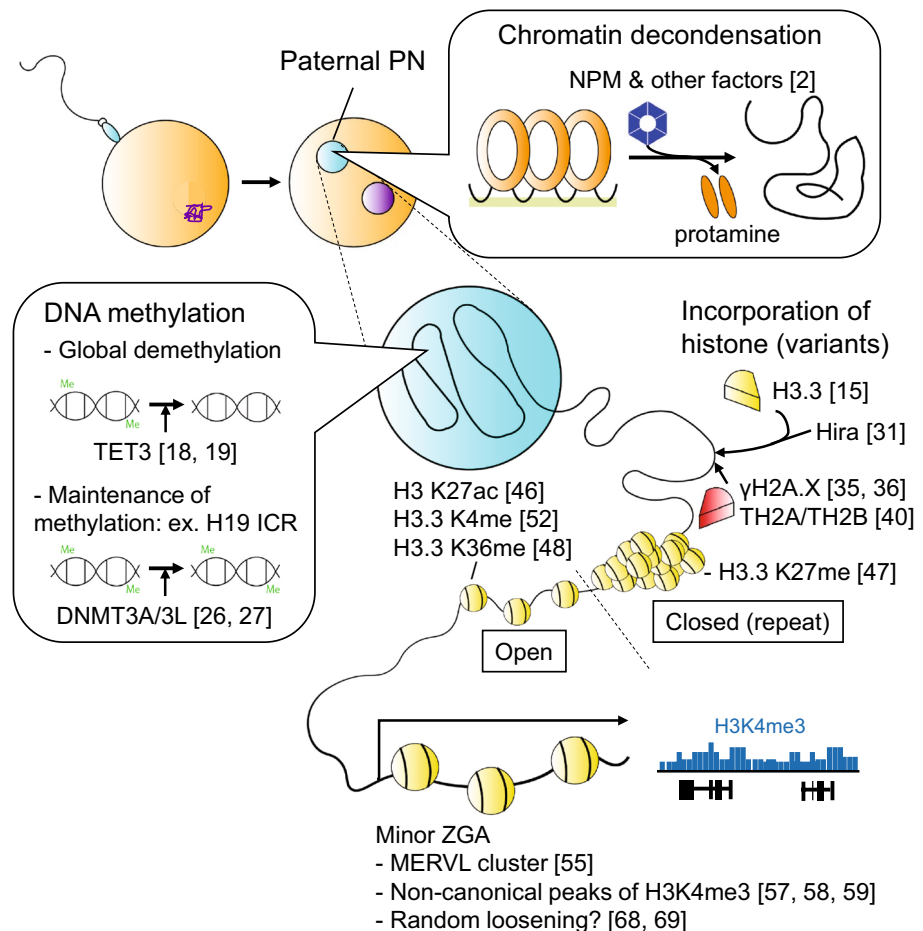
### Chromatin state of zygotic pronuclei

In many animal species, protamine incorporation during spermiogenesis is an essential step for proper chromatin condensation and the full fertility of sperm. In frogs, once the sperm has entered the oocyte, protamines are quickly removed from the paternal chromatin by its chaperon nucleoplasm (NPM), which is stored in oocytes [1]. Similar observations in mice shows that Npm2-deficient

oocytes fail to stimulate sperm chromatin decondensation [2] (Fig. 1). Protamine incorporation and removal are apparently a critical initiator for efficient paternal epigenetic reprogramming, as the efficiency of acquiring pups by round spermatid injection (ROSI) is significantly lower than by injecting mature spermatozoa [3]. This observation also implies that protamine-containing spermatozoa are more capable of being reprogrammed after fertilization. The details of the substantial differences between spermatids and spermatozoa in regard to efficient reprogramming have barely begun to be elucidated. So far, a study has demonstrated a failure of active DNA demethylation in ROSI-derived mouse zygotes [4]. A comparison between sperm- and spermatid-derived frog embryos recently revealed that proper epigenetic alterations such as histone methylations in sperm affect the transcription of a set of developmental genes in early embryos, implying the transgenerational effect [5].

