REVIEW

Epigenetic regulations in mammalian spermatogenesis: RNA-m^{6}A modification and beyond

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Abstract

Emerging evidence shows that m^{6}A, one of the most abundant RNA modifications in mammals, is involved in the entire process of spermatogenesis, including mitosis, meiosis, and spermiogenesis. "Writers" catalyze m^{6}A formation on stage-specific transcripts during male germline development, while "erasers" remove m^{6}A modification to maintain a balance between methylation and demethylation. The different functions of RNA-m^{6}A transcripts depend on their recognition by "readers". m^{6}A modification mediates RNA metabolism, including mRNA splicing, translation, and degradation, as well as the maturity and biosynthesis of non-coding RNAs. Sperm RNA profiles are easily affected by environmental exposure and can even be inherited for several generations, similar to epigenetic inheritance. Here, we review and summarize the critical role of m^{6}A in different developmental stages of male germ cells, to understand of the mechanisms and epigenetic regulation of m^{6}A modifications. In addition, we also outline and discuss the important role of non-coding RNAs in spermatogenesis and RNA modifications in epigenetic inheritance.

Keywords m^{6}A · RNA modification · Spermatogenesis · Sperm RNA · Epigenetic inheritance

Introduction

In mammals, spermatogenesis is a highly sophisticated and complex process that can be divided into four stages: mitosis, meiosis, spermiogenesis, and spermiation [1–3]. In the mitosis phase, A-single (As) spermatogonia are capable of self-renewal, amplifying the stem cell pool, and differentiation to undergo spermatogenesis. The As spermatogonia differentiate into two A-paired (Ap) spermatogonia and then undergo repeated mitotic division to form chains of 4, 8, 16, and even 32 A-aligned (Aal) spermatogonia. These spermatogonia, including As, Ap, and Aal, are connected together through an intercellular bridge arising from incomplete cytokinesis and are defined as undifferentiated spermatogonia. Undifferentiated spermatogonia undergo an irreversible transition to differentiating A1 spermatogonia (A–A1 transition), followed by five synchronized cell divisions to form A2, A3, A4, A-intermediate (AIn) and type B spermatogonia, which differentiate into preleptotene spermatocytes that enter the meiosis phase [2, 4]. The meiosis phase consists of a single round of meiotic DNA replication and two consecutive rounds of chromosome segregation, meiosis I, and meiosis II. Double-strand break (DSB) formation, recombination and synapse of homologs are hallmark events during meiotic prophase I ensuring proper segregation of homologs during meiosis I. Meiotic prophase I can be divided into leptonema (SPO11-induced DSB formation), zygonema (synapsis initiation), pachynema (synapsis completed) and diplonema (desynapsis and chiasmata) according to morphological characteristics [3]. During meiosis II, sister chromatids separate to produce haploid round spermatids and then enter the spermiogenesis stage. There are several major events in the development of highly specialized spermatozoa during spermiogenesis: formation of the acrosome and flagellum, chromatin remodeling, and removal of the residual body. The development of mouse spermatids is subdivided into 16 steps based on the morphology of the nuclei and acrosome [5]. Steps 1–8 are characterized by early round spermatids; whereas, the

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later steps (steps 9–11) are characterized by intermediate and late spermatids with elongating nuclei. In elongated spermatids (steps 12–16), transcription terminates due to chromatin compaction. Finally, mature spermatozoa are released into the seminiferous tubule, through a process termed spermiation [1] (Fig. 1).

Epigenetic regulation (DNA methylation, histone modification, and non-coding RNAs) plays an important role in spermatogenesis, including spermatogonia stem cell amplification to maintain stem cell pools, meiosis to form haploid cells, and spermiogenesis to develop into mature spermatozoa [6, 7]. Similar to DNA methylation, there are various types of methylation modifications on mRNA, such as m^6A, 5-methylcytosine (m^5C), N^7-methylguanosine (m^7G), N^6-methyl-2′-O-methyladenosine (m^6Am), and 2′-O-methylation, which comprise the emerging field "RNA Epigenetics" [8]. m^6A plays an important role in gametogenesis [9–13], embryonic development[14–16], sex determination [17], and response to environmental insults [18] by affecting the splicing [19], translation [20, 21], and stability [22] of mRNA, as well as the maturity and biosynthesis of non-coding RNAs [23–25]. The importance of RNA-m^6A modification and epigenetic regulation mediated by m^6A in spermatogenesis has attracted widespread attention, as discussed in the following sections.

