

Review

N6-Methyladenosine Modifications in the Female Reproductive System: Roles in Gonad Development and Diseases

Hongbei Mu¹, Huiying Li¹, Yu Liu¹, Xiaofei Wang¹, Qiaojuan Mei¹, Wenpei Xiang^{1,2}✉

1. Institute of Reproductive Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.
2. Center of Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

✉ Corresponding author: Wenpei Xiang; Email: wpxiang2010@gmail.com

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Abstract

N6-methyladenosine (m⁶A) is the most prevalent chemical modification in eukaryotic messenger RNAs. By participating in various RNA-related bioprocesses including RNA decay, splicing, transport and translation, m⁶A serves as a pivotal regulator of RNA fate and plays an irreplaceable role in cellular activities. The m⁶A modifications of transcripts are coordinately regulated by methyltransferase “writers” and demethylase “erasers”, and produce variable effects via different m⁶A reading protein “readers”. There is emerging evidence that m⁶A modifications play a critical role in a variety of physiological and pathological processes in the female reproductive system, subsequently affecting female fertility. Here, we introduce recent advances in research on m⁶A regulators and their functions, then highlight the role of m⁶A in gonad development and female reproductive diseases, as well as the underlying mechanisms driving these processes.

Key words: N6-methyladenosine, epigenetics, oogenesis, fertility, Female reproductive system

Introduction

More than 170 kinds of RNA modifications were found to be widely distributed among various organisms [1], which are collectively known as the ‘epitranscriptome’. Substantial studies aimed to uncover the mechanisms and characterizations of these modifications, such as N6-methyladenosine (m⁶A), N1-methyladenosine, 5-methylcytidine and pseudouridine [2], unveiling a brand-new landscape of epigenetic regulatory mechanisms.

Among all of these modifications, m⁶A, which has been reported as early as 1974 [3, 4], was considered to be the most prevalent chemical modification in eukaryotic messenger RNAs [2, 5, 6]. The m⁶A modification is preferentially enriched around stop codons, 5'- and 3'-untranslated regions (UTR), as well as in long internal exons with the consensus motif RRACH (R = A or G, H = A, C or U) [7, 8]. Due to its involvement in various RNA-related

bioprocesses, including RNA decay, splicing, transport and translation [9-12], m⁶A is a pivotal regulator of RNA fate and plays an irreplaceable role in cell differentiation, development, metabolism, stress response and other cellular activities [2, 13-15].

Epigenetic molecules and signaling pathways in the female reproductive system have various effects and are important for female fertility [16-19]. With the deepening of m⁶A research, there is emerging evidence for the involvement of m⁶A in various physiological and pathological processes in the female reproductive system, including oogenesis and diseases, subsequently affecting female fertility. Here, we introduce recent advances in research on m⁶A regulators and their functions, and highlight the role of m⁶A in oogenesis and female reproductive diseases, as well as the underlying mechanisms driving these processes.