After protamine removal, the paternal genome gradually acquires new histones, which are provided from maternal stock, and establishes a new epigenetic state, resulting in the epigenetic asymmetry between paternal and maternal pronuclei [6–15] (Fig. 1). In contrast, maternal chromatin

**Fig. 1** Schematic depiction of “paternal reprogramming”. After fertilization, paternal pronucleus undergoes massive chromatin remodeling including protamine removal, acquisition of new histones and their modifications, and DNA demethylation. These events subsequently trigger minor ZGA. Reference numbers are indicated



basically maintains its modification patterns, although it is highly possible that small amounts of maternal histones are also subjected to be exchanged and acquired new modifications [16].

Among the most obvious asymmetric features between paternal and maternal pronuclei are their DNA methylation and histone 3 lysine 9 (H3K9) methylation state [7, 9, 10]. Genome-wide DNA demethylation occurs predominantly in paternal pronuclei without cell division [7], suggesting that paternal DNA demethylation is active rather than passive in this stage. In 2009, TET family proteins were identified as the first active DNA demethylases [17], and one of the family member TET3 is responsible for paternal DNA demethylation in zygotes [18, 19]. In contrast, H3K9 methylation is exclusively deposited in the maternal pronucleus [20], and is recognized by Dppa3/PGC7/Stella, which prohibit TET3 from demethylating the maternal genome by physical protection [21]. However, maternal DNA to a lesser extent is also targeted for the demethylation by TET3 [22, 23]. In maternal TET3-deficient zygotes, high level of DNA methylation and low level of DNA hydroxymethylation (an oxidation product of 5'-methylcytosine mediated by TET3) are observed in paternal pronuclei concomitant with severely impaired fecundity [18, 21]. Surprisingly, however, the transfer of a male pronucleus harvested from a maternal TET3-deficient zygote to a wild-type recipient demonstrated a comparable fecundity to the control, strongly suggesting that paternal DNA oxidation is dispensable for mouse development [24].

While the majority of the paternal genome is subjected to TET3-dependent global DNA demethylation, DNA methylation in imprinting control regions (ICRs) is faithfully maintained, and several factors including Dppa3/PGC7/Stella, TRIM28, and DNMT1 help to maintain ICR methylation in preimplantation embryos [20, 25, 26]. Interestingly, a recent study of the H19 ICR, where methylation is established by DNMT3A/3L in prospermatogonia and maintained on the paternal allele following fertilization, demonstrated that DNA methylation is re-established after fertilization by maternal DNMT3A/3L, suggesting that the continuous DNA methylation of the paternal H19 ICR is not simply due to the escape from DNA demethylation, and that gametic and zygotic DNA methylations are separable events [27] (Fig. 1).

Paternal histone incorporation in the early zygotic stage is independent of DNA replication. Consistent with the fact, one histone H3 variants called H3.3, whose chromatin incorporation is known to be replication-independent [28], is predominantly incorporated into paternal chromatin in this stage [15, 29]. In contrast, the canonical replication-dependent histones H3.1 and H3.2 do not appear until later zygotic stages even when they are overexpressed in zygotes [30]. Although there are only five amino acid differences

between H3.3 and H3.1 (at positions 31, 87, 89, 90 and 96), these differences are critical for recognition by histone chaperones. The preferential incorporation of H3.3 into paternal chromatin is regulated by Hira, an H3.3-specific histone chaperon [31], which generally ensures the deposition to the transcriptionally active regions [28]. H3.3 is also known to localize to telomeres through the mediation of another H3.3 chaperons called ATRX. However, at least in *Drosophila*, ATRX-dependent H3.3 deposition is not involved in paternal chromatin assembly [32]. Surprisingly, knockdown of Hira in mouse oocytes causes not only deficiency in the incorporation of H3.3, but also a depletion of nucleosome incorporation in paternal pronuclei and subsequently, a failure in nuclear pore complex formation [33, 34]. These results indicate that the incorporation of H3.3 is a scaffold not only for nucleosome formation, but also for the entire pronuclear structure [33].