The functions of m^6A-associated proteins in spermatogenesis

Although m^6A was discovered as early as 1975 [26], its biological function and clinical application have received increasing attention with the development of epigenetics and the application of high-throughput sequencing technology. There are three m^6A-associated proteins: "writer" (methyltransferase), "eraser" (demethylase), and "reader" (decoder). "Writer" is a multicomponent m^6A methyltransferase complex (MTC), which introduces m^6A into mRNAs. The main components of "writer" include METTL3, the core catalytic subunit; METTL14, the scaffold [27, 28]; and WTAP, the regulatory subunit, ensuring the stability and localization to nuclear speckles [29]. "Eraser" includes fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5), which are responsible for removing m^6A modification. The decoder "reader" (YTH family) recognizes m^6A modifications and has complex biological functions [30].

m^6A writers in spermatogenesis

Methyltransferase complexes play an important role in mammalian spermatogenesis, including spermatogonial stem cell

Fig. 1 A schematic diagram of mouse spermatogenesis
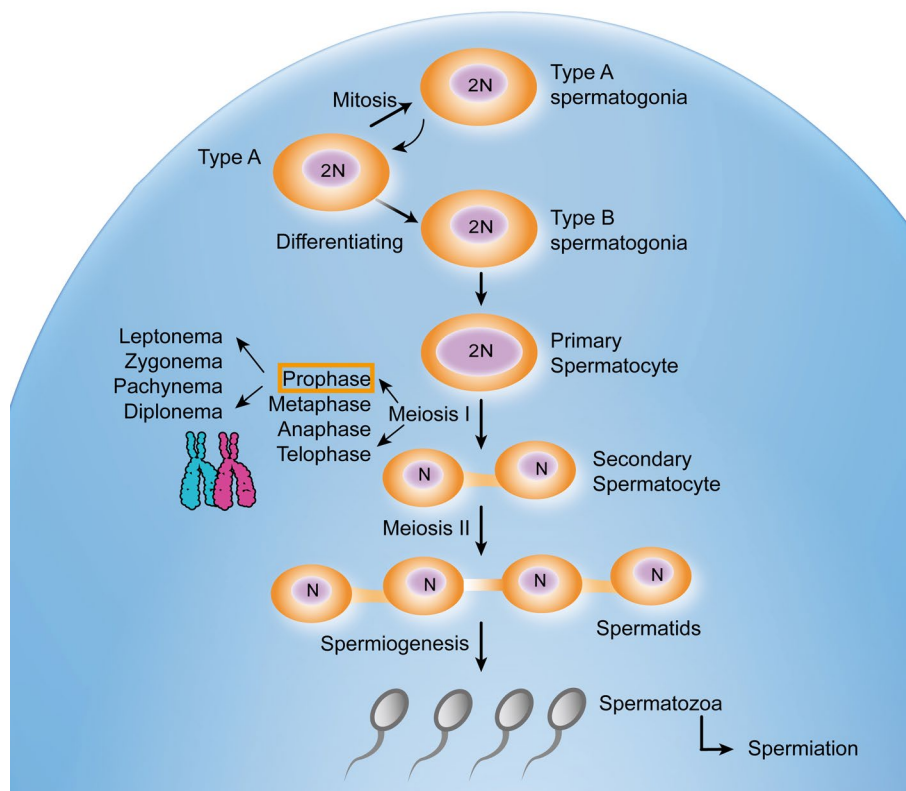
differentiation, meiosis, and spermiogenesis (Fig. 2). *Mettl3* is highly conserved in eukaryotes from yeast to humans. In Arabidopsis [31], inactivation of methyltransferase results in failure of the developing embryo to progress past the globular stage. In yeast [32], the deletion of *Ime4* (homolog of *Mettl3*) causes failure to initialize meiosis and sporulation. In zebra fish [11], *Mettl3* mutation alters the expression profile of hormone synthesis genes, disrupts gametogenesis, and reduces fertility. In mice, inactivation of *Mettl3* or *Mettl14* with Vasa-Cre in early male germ cells causes m^{6}A loss and excessive spermatogonial stem cell proliferation and depletion, disrupting spermatogenesis [12, 33]. *Mettl3 and Mettl14* double-knockout mice showed severely reduced sperm motility, flagellar defects, and abnormal sperm head abnormalities, similar to the human oligo-astheno-teratozoospermia (OAT) syndrome [33]. In humans [34], *METTL3* mediates higher m^{6}A levels in sperm RNA and is considered a high-risk factor for asthenozoospermia.

METTL16 is a non-classical methyltransferase that targets pre-mRNAs and various non-coding RNAs [35], such as MAT2A hairpins and spliceosomal U6 snRNA [36]. METTL16-mediated modifications may play an important role in regulating splicing events. In Caenorhabditis elegans, METT10 (a homolog of METTL16) inhibits the specification of germ-cell proliferative fate [37]. METT10 also promotes vulva, somatic gonad, and embryo development and ensures meiotic development of these germ cells do differentiate. However, whether METTL16 plays an important role in mammalian gametogenesis remains unclear. Additional studies are needed to explore the function and mechanism of "writer" in germ cells.

m^{6}A erasers in spermatogenesis

FTO [38] and ALKBH5 [39] can remove the methyl group of m^{6}A from RNA both in vitro and in vivo. FTO and ALKBH5 are highly expressed in the testis, and both are localized to nuclear speckles [40]. ALKBH5 co-localizes with mRNA processing factors that play an important role in alternative splicing [39]. FTO-dependent demethylation of m^{6}A also regulates mRNA splicing and is required for adipogenesis [41] (Fig. 2). *Alkbh5*-deficient male mice are characterized by impaired fertility resulting from apoptosis, which affects meiotic metaphase stage spermatocytes [39]. Dysfunction may be related to the interaction between ALKBH5 and nuclear speckle proteins to regulate RNA metabolism [39]. Another study showed that ALKBH5-mediated m^{6}A is essential for correct splicing of transcripts

