



Not just heads and tails: The complexity of the sperm epigenome

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Transgenerational inheritance requires mechanisms by which epigenetic information is transferred via gametes. Canonical thought holds that mammalian sperm chromatin would be incapable of carrying epigenetic information as post-translational modifications of histones because of their replacement with protamine proteins. Furthermore, compaction of the sperm genome would hinder DNA accessibility of proteins involved in transcriptional regulation and genome architecture. In this Minireview, we delineate the paternal chromatin remodeling events during spermatogenesis and fertilization. Sperm chromatin is epigenetically modified at various time points throughout its development. This allows for the addition of environment-specific modifications that can be passed from parents to offspring.

Background

Sperm chromatin in mammals is thought to be structurally distinct from that of somatic cells. In somatic cells, chromatin contains epigenetic information in the form of DNA methylation and post-translational modifications on histones, information that is thought to influence chromosome architecture and gene expression. Methylation at the 5-position of a cytosine base pair is an epigenetic mark predominantly located at cytosine–phosphate–guanine (CpG)² dinucleotides and is associated with gene silencing when at the promoter and active transcription when in the gene body (1). These site-specific marks are established in sperm by DNA methyltransferases (DNMT) and ten-eleven translocation methylcytosine dioxygenase (TET) proteins performing active DNA methylation and demethylation, respectively (2). Post-translational modifications in histones come primarily in the form of methylation or

acetylation of amino acids in the N-terminal tails of these proteins, which in turn affect the structure of chromatin (3). These modifications correlate with either transcriptionally active or silenced regions of the genome.

One considerable difference between sperm and all other cells is its size. Although mammalian sperm possess half the DNA than is contained in a typical somatic cell, the nucleus has 40-fold less volume (4). A comparison of mouse sperm nuclei with that of liver cells, taking into account the nuclear volume, density of DNA packaging, and difference in ploidy of these cell types, uncovered a 6-fold compaction of sperm nuclear DNA (4, 5). It has been hypothesized that this compaction is due to the presence of smaller protamine proteins on sperm DNA replacing larger, more complex histone octamers (Fig. 1A) (6). Protamines are basic 50–60–amino acid sequence proteins with DNA-binding domains of 10–11 amino acids. The protamine binds to the major groove of the DNA and neutralizes its phosphate backbone (7). Early studies assumed that the replacement of nucleosomes with protamines is responsible for the tight packaging of DNA in the sperm nucleus; however, certain organisms such as zebrafish (*Danio rerio*) have similarly small, dense sperm nuclei and no protamines (8, 9). Based on these reports, it remains unclear what role protamines play in sperm.

Until recently, it has been thought that the role of sperm in mammals is to deliver the transcriptionally inert paternal DNA, mostly devoid of histones and complexed with protamines, to the egg during fertilization (10, 11). However, recent findings suggest a more conventional view of sperm chromatin, with histones containing typical covalent modifications retained at important genomic sites and a three-dimensional architecture similar to that of somatic cells (Fig. 1B) (12–16). Mammalian sperm may thus be capable of not only carrying epigenetic information, but also passing this information to cells of the early embryo, producing changes that may affect differentiated adult tissues.

Foundations of the epigenetic landscape established in primordial germ cells: Sperm development

Mouse spermatogenesis takes place in three phases, which include self-renewal of spermatogonia through mitosis, followed by meiosis of spermatocytes to form haploid spermatids and transformation of spermatids into spermatozoa by means of spermiogenesis (17). Spermatozoa development begins from primordial germ cells (PGCs) at the base of the emerging allantois in the endoderm of the yolk sac of the mouse embryo (18).

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²The abbreviations used are: CpG, cytosine–phosphate–guanine; ESC, embryonic stem cell; mESC, mouse ESC; DNMT, DNA methyltransferase; TET, ten-eleven translocation methylcytosine dioxygenase; PGC, primordial germ cell; E, embryonic day; 5mC, 5-methylcytosine; miRNA, microRNA; seq, sequencing; ATAC, assay for transposase-accessible chromatin; DHS, DNase I-hypersensitive site; TSS, transcription start site; CTCF, CCCTC-binding factor.