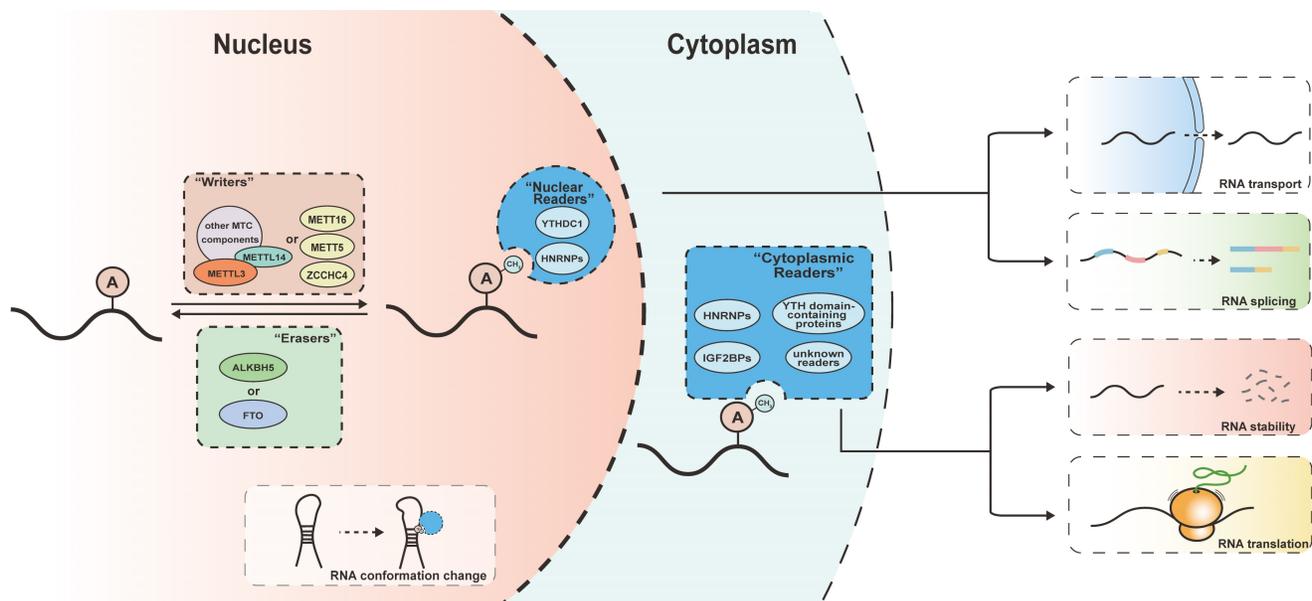


Figure 1. mechanism of m⁶A modifications. The m⁶A modification is catalyzed by writers including multicomponent methyltransferase complex (METTL3, METTL14 and other auxiliary components) and several independent methyltransferase (METTL16, METTL5, ZCCHC4), removed by erasers (ALKBH5 or FTO) and recognized by m⁶A readers (YTH domain-containing proteins, HNRNPs and IGF2BPs). Readers bind to their target RNAs and mediate various downstream effect including RNA transport, splicing, degradation and translation. The m⁶A modifications can regulate the interaction between RNA and m⁶A readers by mediating the conformation changes of RNAs.

Regulators of m⁶A

The m⁶A modification is a reversible process dynamically controlled by m⁶A writers and erasers, and its variable downstream effects rely on the recognition of m⁶A modifications by m⁶A readers (Figure 1).

m⁶A writers

The introduction of the m⁶A modification is accomplished by the multicomponent methyltransferase complex (MTC). The MTC consists of the catalytic subunit METTL3 and auxiliary subunits including METTL14, WTAP, KIAA1429, RBM15 and ZC3H13 [20]. METTL3 serves as the core component of the MTC by installing the m⁶A modification by adding a methyl group to the N6 position of adenine. METTL14 forms a stable heterodimer core complex with METTL3, enabling the latter to recognize its substrates [21-23]. WTAP is indispensable for the correct localization of the METTL3-METTL14 complex in the nuclear speckles, which guarantees the correct continuation of m⁶A-related processes [24, 25]. KIAA1429 and RBM15 maintain the m⁶A levels by recruiting the MTC to specific RNA regions [24, 26, 27]. ZC3H13 and its *Drosophila* homolog Flacc function as a bridge between the MTC and its mRNA targets, and guide RBM15 to WTAP to facilitate m⁶A modification [28, 29]. Hakai is a newly identified member of the MTC in *Drosophila* and human cells that helps stabilize other subunits of the MTC through its ubiquitination domain [30]. Given that METTL3 only produces a part of RNA m⁶A modifications,

there must be other writers mediating the other methyl transfer reactions [31]. METTL16 is a newly identified m⁶A methyltransferase targeting pre-mRNAs and various non-coding RNAs [32, 33]. It consists of two structural domains, whereby the N-terminal methyltransferase domain independently recognizes the 5'-UACAGAGAA motif and the C-terminal vertebrate conserved region binds to internal stem-loops that facilitate the methyltransferase activity of METTL16[34]. In addition, METTL5 and ZCCHC4 were found to be m⁶A writers that respectively act on the 18S and 28S rRNAs in an MTC-independent manner. METTL5 forms a heterodimer with TRMT112 to maintain its metabolic stability and serves as an exclusive m⁶A rRNA methyltransferase.[35]

m⁶A Erasers

Erasers are demethylases that remove m⁶A modifications in a ferrous iron and α -ketoglutaric acid dependent manner, dynamically regulating the m⁶A labeling of mRNAs and other types of transcripts in the nucleus. Erasers determine the m⁶A levels of transcripts together with m⁶A writers, thus modulating the subsequent effects of m⁶A readers. There is evidence that FTO controls RNA splicing by modulating the interaction of RNAs with SRSF2 protein [36]. ALKBH5, another known m⁶A eraser, has been proved to colocalize with mRNA-processing factors in nuclear speckles, and it plays critical roles in RNA transport, synthesis and stability. ALKBH5-dependent demethylation precisely controls the splicing process and length of the 3'-UTR region [37].

m⁶A Readers

Recognition and binding of m⁶A readers to their target RNAs is required for m⁶A modification to execute its important functions in regulating RNA fate. The m⁶A readers discovered to date include YTH domain-containing proteins, heterogeneous nuclear ribonucleoproteins (HNRNPs), insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs) and eukaryotic initiation factor 3 (eIF3) [38, 39].