Fig. 2 The function of m^{6}A-associated proteins in spermatogenesis. "Writers" catalyze m^{6}A formation on stage-specific transcripts during male germline development while "erasers" remove m^{6}A modification to maintain a balance between methylation and demethylation. The different functions of m^{6}A transcripts (splicing, translation, and degradation) depend on their recognition by "readers." Abnormal expression of m^{6}A-associated proteins in different developmental stages results in male infertility
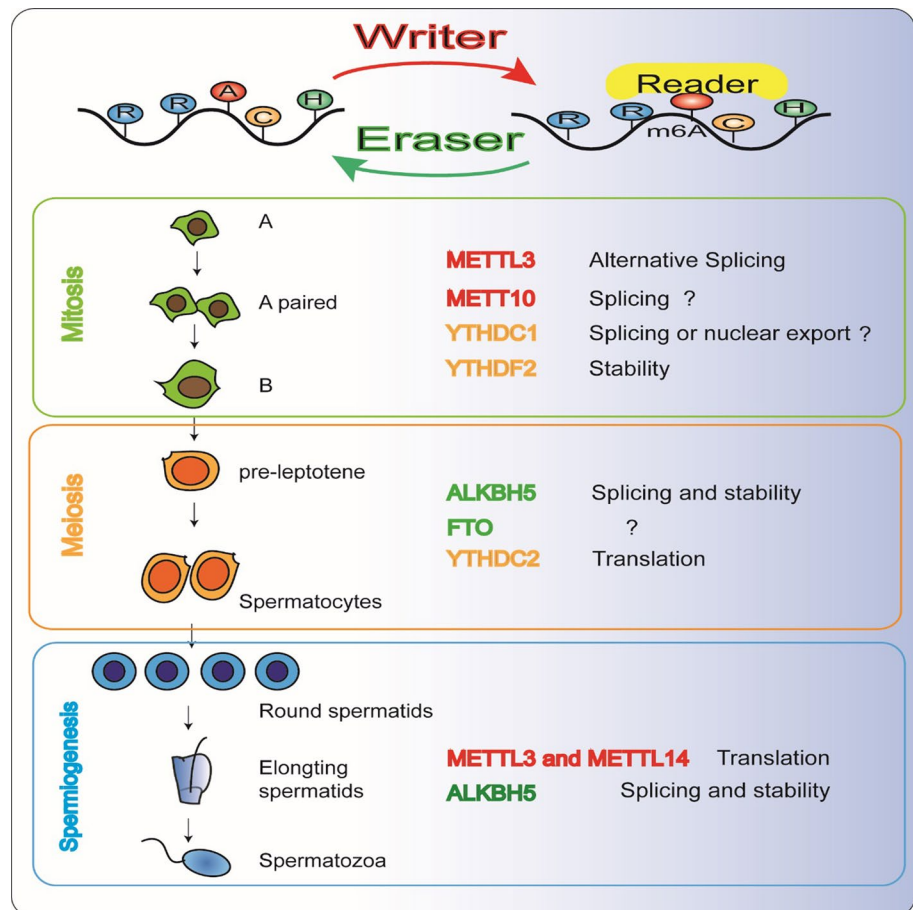
with a longer 3′UTR in spermatocytes and round spermatids [42]. m6A marks the 3′-UTRs of longer mRNAs destined to be degraded during spermiogenesis. Global shortening of 3′-UTRs to enhance translational efficacy and fast turnover through selective degradation of longer 3′-UTR transcripts is essential for spermiogenesis. In short, ALKBH5-dependent m6A is required for the meiotic and haploid phases of spermatogenesis by controlling the splicing and stability of mRNAs. Genetic mutations in human *FTO* are significantly associated with reduced semen quality [43]. The discovery of two missense mutations and a genetic variant of *FTO* suggests that aberrant demethylation of messenger RNA is a risk factor for reduced male fertility [43].

m6A readers in spermatogenesis

The m6A function is mediated by the ''reader'' protein family, which carries a YTH (YT521-B homology) domain. In addition to providing an aromatic cage structure to accommodate m6A modification [30], the YTH protein can also modulate RNA structure (acting as methylation-dependent RNA switch), affecting RNA stability, and splicing [44]. It is worth noting that recent studies have shown that YTHDC2 [45, 46], YTHDC1 [9], and YTHDF2 [47], play an essential role in spermatogenesis (Table 1).