Histone H2A consists of the most diverse variants to be found among histone families. Among these variants, H2A.X is reported to be highly enriched and  $\gamma$ -phosphorylated ( $=\gamma$ H2A.X) in the paternal pronucleus in the absence of aberrant DNA damage in early zygotic stages [35, 36]. Deposition of  $\gamma$ H2A.X is more prominent in the paternal than the maternal pronucleus, which may reflect the fact that this constitutes newly assembled chromatin that needs to achieve a proper nucleosomal configuration [35]. After the first cell division,  $\gamma$ H2A.X quickly decreases and is hardly detectable in the interphase of 2-cell embryos, whereas the H2A.X protein is continuously retained after the 2-cell stages [37]. H2A.Z, another major H2A variant, has been implicated in a functional association with H3.3, forming unstable nucleosomes that ensure transcription [38, 39]. Unlike the quick and preferential incorporation of H3.3 in the paternal pronucleus, however, H2A.Z is hardly detectable in either male and female pronuclei, and this low level persists until the 4-cell stages [16], refuting a cooperative role between H3.3 and H2A.Z in the zygote.

Other histone variants that have recently attracted great recent interest are TH2A and TH2B, which were originally identified as testis-specific H2A/H2B variants [40]. Their expression has also been demonstrated in oocytes and preimplantation embryos [40]. *Th2a* and *Th2b* genes are both located at the largest histone gene cluster, and share the same promoter [41]. Their mRNAs and proteins are abundantly expressed in spermatozoa, mature oocytes, and 1-cell embryos, and decrease after the 2-cell stage upon cell division [40]. *Th2a/Th2b*-double knockout (dKO) oocytes exhibits impaired preimplantation development after fertilization with wild-type sperm due to a failure of paternal genome activation during the zygotic period, and testis from the dKO males exhibit impaired spermatogenesis due to, respectively, the altered release of cohesins and

incorporation of transition proteins [40, 42]. In contrast, oocytes obtained from TH2B-less females can develop without any noticeable defects after fertilization, and depletion of TH2B maintains normal spermatogenesis although functional compensation by canonical H2B, which would be expected to be replaced by TH2B in the early stage of spermatogenesis [43], suggesting that the phenotype of the *Th2a/Th2b* dKO mice is either a single effect of TH2A, or their compensatory effect. Importantly, exogenous expression of TH2A/TH2B together with phosphorylated NPM significantly enhances iPSC generation from mouse embryonic fibroblasts, demonstrating that TH2A and TH2B are embryonic histones that contribute to reprogramming [40].

Recent in vitro study successfully reconstituted mitotic chromatids using frog sperm as a substrate, requiring only six defined factors, including histone chaperones [44]. Although actual transition from sperm to embryonic chromatin in the zygote seems more complicated and involves many other factors, this finding provided a fundamental aspect of understanding the molecular basis for the protamine-histone transition. Related graphical illustrations are presented in Fig. 1.

### Analysis of transcription pattern in minor ZGA

Mammalian preimplantation development is initiated by replacing maternal transcripts with zygotic ones, a process called the maternal-to-zygotic transition. Consistent with the asymmetric chromatin states of paternal and maternal pronuclei, transcriptional regulation in the late 1-cell stage, designated as minor zygotic gene activation (ZGA), is also known to be asymmetric. It is demonstrated that the initiation of minor ZGA in the paternal pronucleus occurs a few hours earlier than in the maternal pronucleus [45]. For decades, however, exactly how this asymmetric transcriptional regulation was established was largely unknown. It was thought that paternal chromatin is more susceptible to transcriptional activation than maternal chromatin, presumably due to the lesser extent of suppressive histone marks such as H3K9 and H3K27 methylations [13]. Indeed, active histone marks including H3.3 and H3K27 acetylation clearly exhibit a preferential deposition in the late paternal pronucleus [46]. H3K4 methylation, the most representative active histone mark, is also established in the paternal pronucleus in the middle to late zygotic stage [9].