These m⁶A readers take part in different RNA metabolic processes and affect different aspects of RNA behavior. YTH domain-containing proteins, the most researched m⁶A readers, recognize m⁶A modifications through the YTH domain and subsequently participate in various RNA-related processes [40]. YTHDF1 positively regulates the translation of its targeted RNAs in a m⁶A-dependent manner [11, 41], YTHDF2 selectively induces the degradation of m⁶A-modified mRNAs, decreasing the abundance of its targets [12, 42], while YTHDF3 facilitated decay and translation of RNAs by interacting with YTHDF1 and YTHDF2 [43, 44]. As a nuclear m⁶A reader, YTHDC1 mediates nuclear RNA export and is required for alternative splicing [45, 46]. YTHDC2 promotes translational activities and also accelerates the degradation of its targets, showing a double-sided effect [47]. HNRNPs and IGF2BPs are also protein families that function as m⁶A readers. Specifically, the binding affinity of HNRNPC for transcripts can be increased by m⁶A-mediated RNA structure alteration, leading to the m⁶A-switch effect [48]. HNRNPA2B1 binds RNA through its RNA recognition motif and recognizes m⁶A-modified RNA in a non-specific manner, enhancing METTL3-dependent microRNA processing and production [49, 50]. HNRNPG interacts with the phosphorylated carboxy-terminal domain of RNA polymerase II through its RGG motifs and co-transcriptionally regulates alternative splicing [51]. IGF2BPs are a group of RNA binding proteins that selectively bind m⁶A-modified RNA and share overlapping RNA targets. IGF2BPs enhance mRNA stability and promote mRNA translation, thus upregulating gene expression [52-54].

Biological functions of m⁶A signals

The introduction of m⁶A into transcripts mainly occurs in the nucleus, making it a nuclear imprint of RNA fate [55, 56]. The m⁶A levels determined by the m⁶A MTC and m⁶A erasers are closely related to RNA metabolism outcomes in the cytoplasm. Through reversible and dynamic methylation of adenine residues, m⁶A regulates the stability, splicing, transport and translation of RNAs [21, 22], playing an

important role in a variety of physiological and pathological processes.

m⁶A in mRNA stability

Possible involvement of m⁶A modification in regulating RNA stability was first explored in 1978. Researchers deduced that rapid elimination of m⁶A in the cytoplasm may be caused by the shorter half-life of mRNAs with a high m⁶A content [57]. This hypothesis that mRNAs harboring more m⁶A were susceptible to degradation has been proved later, and *Mettl3* knockout, which significantly decreased the m⁶A levels of RNAs, remarkably increased their half-life [58].

There is substantial evidence that m⁶A readers are the main regulators of RNA stability. RNA decay takes place in cytoplasmic processing bodies (P bodies) [59], and YTHDF2 was found to colocalize with P bodies and its cognate mRNAs were intended to have shorter half-lives, demonstrating YTHDF2's role in destabilizing its mRNA targets. The deadenylation-dependent decay pathway was also a part of mechanism driving YTHDF2-mediated RNA decay [12, 42]. YTHDF3 shares targets with its family members and promotes RNA degradation in cooperation with YTHDF2 [44]. Conversely, IGF2BPs enhance mRNA stability by recognizing m⁶A modifications, increasing the abundance of its mRNA targets. IGF2BPs silencing led to comprehensive downregulation of its target genes [54, 60].