YTHDC2 is the largest YTH domain-containing protein and the only member of the family that contains helicase domains. YTHDC2 is highly expressed in testicular germ cells and is essential for meiosis because it promotes translation by recognizing m6A [45]. *Ythdc2* missense mutations in germ cells cause germ cells to enter meiosis, but proceed prematurely to aberrant metaphase and apoptosis

[48]. In addition, YTHDC2 has been reported to interact with the essential meiosis-specific protein MEIOC [49]. Bgcn-Bam (YTHDC2-MEIOC homolog in Drosophila) is required autonomously for mitotically dividing spermatogonia to stop meiosis initiation and spermatocyte differentiation [48]. This indicates that gene expression regulated by the YTHDC2-MEIOC complex is an evolutionarily ancient strategy that controls germline transition into meiosis.

The nuclear m6A reader YTHDC1 is essential for maintenance of male spermatogonia development in mice [9]. *Ythdc1* knockout mice showed a Sertoli-only phenotype in seminiferous tubules. In addition, YTHDC1 facilitates the nuclear export of m6A-containing mRNAs through SRSF3 and NXF1 [50]. Tyrosine phosphorylation of YTHDC1 regulates its intra-nuclear localization, thereby modulating its effects on alternative splicing [51]. The role of YTHDC1 in regulating spermatogenesis requires further investigation.

In recent years, YTHDF2 has been reported to play an important role in neural cancer development [52], cancer progression [53], and maternal mRNA clearance [54]. Recently, an in vitro experiment indicated that YTHDF2 may regulate spermatogonia migration and proliferation by affecting the stability of m6A-containing transcripts [47]. In addition, miR-145 modulates expression of YTHDF2 by targeting its mRNA 3′-UTR, which inhibits the proliferation of liver cancer cells [53]. The regulatory effect of microRNA (miRNA) on the "reader" undoubtedly increases the complexity of the functional study of m6A.

Table 1 The functions of m6A-associated proteins in spermatogenesis

m6A-associated proteins	Species	Function in germ cells	References
METTL3/METTL14	Arabidopsis	METTL3 and METTL14 ensure the developing embryo to progress past the globular stage	[31]
	Yeast	IME4 (homologue of METL3) ensures initialization of meiosis and sporulation	[32]
	Zebra fish	*Mettl3* regulates hormone synthesis in gametogenesis and protects fertility	[11]
	Mouse	METTL3 and METTL14 ablation showed spermatogonial stem cells excessive proliferation and disrupted spermatogenesis	[33]
	Human	Higher m6A methylation levels in sperm RNA are considered a high- risk factor for asthenozoospermia	[34]
METTL16	Caenorhabditis elegans	METT10 (a homolog of METTL16) inhibits the specification of germ-cell proliferative fate	[37]
ALKBH5	Mouse	ALKBH5-dependent m6A is required for meiotic and haploid phases of spermatogenesis by controlling both splicing and stability of mRNAs	[42]
FTO	Human	*FTO* genetic mutations are significantly associated with reduced semen quality	[43]
YTHDC2	Mouse and Drosophila	YTHDC2-MEIOC complex is essential for meiosis by promoting translation	[48]
YTHDC1	Mouse	*Ythdc1* knockout mice showed a Sertoli-only phenotype in spermatogenic tubules	[9]
YTHDF2	Mouse	YTHDF2 regulates spermatogonia migration and proliferation by affecting the stability of m6A-containing transcripts	[47]

m⁶A function in testicular somatic cells

In addition to playing an essential role in spermatogenic cells, m⁶A can also participate in spermatogenesis by affecting the function of testicular somatic cells. In Sertoli cells, m⁶A and its "writer" WTAP are essential for sustaining the spermatogonial stem cell pool [55]. Loss of WTAP in Sertoli cells results in infertility and progressive loss of spermatogonial stem cell population [55]. In addition, exogenous Vitamin C supplementation decreases the level of global nucleic acid methylation (including DNA methylation and m⁶A RNA modification) in porcine immature Sertoli cells, which promotes the reproduction function [56]. In Leydig cells, m⁶A mRNA methylation was reported to regulate testosterone synthesis by modulating autophagy [57]. Furthermore, studies have shown that m⁶A and eraser "FTO" may be involved in environmental toxin-induced Leydig cell apoptosis [18, 58].

Non-coding RNAs in spermatogenesis

Non-coding RNAs, as epigenetic regulators, play an important role in spermatogenesis. Among them, miRNAs, circular RNAs (circRNAs), PIWI-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNAs), and rRNA-derived small RNAs (rsRNAs) exert their functions to control the normal development of male germ cells at the transcriptional, post-transcriptional, and translational levels. We will summarize and discuss the functions of those non-coding RNAs in spermatogenesis in the following sections:

miRNA

The classic processing of mature miRNA s (22nt) includes two steps: an original long transcript (primary miRNA) is processed in the nucleus by a microprocessor complex consisting of the RNA-binding protein DGCR8 and ribonuclease type III DROSHA to form a 60–70nt stem-loop structure (pre-miRNA). The pre-miRNA is cut by DICER in the cytoplasm to form one mature miRNA, which is incorporated into the miRNA-induced silencing complex (miRISC) to recognize the 3′-UTR of target mRNA and promote mRNA degradation or inhibit translation [59]. In recent years, miRNAs have been found to regulate various biological processes, such as cancer, in a tissue- and developmental-specific manner [60]. Notably, as an important physiological process, spermatogenesis is also regulated by miRNAs to some extent [61]. miRNAs are abundantly enriched during the active transcription of meiotic genes in male germ cells, especially in pachytene spermatocytes and round spermatids [62]. Many genes, such as *Rsbn1*, participate in spermatogenesis and are involved in the regulation