The importance of histone methylation has been widely verified by employing loss-of-function studies with histone lysine-arginine (K-R) mutants. As in the following examples, overexpressing exogenous histones carrying K-R mutation in potentially methylated lysine residues successfully demonstrated not only the critical role of methylation of specific lysine residues, but also a distinct, incorporation-dependent role of H3.3 in early preimplantation

development. For instance, exogenous expression of H3.3-K27R-GFP but not H3.1-K27R-GFP mRNA in zygotes compromised their preimplantation development concomitant with altered pericentromeric transcription and defects in chromosome segregation, suggesting an important role of H3.3 K27 methylation in pericentromeric silencing [47]. Similarly, injection of morpholino against H3.3 into zygote induced developmental arrest of preimplantation embryos, which could be rescued by the overexpression of wild-type H3.3 but not wild-type H3.1 or H3.3 K36R [48]. Although it remains unknown whether this phenotype was related to transcriptional elongation, it suggests a specific role of K36 methylated H3.3 at this stage [48]. More recently, a lysine-methionine (K-M) mutation in histone H3 was identified from pediatric glioma patients [49, 50]. In studies of cells carrying the K-M mutation, not only the exogenous mutant H3.3 but also endogenous H3 proteins, including canonical forms, were unmethylated due to the inhibition of the enzymes responsible for methylating the mutated sites [51]. By utilizing the K-M mutation instead of K-R mutation, H3.3-K4 methylation was found to be involved preferentially in the minor ZGA preferentially in paternal genome, as well as the subsequent developmental arrest [52]. This work further demonstrated that MLL3/4 is responsible for the H3.3-K4 methylation concomitant with H3K27 acetylation during the zygotic period, implying a role of enhancer activation in the paternal minor ZGA [52, 53]. However, the question of dependence for enhancer at the zygotic stage remains open as a previous study reported that transcription in the paternal pronucleus occurs independently from the enhancer [54]. More direct and concrete evidence such as Chromatin immunoprecipitation sequencing (ChIP-seq) analyses is required to argue the involvement of enhancers in zygotic transcription (ChIP-seq analysis in zygote will be mentioned in the later section).

Recently, Assay for Transposase-Accessible Chromatin with high throughput sequencing (ATAC-seq) has been applied for further qualitative and quantitative characterization of the minor ZGA, and it was found that open chromatin regions are formed as clusters in several regions, especially near the murine endogenous retrovirus-L (MERVL) gene [55]. This study also confirmed that these ATAC-seq peaks disappear with  $\alpha$ -amanitin (an inhibitor of transcription) treatment, suggesting that they are transcription-dependent, although this study did not differentiate between paternal and maternal chromatin [55]. Similarly, genome-wide NGS study of preimplantation embryos using a low-input DNase I hypersensitivity site (DHS) mapping demonstrated that the DHS profile of the paternal genome becomes similar to that of maternal genome as early as at pronuclear stage (PN) 3, approximately 7.5 h after fertilization [56]. As PN3 is the time of the minor ZGA initiation in paternal pronuclei, this observation indicated that the

paternal reprogramming for subsequent zygotic events is almost completed by this stage.

Recently, ChIP-seq was successfully adapted to allow the analysis of extremely small number of cells (~a few hundreds), and this technical development enables to directly examine the genomic localization of several histone modifications in oocytes, zygotes, and early preimplantation embryos [57–59]. Unexpectedly, these results consistently demonstrated the “non-canonical” pattern of H3K4me<sub>3</sub>, which is enriched at low levels across large genomic region (~10 kbp), and distant from transcription start sites [57–60]. This unusual epigenetic pattern is conserved from oocytes to early 2-stage embryos mainly in maternal chromatin, but paternal chromatin also exhibits similar broad distribution of H3K4me<sub>3</sub> [59]. Interestingly, genes associated with the non-canonical H3K4me<sub>3</sub> are related with oocyte growth and major ZGA, suggesting the role as an inherited epigenetic memory of the transcriptional state [58, 59]. However, it is also shown that the depletion of H3K4me<sub>3</sub> in oocytes results in increased transcriptional activation, implying the role of H3K4me<sub>3</sub> in transcriptional silencing [59]. Collectively, these genome-wide sequencing analyses clarify the transcriptional regulation of minor ZGA more unique and mysterious, thus further work is required to fully understand the precise molecular mechanisms [60]. Related graphical illustrations are presented in Fig. 1.