It is worth mentioning that m⁶A modifications are also deposited in the RNAs of pseudogenes. These RNAs have higher m⁶A levels than their cognate mRNAs, which facilitates the degradation of processed pseudogene transcripts in the cytoplasm. Given that processed pseudogenes cannot be recognized and cleaned by canonical nonsense-mediated decay process, m⁶A-mediated cytosolic elimination of these nonfunctional RNAs provides a novel mechanism of RNA surveillance [61].

m⁶A in mRNA splicing

The m⁶A modification plays a key role in sex determination in *Drosophila* by modulating the splicing of Sex lethal transcripts [62-64], revealing a novel posttranscriptional regulatory mechanism of gene expression. However, the effects and functions of m⁶A modification in RNA splicing in mammals have not been fully elucidated. Some researchers proposed that m⁶A does not have a major effect on splicing [58]. However, there is accumulating evidence that m⁶A modification is involved in RNA splicing through multiple mechanisms. *Mettl3*-depletion partially affected RNA splicing in terms of

frequency and type of alternative splicing events [15]. The demethylases FTO and ALKBH5 control this process by regulating m⁶A levels in alternative exons cassettes and coding sequences, respectively [36, 65]. The nucleus-located m⁶A reader, YTHDC1, participates in the processing of transcripts together with splicing regulators such as CPSF6, SRSF3, and SRSF7 [10, 66].

Mechanistically, the distribution pattern of m⁶A modifications may be a key factor driving the impact of m⁶A on alternative splicing. It was found that m⁶A is preferentially located in long exons, near the stop codons, and in 3'-UTR [48]. Yet another study found that m⁶A was enriched near splice sites in both exonic and intronic regions [67]. This differential distribution may lead to region-specific binding of splicing-related proteins, thereby mediating alternative splicing. The indirect effect of m⁶A on RNA splicing should also be taken into consideration. By controlling the expression of highly m⁶A-modified splicing regulators such as SON44, HNRNPC38 and HNRNPF [68], m⁶A may also alter RNA splicing outcomes without direct participation.

m⁶A in mRNA transport

Correct subcellular localization of transcripts is closely related to their function, making m⁶A modification an important regulator of RNA fate by affecting nuclear RNA export. A conditional knockout of *Mettl14* led to nuclear retention of m⁶A-modified mRNAs by impairing the preferential binding of FMRP [69]. *ALKBH5*-deficient cells with increased m⁶A levels showed accelerated nuclear export and massive cytoplasmic accumulation of mRNA, leading to significant changes in the ratio of nuclear and cytoplasmic mRNA content [9]. The m⁶A reader YTHDC1 was found to enhance the binding of m⁶A-modified RNAs to nuclear export adaptor protein SRSF3 and mRNA export receptor NXF1, which facilitates the nuclear export of its target mRNAs [46]. These studies proved the involvement of m⁶A in nuclear RNA export, but its effect on the whole transcriptome needs to be further elucidated.

m⁶A in mRNA Translation

Some researchers have shown that m⁶A facilitates mRNA translation by interacting with eukaryotic translation initiation factor eIF3 via two different pathways [11, 70]. As one of the most important translation initiation factors in eukaryotic cells eIF3 is usually bound to the 5' untranslated regions of mRNAs and recruits the small ribosomal subunit around its binding sites, thereby regulating translation initiation [71]. It has been shown that the effect of the m⁶A reader YTHDF1 on protein

expression is dependent on the eIF3-related translation machinery. YTHDF1-binding mRNAs showed a higher ribosome-bound fraction and translation efficiency [11]. Conversely, *YTHDF1* silencing led to significantly downregulated protein production of its target mRNAs. Since YTHDF1 usually binds RNA close to stop codons in mammalian cells, the effect of 3'UTR-enriched YTHDF1 was based on eIF4G-induced spatial proximity to translation initiation sites [11]. Direct eIF3 recruitment around 5'UTR also initiated mRNA translation. Cell-stress associated mRNAs contain m⁶A modifications around the 5'UTR region and exhibit enhanced cap-independent translation [70]. Additionally, YTHDF1 was found to augment the translation of EIF3C in an m⁶A-dependent manner and form a YTHDF1-EIF3C axis promoting general protein synthesis [41].

Furthermore, METTL3, which was generally known as a methylase acting in the nucleus, also took part in mRNA regulation by interacting with the translation initiation machinery. Cytoplasmic METTL3 physically and functionally associates with eIF3 subunits, bridging its binding sites to the 5'-end of the mRNA to mediate mRNA circularization [72, 73]. METTL3 depletion remarkably downregulated the expression of its target mRNAs [73].