of translation by miRNAs [62]. In addition, miRNA clusters (miR-34b/c and miR-449a/b/c) are indispensable for spermatogenesis and male efferent ductule ciliogenesis [63, 64]. Proteins involved in miRNA processing, such as DGCR8 and DROSHA, are essential for spermatogenesis and male fertility in mice [65]. There is increasing evidence that abnormal expression of miRNAs is closely related to spermatogenic disorders in humans. For instance, compared to fertile men, the expression of miR-141 (targeting CBL and TGFβ2) and miR-7–1-3p (targeting RBL and PIK3R3) are significantly increased in non-obstructive azoospermia (NOA) patients[66], and may serve as clinical biomarkers in the future.

In addition to miRNAs, endogenous small interfering RNAs (endo-siRNAs) can serve as epigenetic regulators and be involved in spermatogenesis [65]. Although there are many similarities between endo-siRNA and miRNA, such as 22nt length, requiring DICER for processing and the same function, the endo-siRNA processing is independent of the microprocessor. Thus, the role of endo-siRNA in mammalian spermatogenesis requires further study.

circRNA

Circular RNA (circRNA), a new class of non-coding RNA, is characterized by a back-splicing mechanism without poly-A tails and 5′ caps. The biosynthesis efficiency of circRNA is closely related to the integrity and extension speed of RNA polymerase II [67]. Compared with miRNAs, circRNAs are more conservative and tissue specific. As early as 1979, circRNAs were observed through electron microscopy, but these molecules have always been considered as "junk" products of abnormal splicing [68]. In 1993, the testicular-specific circRNA derived from the sex-determining region (SRY) gene was found to be functional [69]. With the development of next-generation sequencing technology, thousands of circRNAs have been detected in the testes. Most testicular circRNAs are derived from the exon regions of genes and are widely distributed on chromosomes (including the mitochondrial genome). CircRNAs exert their biological functions in various ways: 1) weakening the repressive effects of miRNAs on mRNA translation as "miRNA sponges"; 2) acting as decoys/transporters for factors or serving as a protein scaffold; and 3) being translated through the internal ribosome entry site (IRES) [70]. CircRNA levels increase with the progression of spermatogenesis, especially when late pachytene spermatocytes develop into round and elongating spermatids [71]. Interestingly, the differentially expressed circRNAs were detected in high-quality and low-quality human spermatozoa [72]. In addition, a large number of abnormally expressed circRNAs, such as hsa_circRNA_0023313, have also been detected in patients with NOA [73]. CicrRNAs generated

from the conserved reproductive gene BOULE protect the fertility of males under heat stress [74]. In addition, the level of circBoule RNAs in asthenozoospermic sperm is lower [74]. Thus, sperm-derived circRNAs are potential modulators of sperm quality and can be used as a new non-invasive biomarker for male fertility.

piRNA

piRNA, another member of the non-coding RNA family, was discovered in 2006 and has high specificity in germ cells. piRNA can be divided into two categories according to piRNA expression in germ cells: pre-pachytene piRNAs and pachytene piRNAs. The length of piRNAs is mainly 24–31nt, which is longer than that of other small non-coding RNAs. piRNAs interact with PIWI subfamily members, represented by MIWI, MILI, and MIWI2. piRNA and PIWI proteins may inhibit transposable elements, regulate translation, participate in germline stem cell maintenance, regulate RNA degradation, and influence cellular defence at the transcriptional or post-transcriptional level [59]. Deficiency in any of the PIWI proteins (MIWI, MILI, and MIWI2) in mice causes aberrant piRNA production and spermatogenesis arrest [75–77]. In addition, a recent study revealed that the assembly of PIWI proteins and piRNAs can assemble to form germ granules, which are products of liquid–liquid phase separation (LLPS), are filled with amorphous fibrous material mixed with RNA [78], and protect germline transcripts from inappropriate piRNA-induced silencing [79]. piRNAs establish intergenerational responses to environmental stress in the nematode *Caenorhabditis elegans* [80]. In addition, recent research shows that piRNA-30473 contributes to tumourigenesis by regulating m^6A RNA methylation in diffuse large B-cell lymphoma [81], which may provide new insights into piRNA-mediated multi-generational epigenetic inheritance through m^6A.

tsRNAs and rsRNA

In recent years, a novel class of tRNA-derived small RNAs (tsRNAs) has been identified in mature mouse sperm [82]. In contrast to other small RNAs, the length of tsRNA (also known as tRNA-derived fragments, tRFs) is mainly 30–40nt. Sperm tsRNAs have been reported to play an important role in transmitting paternal high-fat-diet (HFD)-induced impaired glucose-tolerance phenotype in the progeny [83]. Further studies showed that deletion of tRNA methyltransferase abolished the transmission of HFD-induced metabolic disorder, implicating sperm RNA modifications as an additional layer of paternal hereditary information [84]. There are two independent pathways for tsRNAs biogenesis: one involving the specific cleavage in the T-loop of mature nuclear tRNAs and the other involving the mitochondrial tsRNAs [85]. tsRNAs are scarce in testicular sperm, but are abundant in mature sperm in the epididymis, suggesting the important role of exosome transfer [86]. The transcription of mitochondrial small RNAs is rarely understood.