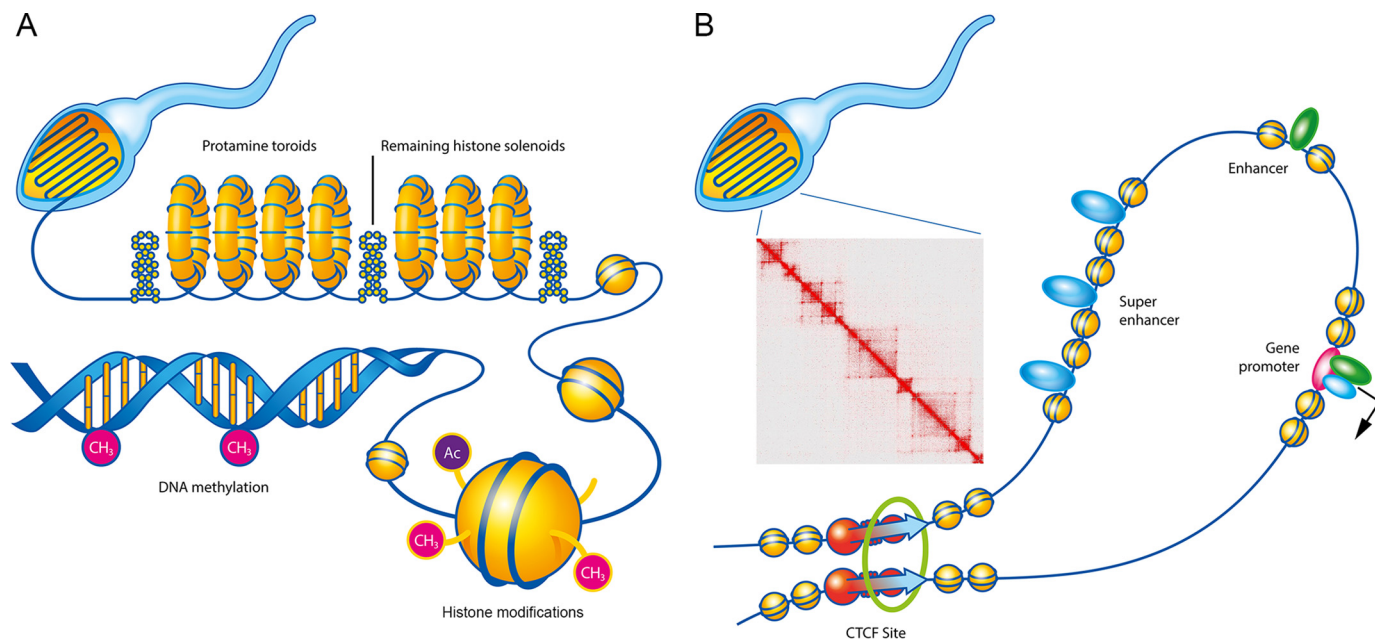


Figure 1. Old and new views of chromatin structure in mature sperm. *A*, most sperm DNA was thought to be packaged in protamine toroids (yellow rings) and remaining histone octameres (yellow spheres). DNA is methylated at 5mC (pink), whereas histones are methylated or acetylated in their N-tails. *B*, recent results suggest the retention of more nucleosomes than previously thought in mouse sperm. These nucleosomes contain most or all histone modifications found in somatic cells (data not shown). Well-positioned nucleosomes flank TSSs and transcription factor-binding sites, including CTCF. Many enhancers and super-enhancers active in embryonic or adult tissues are already specified in the sperm epigenome. CTCF and cohesin mediate long-range chromatin interactions and establish the three-dimensional architecture of the chromatin. This is represented by a Hi-C heatmap displaying sperm chromatin organization into compartments and domains similar to those found in other cell types.

PGCs migrating into the genital ridges between E 7.5 and E13.5 (19) undergo extensive epigenetic reprogramming in the form of global demethylation. PGC methylation level reaches its minimum between E10.5 and E12.5 (20). The process of sexual dimorphic development of the post-migration PGCs into male gametes begins at E13.5 and involves extensive remethylation of sperm DNA. Around day 5 after birth, some prospermatogonia within the basement membrane of the pup's seminiferous tubule resume mitotic division to form primary spermatocytes. Secondary spermatocytes are formed by meiotic division, and they later divide once more to become haploid spermatids. The remaining prospermatogonia not forming spermatids will continue to divide mitotically and produce spermatogonial stem cells, to ensure the continuation of spermatogonia supply. Finally, spermiogenesis involves microtubule growth, tail formation, and the tight packaging of DNA into condensed, protamine-bound chromatin (21).