### Putative chromatin regulators in minor ZGA

Despite such accumulation of epigenetic knowledges of the pronuclear chromatin state, the physiological importance and molecules that control the minor ZGA is not yet fully understood. In addition to MLL3/4 (mentioned previously), TIF1 $\alpha$  has also been reported to modulate zygotic gene expression through BRG1/SNF2 in both male and female pronuclei [29]. The DHS study also identified several transcription factors, whose promoter regions are open as early as PN3 [56]. Interestingly, in these cases, knockdown of their identified factors (i.e. MLL3/4, TIF1 $\alpha$ , and Nfya) by the siRNA injection induced developmental arrest a few stages later than their actually expressed stages, whereas complete block of transcription by  $\alpha$ -amanitin treatment stops the development at the next embryonic stage. This implies at least two possibilities; (1) that several molecules are involved in the minor ZGA, and that inhibition of single molecule is thus insufficient to cause immediate arrest, or (2) siRNA injection is inadequate to completely suppress the target genes due to the abundance of maternally inherited mRNA/protein or limited timing of injection. Similar observation was reported in the case of somatic cell nuclear transfer (SCNT) [61]. In this study, erasure of H3K9me<sub>3</sub> by exogenous expression of the demethylase significantly increases the efficiency of SCNT, and 49 genes

were identified as their overexpression possibly improves the efficiency. One of the top-ranking genes, Zscan4d, however, failed to increase the developmental rate of SCNT embryos when it was administered in the embryos, also suggesting that complicated gene networks rather than a sole specific factor is responsible for proper preimplantation development [61].

Transcription during the minor ZGA is subtle but substantial. However, there is little translation from these transcripts [62], suggesting the possibilities that the role of minor ZGA is not to express functional proteins, or only a few transcripts, which are critical for subsequent embryonic development, are selectively translated. Later, genome-wide transcriptome analyses in preimplantation embryos by microarray identified substantial change of transcription in 1-cell embryo, which was dramatically altered by cycloheximide (CHX: an inhibitor of translation) and  $\alpha$ -amanitin, whereas aphidicolin (an inhibitor of DNA replication) exhibited a little effect [63]. Interestingly, there was a remarkable overlap of transcripts, which expression were commonly affected by CHX and  $\alpha$ -amanitin, indicating that translation of maternally stored transcripts or translation of ZGA transcripts themselves is essential for the minor ZGA [64]. Further, large-scale transcriptome analysis by RNA-seq identified five transcription factors expressed in 1-cell but not in parthenogenic embryos, which possibly involved in transcriptional initiation in fertilized oocytes [65].

Interestingly, another RNA-seq analysis demonstrated that during the minor ZGA, intergenic regions are extensively expressed, and thousands of genes are transcribed at comparably low levels accompanied by inefficient mRNA splicing [66]. However, inhibition of RNA polymerase II by DRB (5,6-dichlorobenzimidazole riboside) from 1-cell to early 2-cell stages results in the developmental arrest at the 2-cell stage, suggesting that transcription at the 1-cell stages is essential for further development. These confusing observations may be interpreted as a suggestion that the transcription itself but not the products (i.e. the mRNAs or proteins) possesses a role in early embryonic development such as transcription-induced alteration of chromatin structure including the nucleosome re-positioning, histone replacement, and histone modifications required for the major ZGA at the 2-cell stage [66]. Further analysis by ATAC-seq at the early 2-cell stage corresponding to the minor ZGA also demonstrated noisy peaks [55]. However, the ATAC-seq demonstrated that open chromatin is enriched in repeat regions, especially MERVL clusters, which are highly transcribed during the minor ZGA [55], which also suggests that an open chromatin structure is one of the key factors for transactivation.