In addition to tsRNAs, an underestimated "housekeeping RNA-derived" small RNA family (rsRNAs) is enriched in mature sperm and various somatic tissues [87]. Similar to tsRNAs, rsRNA-28 s are more abundant in mature sperm in the epididymis than in testicular sperm. Recent studies have suggested that rsRNA-28 s and tsRNAs are actively involved in the acute phase inflammation in mice [87]. The potential existence and function of these small RNAs in mature sperm increase understanding of mechanisms involved in reproductive health.

RNA-m^6A modifications and spermatogenesis

mRNA-m^6A modifications in spermatogenesis

m^6A is the most abundant modification of mammalian mRNAs. To date, more than 12,000 m^6A sites have been identified in more than 7000 genes using m^6A affinity purification and sequencing (m^6A-seq). Each mRNA contains an average of 3–5 m^6A sites, which are synthesized co-transcriptionally and depend on the dynamics of RNA polymerase II [88]. The abundance of m^6A in nascent transcripts is higher than that in mRNA in the nucleoplasm or in the cytoplasm [89, 90]. Histone H3 trimethylation at lysine 36 (H3K36me3), a marker for transcription elongation, guides m^6A modification co-transcriptionally [89]. m^6A modification is enriched near H3K36me3 peaks and reduced globally when cellular H3K36me3 is depleted. In addition, when the histone methyltransferase SETD2 is silenced, the target gene loci modified by the classic methyltransferase complex (METTL3 / METTL14 / WTAP), especially the CDS and 3′UTR regions, are hypomethylated. Further research shows that METTL14 directly recognizes and binds H3K36me3, promoting the transcription of nascent RNA with m^6A modification [89]. The information exchange (cross talk) between histone modification and m^6A modification provides a new concept for the biosynthesis of mRNA-m^6A.

During spermatogenesis, many genes are regulated by m^6A modification in spermatogonial differentiation, meiosis, spermiogenesis, and other processes. m^6A modification is highly enriched in pachytene/diplotene spermatocytes and round spermatids. Many m^6A-target transcripts in male germ cells have been identified as the result of the development of high-throughput sequencing [4, 12, 33, 39, 42] (summarized in Table 2).

Table 2 m⁶A-target genes in spermatogenesis

Stage-specific events	m⁶A-target genes
Spermatogonial stem cell maintenance	*Dazl, Ddx4, Plzf, Pax7, Nanos2, Id4, Pou3f1, Taf4b, Bcl6, Etv5, Bcl6b, Lhx1, Pou3f1, Gfrα1, Ret, Pou5f1, Foxo1, Lin28a, Plzf, Sox3, Taf4b, Stat3*
Spermatogonial differentiation	*Sohlh1, Sohlh2, Neurog3, Kit, Dmrt1, Sox3, Stra8, Kit, Sohlh2, Dnmt3b, Ccnd2*
Meiosis	*Stra8, Sycp1, Sycp3, Spo11, Rad18, Dmc1, Rec8, Mlh1, Smc1b, Prdm9, Spo11, Gm960, Rec114, Mei4, Hormad1, Rpa1, Brca1, Brca2, Dmc1, Rad51, Rec8, Atm, Msh4/5, Mlh1/3, Marf1*
Spermiogenesis	*Brd7, Cstf2t, Jmjd1c, Parp11, Lmtk2, Tdrd12, Gopc, Pick1, Csnk2a2, Spaca1, Setd2, Gba2, Agfg1, Spaca1, Prm1, Camk4, Pygo2, Crem, Prm2, Foxj1, Dnnaf3*

miRNA-m⁶A modifications in spermatogenesis

During the process of maturation of miRNA, the pri-miRNA is methylated by METTL3, allowing its recognition by HNRNPA2B1 and processing by DGCR8 [91, 92]. HNRN-PA2B1 is recruited by "m⁶A switch" [93] and combined with DGCR8 promotes pri-miRNA processing [92]. For example, METTL3 interacts with DGCR8 to regulate the processing of pri-miRNA-221/222 in an m⁶A -dependent manner and downregulate the tumor-suppressor gene PTEN in bladder cancer [94]. The effect of METTL3 depletion on miRNA processing is not restricted to a particular cell line but is also observed in multiple cell types [95]. Additionally, HNRNPA2B1 can also regulate miRNA production and exert biological functions, such as endocrine resistance in breast cancer [96].