Chromatin state in mature sperm

Methylation

Mature sperm are highly methylated on a global level as compared with somatic cells (Fig. 1A) (22). This methylation, established by DNMTs during spermatogenesis, is necessary for normal embryo development (23, 24). Several groups have found that patterns of paternal methylation are maintained through the early embryonic stages, suggesting that this methylation continues to serve a regulatory role during embryo development (23–25). Furthermore, several oocyte-derived genes in the developing embryo adopt a methylation pattern similar to that of sperm, namely gaining methylation in genes involved in germline specification (25). It appears that the dense global methylation on sperm is highly gene-specific, which may play a role in directing transcription in the early embryo.

Equally important as site-specific methylation in the sperm genome is the absence of this modification from specific sequences. Sites in sperm that are demethylated despite the dense global methylation levels lie mostly in CpG islands, regions of high CpG density, found mostly near promoters (26).

One comparison study between human sperm and embryonic stem cells (ESCs) found very similar distributions of methylation in each cell type. They found an enrichment of hypomethylated regions at promoters and highly methylated repeat elements (27). This group also found that sperm-specific hypomethylated regions are located within genes related to germ cell development. Another group confirmed these findings in a comparison study with oocytes and early embryos. They found hypomethylated sequences enriched at high-density CpG promoters, enhancers, and exons. Sperm exhibits a high density of methylation at intergenic regions (28). There is evidence of parent-of-origin-specific methylation patterns in the early embryo, with the male sperm contributing primarily differentially methylated sites in intergenic regions (29). DNA methylation is globally erased and re-established both during spermatogenesis and after fertilization, suggesting that 5mC may not be a good candidate, on its own, to carry epigenetic information between generations in mammals. However, evidence of site-specific methylation in mature sperm suggests there must be complementary or alternative pathways by which epigenetic information can be altered and transmitted through the paternal germline. Other candidate mechanisms are discussed below.

Histone modifications

In 1977, Balhorn *et al.* (30) examined histones retained in human and mouse sperm by electrophoretic analyses of HCl-extracted proteins from sperm chromatin. They determined that only 1% of the mouse sperm genome is associated with histones (Fig. 1A), and these histones were said to be related to developmental and/or housekeeping genes important for embryo development (31). However, more recent studies using micrococcal nuclease DNA digestion and ATAC-seq (assay for transposase-accessible chromatin using sequencing) techniques revealed that 7.5% of histones present in diploid somatic cells are retained in sperm nuclei when taking ploidy into account. The distribution and location of these histones in the paternal genome are actively debated, with some reports suggesting histones are distributed primarily within distal intergenic and intronic regions, and others have identified enrichment in imprinted genes (14, 15). This discrepancy may be explained by differences in methodology (14).

Results from ATAC-seq and ChIP-seq experiments suggest that sperm histones located in promoter regions possess post-translational modifications that are both a consequence of the transcriptional state of the preceding round spermatid stage as well as a prelude to expression patterns observed in ESCs and adult tissues (Fig. 1B). Modifications on histones at promoters can either be active, repressive, or bivalent. Approximately 60% of sperm promoters are in an active epigenetic state, and the TSSs of these promoters are flanked by three to four nucleosomes upstream and five to six nucleosomes downstream (16). It has been shown that 28% of promoters containing H3K27me3 also have H3K4me2, bivalent marks indicative of a poised promoter state (13). These are surprising findings considering the transcriptionally inert state of mature sperm. However, current studies have found that genes encoding for proteins involved in embryo development, HOX, SOX, FOX, TBX, PAX, CDX, and GATA family transcription factors, are the ones whose promoters possess these bivalent marks (12, 13, 33–35). Perhaps these histone modifications contain epigenetic information that affects transcription in the early embryo.