Interestingly, this idea seems to be supported by another line of evidence. There is a rare transient cell population within cultured mouse ES cells, which express high levels



of transcripts found in 2-cell embryos including MERVL, designated as 2 cell-like cells (2CLCs) [67]. The 2CLCs lack the expression of pluripotent factors such as Oct4 and Sox2, and have acquired the ability to contribute to both embryonic and extraembryonic tissues, suggesting that they functionally resemble 2-cell embryos. Transcriptome analysis further identified that the knockdown of heterochromatic factors such as G9a, KAP1, and HDACs ensure the conversion from ES cells to 2CLCs, implying the link between loosened chromatin structure and 2C-like state, although it is not clear whether this is a global or target gene-specific effects [67]. Later, knockdown of CAF-1, a somatic histone H3 chaperon, in ES cells was also demonstrated to ensure the conversion to 2CLC [68], supporting the contribution of structural modulation of chromatin rather than gene-specific transcriptional regulation to the acquisition of 2C-like state. Consistently, an examination of chromatin stability in early embryos by fluorescent recovery after photobleaching (FRAP) demonstrate that highly relaxed chromatin structure is involved in totipotency of 1-cell embryos, and this looseness is lost upon differentiation during preimplantation development [69]. Further analyses and combined knowledge of early embryos and 2CLC may enable the acquisition of totipotent cells in vitro. Related graphical illustrations are presented in Fig. 1.

### Sperm-retained histones: where are they?

As described above, it can be easily imagined that the unique chromatin remodeling in the paternal pronucleus is tightly associated with sperm chromatin structure. In fact, this hypothesis is strongly supported by a recent study on *Xenopus* demonstrating the critical role of sperm-retained histones in the regulation of gene transcription in embryos [5]. On the other hand, very little histones are retained in sperm chromatin after histone-protamine replacement, although the extent varies between animal species [70]. Thus, the major question that emerges is whether histones are retained precisely within the sperm genome, and if so, where. This is particularly germane when we interpret the effects of sperm-retained histones on subsequent generations, as described in the next section.

Retention of histones in spermatozoa/mature sperm has been described in several mammalian species including human and mouse; approximately 10–15% of histones in human and 1% in mouse are retained as a heterogeneous mixture of protamines and other basic proteins [70–72]. In human sperm, the discrete structures of nucleohistones and nucleoprotamines have been demonstrated, and unique toroid formation of nucleoprotamin was later suggested [71, 73]. Immunohistochemical analyses of sperm demonstrate the accumulation of histones in DAPI-dense regions,

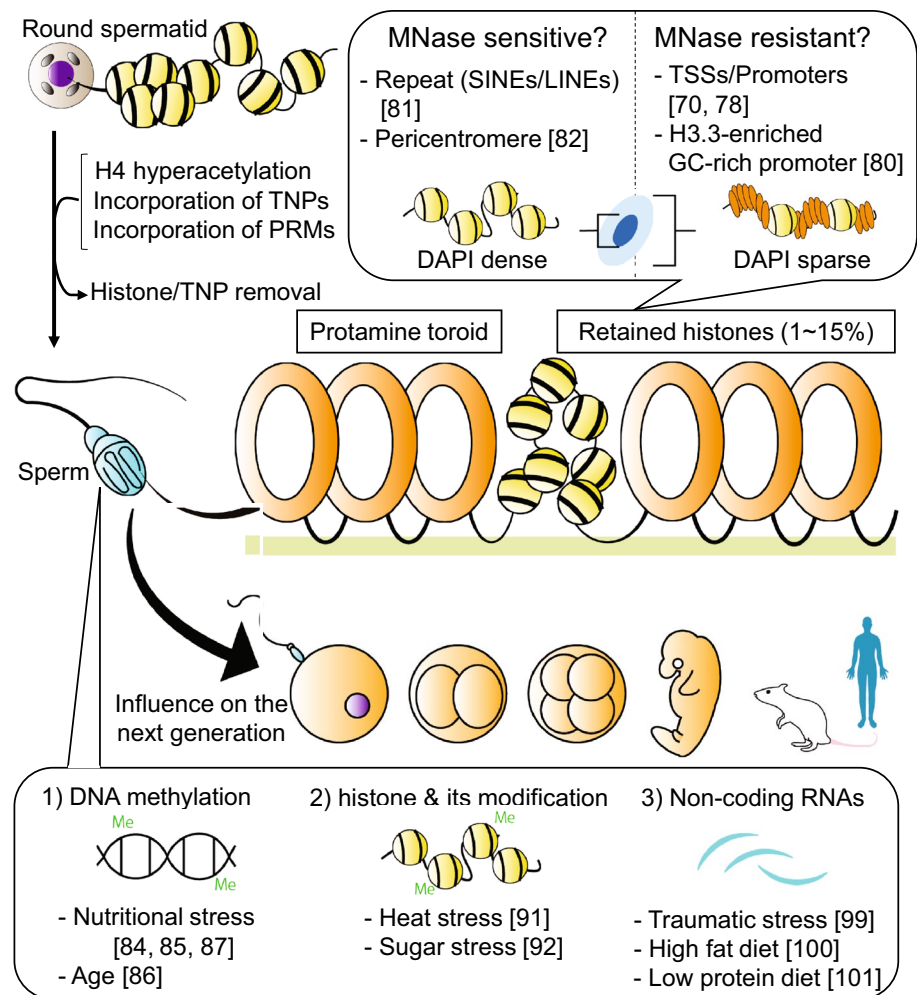
which correspond to the major satellite and pericentromeric heterochromatin demonstrated by fluorescent in situ hybridization (FISH), concomitant with hyperacetylated H4 in mouse and H3K9 trimethylation in human, suggesting that the preferential retention of sperm histones in pericentromere/repeat sequences including telomeres [74–77].