In spermatogenesis, miRNAs are expressed in a cell-specific or stage-specific manner and inhibit translation or promote mRNA decay at the post-transcriptional level [97]. miRNAs can also serve as epigenetic markers to participate in prenatal and postnatal male germ-cell development. However, the function of miRNA-m⁶A modifications in spermatogenesis is largely unknown. Owing to the recent discovery of the role of m⁶A in the promotion of mature mRNA processing, future research will focus on the understanding of the biological functions of m⁶A during germ-cell development.

CircRNA-m⁶A modifications in spermatogenesis

Recently, researchers proposed that m⁶A mediates the biogenesis of circRNAs [71, 98]. Junction sequences of these circRNAs appear to be enriched in m⁶A, which is usually located around the stop codons in linear mRNAs. Elevated m⁶A levels enhance circRNA production.The amount of circRNA in METTL3 deleted testes is significantly reduced compared to that in the wild type [71]. In contrast, the amount of circRNA in spermatogenic cells lacking ALKBH5 is significantly higher than that in wild-type cells [71]. For example, specific m⁶A promotes the synthesis of circ-ZNF609 through METTL3 and YTHDC1 in HeLa cells [99]. Although YTHDC1 was previously reported to play a role in the export of circRNAs, the export or stability of circ-ZNF609 was not found to be affected in this study [99]. This indicates that YTHDC1 on circ-ZNF609 is rate-limiting for the back-splicing reaction.

CircRNAs increase with a decrease in linear mRNAs during spermatogenesis. The number of circRNAs in mature sperm is approximately 50–100 times higher than that in spermatocytes and spermatids [71]. Further investigation indicates that circRNA encodes stable and long-lasting proteins, which compensates for the massive degradation of linear mRNAs during late spermiogenesis and maintains protein levels during the chromatin concentration stage [71]. ORF-containing circRNAs regard the specific m⁶A modification site as a ribosome entry site (IRES) to facilitate translation [98]. For example, specific m⁶A modifications promote the translation of circ-ZNF609 through YTHDF3 and eIF4G2 in HeLa cells [99]. The study of circRNA m⁶A in spermatogenesis is in its infancy, and additional studies are expected to reveal the role of m⁶A in non-coding RNA. In addition, studies have also shown that circRNAs reversely regulate m⁶A modification by binding to m⁶A-related proteins. By capturing the "eraser" ALKBH5, circSTAG1 inhibits ALKBH5 transport into the nucleus, resulting in enhanced FAAH m⁶A modification levels in astrocytes and reduced depression-like behavior in mice [100]. Thus, this type of feedback loop is worth exploring.

RNA modifications and epigenetic response

Environmental effects on RNA-m⁶A modifications

There have been several reports that external factors, such as environmental toxins [18], drugs [101], cigarette smoke [102], carbon black particles [103], ultraviolet [104], heat shock [105, 106] and PM2.5 [107], can regulate the levels of m⁶A modification of RNAs. For example, cigarette smoke condensate (CSC) leads to hypomethylation of DNA in the METTL3 promoter region and increases m⁶A levels, leading to the excessive maturation of miR-25-3p and pancreatic cancer [102]. In response to ultraviolet-induced DNA damage, m⁶A modification of mRNA is rapid (within

2 min) and induced at the site of DNA damage [104]. Under acute temperature stress, RNA-binding protein (DGCR8) and METTL3 are relocated to heat-shock genes, which in turn play co-transcriptional effects, affecting mRNA-m^{6}A modifications, thus promoting mRNA degradation [106]. In addition, under heat-shock stress, the increased m^{6}A modification in the 5′-UTR of mRNAs promotes cap-independent translation initiation, providing a mechanism for selective mRNA translation [105]. A recent study has shown that in humans with a higher PM2.5 exposure group, the expression levels of m^{6}A writers (METTL3 and WTAP), erasers (FTO and ALKBH5), and readers (HNRNPC) are significantly higher than in the lower PM2.5 exposure control group [107]. In addition, the microbiome has a strong effect on host m^{6}A mRNA modification by regulating the expression of both m^{6}A writers and erasers [108]. Changes in m^{6}A modifications induced by environmental factors may serve as molecular markers for monitoring and early diagnosis of adverse health outcomes from environmental exposure.

RNA modifications and epigenetic transgenerational inheritance

In mammals, the epigenetic response (DNA methylation, histone modifications or non-coding RNAs) plays a vital role in gametogenesis and embryonic development [6, 7]. Although sperm RNA is thought to play a minimal role in spermatogenesis, recent studies have shown that it can also be transmitted into the zygote during fertilization [109]. Mature spermatozoa are enriched in a wide range of larger RNAs (mRNA, long non-coding RNA, and circRNA) and small RNAs (miRNAs, tsRNAs, rsRNAs, and piRNAs) [82, 86, 87, 110]. Several compelling independent studies have corroborated that epigenetic modifications can be transmitted from the father to the offspring via paternal RNAs. Both coding RNA and non-coding RNA, as regulatory elements of gene expression and chromatin structure, can serve as targets of epigenetic programs induced by environmental factors, acting on the reproductive system and being transmitted from generation to generation [111]. Environmental exposure-induced epigenetic transgenerational inheritance is defined as the germ-line transmission of changed epigenetic information between generations without sustained environmental exposures [112]. When an F0 father is exposed to environmental insults, the effect on F1 (♂) is mediated by intergenerational inheritance, and the effect on F2 (♂) generation is defined as transgenerational inheritance (Fig. 3).