Sperm not only have promoters in a primed state that correlates with expression in mESCs, but they also appear to have enhancers in a similar state (Fig. 1B). Sperm enhancers were defined by the presence of ATAC-seq signal and the presence of H3K4me1 and H3K27ac (16). Results from ATAC-seq experiments suggest the presence of around 58,000 transposase-hypersensitive sites that may be bound by specific transcription factors, 10,240 of which correspond to enhancers previously identified in embryonic or adult tissues. In addition to typical enhancers, the sperm genome also contains around 645 super-enhancers, most of them in common with mESCs or specific cell lineages found in the adult organism. Interestingly, super-enhancers are present in loops formed by CTCF and cohesin to create insulated neighborhoods as found previously in mESCs (Fig. 1B) (36). These results suggest that enhancer elements are already specified in the sperm and may be primed for subsequent function during embryogenesis and in the establishment of specific cell fates during the formation of differentiated tissues (16).

3D organization of sperm chromatin

The dense compaction of sperm chromatin was thought to result in the establishment of a 3D architecture very different from that of somatic cells. However, recent studies suggest that the three-dimensional organization of sperm chromatin is very similar to that of other cells, in particular the ESCs (16, 37). CTCF is an architectural protein responsible, at least in part, for this organization (38), and binding motifs for CTCF can be detected at micrococcal nuclease and ATAC-seq sites on sperm DNA, suggesting that this protein may be retained in the sperm nucleus (Fig. 1B) (14, 16). The presence of CTCF at sequence motifs in accessible sperm DNA was confirmed via ChIP-seq experiments (16). CTCF has been found to be essential for normal spermiogenesis, sperm fertility, and histone retention in mature sperm (39). Genome-wide Hi-C mapping experiments have uncovered the presence of compartments, CTCF-mediated loops, and topologically associating domains in sperm chromatin similar to those found in somatic cell lines and embryonic stem cells (16, 37). Recent single-nucleus Hi-C experiments have shown that a similar higher-order chromatin organization also exists in the female gamete at the germinal vesicle stage but not in the MII stage, at which time the oocyte is arrested in metaphase (40).

Epigenetic remodeling after fertilization

Histone replacement with protamines

It is now understood that mature sperm DNA is both highly methylated and bound by protamines, modified histones, and transcription factors when it first encounters an oocyte. Fertilization begins with the binding of the sperm head to the oocyte zona pellucida. Sperm fusion with the membrane activates the oocyte and initiates completion of meiosis II. The sperm head and its contents are then engulfed by the oocyte. While the oocyte completes meiosis II, the sperm nucleus undergoes several changes. Its chromatin is primed for later nuclear syngamy and transcription in two ways: the first is through the replacement of protamines with histones, and the second is through active demethylation. Decondensation of the sperm nucleus occurs ~45–60 min after fertilization. Disulfide bonds, which allow protamines to attach to sperm DNA, are broken upon exposure to oocyte-produced chemicals such as GSH (41); however, it is unclear whether chromatin decondensation is initiated by protamine detachment. Experiments showing a depletion of radiolabeled protamines occurring after chromatin decondensation (41, 42) may be explained by unbound protamine degradation. The paternal chromatin is then loaded with histones produced by the oocyte prior to fertilization, as suggested by immunofluorescence data showing the appearance of histones in the male pronucleus following protamine depletion (43). These histones carry modifications associated with newly-synthesized histones (44), including histone H4 Lys-5 and Lys-12 acetylation (45). One study detected the presence of H3K4me1 in the male pronucleus beginning at stage PN1, and H3K4me3 beginning at stage PN4. Researchers hypothesized that these observed modifications can be explained by histone methyltransferase activity in the zygote (46). Alternatively, the presence of H3K4me1 and H3K4me3 in mature sperm (12, 16)

suggests that histone modifications detected in the early embryo may be paternally derived and maintained during fertilization and early embryogenesis.