Inconsistent with these observations, however, genome-wide analyses of human sperm genome by NGS demonstrated that nucleosomes are preferentially retained in gene coding regions [78]. Interestingly, the enrichment of nucleosomes are significantly accumulated in developmental genes rather than gene-poor regions, although it is technically difficult to access pericentromere/repeat regions by NGS [70, 78, 79]. Subsequent studies of mouse sperm also revealed the enrichment of sperm nucleosomes, especially H3.3-containing nucleosomes, in promoters containing GC-rich sequences with lower DNA methylation [80]. These results also imply the participation of sperm epigenome to the transgenerational effects, especially transcriptional regulation. In contrast, similar NGS analyses performed in two other studies found that sperm nucleosomes are enriched in gene-poor regions including the centromere and repeat regions, which was further supported by the agreement of immunostaining of histones [81, 82]. This controversy may be caused by the complex sensitivity to micrococcal nuclease (MNase) treatment [82]. In addition, it has also been pointed out that inappropriate sequence data analysis may have influenced on the interpretation [83]. Further multi-dimensional investigations are required to fully understand the sperm chromatin structure, and the regularity of histone-protamine replacement during spermiogenesis. Related graphical illustrations are presented in Fig. 2.

### Transgenerational effects of paternal epigenome

The transgenerational inheritance of sperm epigenetic state has been receiving a large amount of attention in the field, despite the confusing situation regarding sperm chromatin structure described above. In 2010, influence of paternal diet on the gene expression and metabolism in the progeny, also designated as paternal-diet-induced intergenerational metabolic reprogramming (IGMR), was first demonstrated in rodent, strongly suggesting the non-genetic intergenerational transmission of metabolic sequelae of paternal diet through sperm [84, 85]. Regarding the molecular mechanisms, at least three possible causative factors so far have been claimed as responsible for sperm-mediated epigenetic inheritance: DNA methylation, histones (and their modifications), and RNAs. Among these, DNA methylation has been regarded as the most promising factor based on the high level of DNA methylation in sperm, as well as