Environmental exposure, such as HFD [83], high-fat and high-sugar diet [113], traumatic stress [114], and depression, alter the RNA profiles in sperm and have a corresponding impact on future generations. Several sperm miRNAs and piRNAs exhibited different expression profiles in F0 males of the depression-like model and reproduced paternal
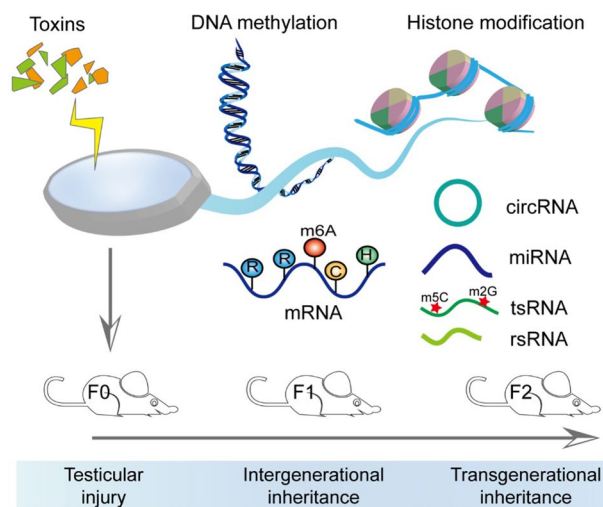
Fig. 3 A schematic diagram of epigenetic response to environmental insults. Epigenetic alterations (DNA methylation, histone modification, m^{6}A modification, and non-coding RNAs) induced by an environmental exposure can substantially affect the sperm function and have an inter-/transgenerational inheritance effect

depressive-like phenotypes in F1 offspring [115]. Furthermore, when a combination of miRNA antisense strands was injected at the zygote stage to neutralize the abnormal miRNAs, successfully rescue the depressive-like phenotype in F1 offspring successfully [115]. In addition, m^{5}C and m^{2}G on sperm tsRNAs are involved in the intergenerational inheritance of HFD-induced metabolic disorders [83, 84]. In addition to small RNAs, large sperm RNAs could also be involved in transmitting of traumatic experiences from parent to offspring, but the mechanism is not clear [116]. Different sperm RNA fractions contain distinct profiles of RNA modifications, which add new dimensions of complexity regarding RNA structural and functional diversities (Fig. 3).

Conclusions and perspectives

Studies on the m^{6}A of mammalian spermatogenic cells and knockout models of m^{6}A-associated proteins have revealed the importance of m^{6}A in spermatogonial stem cell maintenance, spermatogonial differentiation and meiosis. However, studies on the role of m^{6}A in non-coding RNAs (such as miRNAs and circRNAs) in spermatogenesis are still very preliminary, and further research is required to enrich it. During spermatogenesis, the role of other methyltransferases, such as RBM15 (anchoring MTC in nuclear speckles and U-rich regions in mRNAs) [117], ZC3H13 (a bridge for WTAP and RBM15) [118], and KIAA1429(known as VIRMA, serving as a scaffold to guide m^{6}A modification in the 3′-UTR around the stop codon) [119] needs also further exploration.

Crosstalk among writers, erasers, and readers of m^{6}A regulates the homeostasis of biological processes. As key components of the methyltransferase complex, METTL3 and METTL14 are known to regulate each other's stability at the protein level [15]. Furthermore, the "reader" YTHDF2 has been reported to preserve 5′UTR methylation of transcripts by limiting the "eraser" FTO under heat-shock stress [105]. In cancer cells, the "eraser" ALKBH5 and the "writer" METTL14 constitute a positive feedback loop to regulate the stability of m^{6}A-target transcripts with the involvement of RNA stability factor HuR and miRNA silencing pathway [120]. Cooperation among writers, erasers, and readers ensures the appropriate m^{6}A and proper mRNA processing.

In addition to the m^{6}A modification, another major functional modification located on RNAs is m^{5}C, which is abundant in tsRNAs, rsRNAs, and mRNAs. Interestingly, methylation of m^{5}C by NSUN2 facilitates the methylation of m^{6}A by MTC, which affects protein expression in a coordinated manner [21]. m^{5}C also plays an important role in facilitating mRNA export [121] and preventing mRNA decay [122]. Similar to m^{6}A, m^{5}C modification of mRNA serves as a DNA damage code to promote homologous recombination [123]. The similarity and synergy between the various modifications in RNAs increase the charm of "RNA Epigenetics".

The changes in the sperm RNA profile in response to environmental exposure initially revealed the role of RNA modification in coping with external pressure and its important role in epigenetic regulation. It would be promising to use the sperm RNA profile to assess the disease susceptibility of offspring. Further development of RNA sequencing technology will promote their application in translational medicine.

Declarations

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