Demethylation of the paternal pronucleus

It was previously thought that both female and male pronuclear DNA is passively demethylated once replication commences. This is shown to be true of the maternal nucleus (47, 48); however, evidence suggests there is active demethylation of the male pronucleus before the start of DNA replication (49, 50). Immunofluorescence and bisulfite sequencing data show a sharp decrease in methylation in the male pronucleus within 4 h of fertilization (48, 50). Replication begins 7–9 h into the first cell cycle, when pronuclei enter the PN3 stage (51). Therefore, any demethylation occurring prior to the start of replication must be active. One proposed mechanism for this demethylation is the oxidation of 5-methylcytosine by TET proteins present in the oocyte cytoplasm (52–54). One study confirmed the presence of 5-hydroxymethylcytosine (5hmC) in the mouse male pronucleus using antibody staining and the specific expression of TET protein in the early embryo (52). The 5hmC signal increases significantly between stage PN0 and PN5, and a deficiency of Tet3 prevents the oxidation of 5mC into 5hmC and results in developmental abnormalities (55). Another model suggests the presence of sperm chromatin-specific demethylases in the oocyte mediating pronuclear demethylation (42). Enrichment of 5hmC in sperm highlights the differences in the methylation state of male and female pronuclear chromatin before the start of nuclear syngamy.

Recent studies explore the apparent asymmetry in the epigenetic states of male and female pronuclei and its implications in the later stages of embryonic development. Inoue *et al.* (56) used DNase-sequencing techniques to compare DNase I-hypersensitive sites (DHSs) in the maternal and paternal mature gametes, pronuclei, morula, and blastula stage embryos. In searching for allele specificity and proof of parent-specific inheritance of epigenetic marks, they discovered that most DHSs in the early embryo are maternally derived and prime differential gene expression at the time of zygotic genome activation (56). Certain aspects of the epigenetic state of sperm and oocyte chromatin are maintained throughout early stages of embryonic development, suggesting a role for the transfer of information from the gamete to the embryo.

Conclusions

Current research reveals that sperm chromatin contains far more complex epigenetic information than was previously recognized. The sperm nucleus contains sex-specific methylation patterns, nucleosomes at promoters carrying both active and silencing histone modifications, putative enhancer and super-enhancer elements flanked by nucleosomes and possibly bound by transcription factors, and CTCF/cohesin bound at specific sites to establish highly-organized chromatin interactions within the three-dimensional nuclear space. It is difficult to imagine that the presence of this wealth of information in the sperm is not used during early embryonic development to guide the initial steps controlling gene expression after fertilization. DNA methylation, an obvious candidate to explain the inheri-

ance of epigenetic information through generations, is erased and re-established during spermatogenesis and after fertilization and thus has been discounted as a plausible mechanism underlying transgenerational effects (57). However, the erasure of 5mC in the paternal genome is only partial, suggesting the existence of mechanisms that maintain some of the epigenetic information established by DNA methylation. It is possible that the presence of DNA-bound transcription factors and histone modifications in specific regions of the genome may serve to guide re-methylation of the DNA after the demethylation of the paternal chromosomes that takes place immediately after fertilization. Under this model, although part of the epigenetic information specified by DNA methylation of the paternal chromosomes may be erased after fertilization, some information may be maintained by the interaction of specific DNA-bound transcription factors that preserve a memory of critical regulatory functions encoded by enhancers, super-enhancers, and 3D organization (16).

The information carried in the sperm epigenome has the potential to be transferred transgenerationally, explaining the occurrence of experience-based transmission of epiphenotypes. Environmental factors causing novel transcription during development of the paternal germline may result in the persistence of DNA-binding proteins at new sites in the genome, which in turn would modulate DNA re-methylation after fertilization and transcription in the early embryo. Other possible products of altered transcription as a consequence of environmental signals are small RNA fragments, including tRNAs and miRNAs, whose presence in sperm has also been shown to mediate the transfer of epigenetic information between generations (58–60). Skinner and co-workers (61) made the important discovery that exposure to endocrine disruptors *in utero* can lead to several phenotypes that persist as far as the F3 generation. Phenotypes include testicular and kidney diseases, tumors, and obesity, among others. Transgenerational transmission of these phenotypes may be mediated by epigenetic changes occurring in gametes upon exposure to these toxins. Other environment-altering elements such as diet, stress, smoking, and even certain medications have been found to have inter- or transgenerational phenotypic implications (32, 62–64). Epigenetic information carried in sperm in the form of DNA and histone modifications, transcription factors, noncoding RNA, and chromatin 3D architecture is one potential explanation for this phenomenon.

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