**Fig. 2** Schematic depiction of “paternal transgenerational effect”. During spermiogenesis, sperm histones are replaced by PRMs, while 1–15% of histones are still retained in sperm chromatin. A current question is whether these histones are retained in sperm genome, and whether they have substantial roles in the next generation. Other than histones, DNA methylation and non-coding RNAs are possibly transmitted from sperm to offspring, and their involvement in the next generations are also suggested. Reference numbers are indicated



the more minor effect of histone-protamine replacement. Indeed, clinical cohort studies have indicated relationship between the age-associated alteration of sperm DNA methylation and the risk of certain neuropsychiatric conditions [86]. Nutritional stress also affects DNA methylation in sperm and subsequently the health condition of the offspring [87]. In one case, altered DNA methylation status in *Olf151* gene is demonstrated to be transferred to subsequent generations as “a paternal fear memory”, an odor (acetophenone)-linked fear conditioning [88]. *Olf151* is a known odorant receptor activated by acetophenone. In this study, it was demonstrated that progenies of the fear-conditioned male by acetophenone exhibit traumatic response, when they sense acetophenone without actual fear experience. This phenomenon is linked to the CpG hypomethylation in the *Olf151* gene, which is inherited from the fear-conditioned male through sperm [88]. Moreover, in zebrafish, the paternal DNA methylation pattern rather than the maternal pattern is maintained throughout early embryogenesis, and is critical for reprogramming during ZGA [89, 90].

The second factor, histone modifications, may be involved in sperm-derived transgenerational effects. In 2011, a study in *Drosophila* demonstrated that the stress-induced disruption of heterochromatin in gametes was transferred to subsequent generations, implying the importance of chromatin structure for epigenetic inheritance [91]. Furthermore, an IGMR study in *Drosophila* successfully demonstrated the link between the altered gene expression in IGMR and Polycomb/heterochromatin machinery [92]. In *C. elegans*, increased longevity observed in *Wdr5*-deficient worm can be inherited by their wild-type progeny, and multiple chromatin-modifying factors related to H3K4 and H3K9 methylations regulate transgenerational effects on fertility [93, 94]. In rodent, wild-type mouse progeny derived from sperm carrying an *LSD1*-overexpression allele exhibited morphological abnormalities and reduced survival rates [95]. However, there were no obvious alterations of major histone modifications in the abnormal wild-type progeny, suggesting that at least in this case, impaired histone modifications were not responsible for the sperm-mediated transgenerational abnormalities [95].

The third factor, RNA, has been in the limelight for the last few years. Initial report was demonstrated in RDE-4 deficient *C. elegans*, which are sensitive to flock house virus infections due to the lack of small RNA biogenesis. Surprisingly, RDE-4 deficient *C. elegans* can acquire an anti-viral response through viRNAs transferred from a wild-type ancestor to the progenies through their sperm [96]. Subsequently studies in *C. elegans* further demonstrated that the transgenerational inheritance of starvation-induced developmental arrest is also mediated by small RNA-induced gene silencing [97]. The lifespan in the F3 offspring of starved animals was also increased, suggesting that calorie restriction-induced longevity can become a transgenerational memory [97, 98]. Similarly, microRNA in mice can be a vector transmitting the effects of traumatic stress experienced by the father from the sperm to the progeny, and this can affect children's behavior [99]. More recently, fragmented transfer RNAs produced and supplied by the epididymal epithelium were found to be the causative agent of the transgenerational effects of diet-induced nutritional stress [100, 101]. Importantly, these effects in mice could be reconstituted experimentally by the injection of RNA harvested from the sperm of stress-exposed fathers, although how particular transfer RNAs influence the metabolism of the offspring, and how the abnormal condition persists over the long term both remain questions to be answered. Related graphical illustrations are presented in Fig. 2.

## Conclusion

Advancements in technologies for epigenetic studies, especially NGS and technical improvement in analyzing small-scale samples, have made study of the dynamic epigenetic transitions from gamete to embryos more accessible than ever. Genome-wide sequencing analyses in oocytes and early preimplantation embryos in particular have provided critical information for understanding reprogramming at the molecular level. However, this has also led to some confusing findings regarding sperm chromatin structure, a comprehensive understanding of which seems essential for further investigation and for understanding the transgenerational inheritance of the paternal epigenome, as well as for subsequent clinical and bioengineering applications. It is also obvious that a better understanding of paternal reprogramming will provide useful information for the technical improvement of regenerative medicine and somatic cell nuclear transfer, in the latter of which the donor nucleus is reprogrammed in the ooplasm, as similar molecules may participate in both cases.

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