m⁶A in non-coding RNA

In addition to mRNAs, the deposition of m⁶A modifications also affects the fate of modified non-coding RNA (ncRNA) in various ncRNA-related bioprocesses [74]. METTL3 induced m⁶A hyper-methylation facilitated stabilizing lncRNA, thus enhancing its expression [75, 76]. FTO decreased the m⁶A level on the LINC00022 transcript and inhibited its degradation via the m⁶A reader YTHDF2 [77]. HNRNPA2B1, a nuclear m⁶A reader, showed a METTL3-like effect in RNA splicing and promoted primary miRNA processing by interacting with the microRNA microprocessor complex protein DGCR8 [50]. YTHDC1 was involved in the circRNA transport process. *YTHDC1* silencing led to significant nuclear retention of circNSUN2, which was rescued by enforced expression of wild-type YTHDC1 [78]. Moreover, the m⁶A modifications can initiate circRNA translation through YTHDF3-mediated recruitment of the translation initiation complex [79].

The m⁶A modification plays a key role in modulating interaction between ncRNAs and their targeted RNAs. For example, m⁶A modification promoted miR-133a binding to its targets and enhanced the miR-133a repression [80]. LncRNA ILF3-AS1 recruited METTL3 to ILF3 mRNA, which increased its m⁶A level and strengthened its stability

[81]. The m⁶A-dependent RNA structural changes regulate the accessibility of RNA binding motifs of proteins to affect RNA-protein interactions, which is termed as “m⁶A-switch”. The m⁶A modification destabilizes hairpin stem structure of lncRNA MALAT1, thus making it more accessible for the RNA binding proteins [48].

m⁶A in female gonad development and gametogenesis

Establishment of gonadal structure and function is dependent on the development of both germline cells and somatic cells. The oocyte and its surrounding somatic cells form the basic functional unit of the ovary, the follicle. Normal follicle development and gametogenesis are prerequisites for female fertility.

Follicle development, also known as folliculogenesis, is a long and complex process in which the recruited primordial follicle either develops into a mature Graafian follicle to ovulate or undergoes atresia. Folliculogenesis is synchronized with the growth and maturation of recruited oocytes, providing necessary sex steroids and establishing an appropriate microenvironment for oocytes.

In a broad sense, oogenesis is a highly sophisticated and coordinated process by which primordial germ cells (PGCs) develop into mature oocytes to prepare for fertilization. In humans, PGCs are specified as early as the peri-implantation window of the embryo at day 12 post-fertilization [82]. PGCs exist transiently and soon differentiate into oogonia after undergoing genome-wide epigenetic reprogramming [83]. The primary oocytes derived from diploid oogonia then enter meiosis and remain arrested in prophase I at birth. The prophase I oocytes have an intact germinal vesicle (GV) and remain dormant until gonadotropin stimulation in puberty, which can lead to germinal vesicle breakdown (GVBD). Following GVBD, the oocytes continue to complete meiosis I with formation of metaphase I spindles and separation of paired homologous chromosomes. Afterwards, the oocytes directly enter meiosis II and arrest for the second time in metaphase II (MII) until fertilization triggers the completion of meiosis.

Gene expression is precisely and dynamically controlled during the whole female gonad developmental process. In follicles, proliferation and differentiation of somatic cells relies on the adequate alteration of gene expression patterns, while in oocytes, the changes are even more intricate. Transcription of oocyte genes is active in the early stage of follicle formation and ceases at the GV

stage[84]. Maternal mRNAs synthesized in this period dominate the subsequent developmental phases of oocytes and early embryos until zygotic genome activation. As the oocytes emerge from meiotic arrest, maternal mRNAs are constantly degraded and decrease to the lowest level in the two-cell embryo [85]. All these processes largely rely on the post-transcriptional regulation of mRNA metabolism and expression. There is wide-ranging evidence for the importance of epigenetic modification in folliculogenesis and oogenesis [31, 86, 87], and the roles of m⁶A modification in these processes have begun to be partially elucidated in recent years. The m⁶A level was found to generally increase along with the development of the mouse ovary, reaching the highest point at the luteal phase. The m⁶A associated regulators also demonstrated a similar trend in accordance with the m⁶A levels, whereby m⁶A writers increased and erasers decreased from 12.5 days post coitum to the mature phase in the ovary, suggesting the potential impact of m⁶A modification during female gonad development [88]. There is increasing evidence for the involvement of m⁶A in specific stages and aspects of this process (**Figure 2**).

Follicle development

Follicle development is a dynamic and continuous process that happens simultaneously with oocyte growth. Before the resumption of meiosis, oocytes remain in prophase I stage and continue accumulating water, ions, carbohydrates and lipids until GVBD. Follicles provide a developmental microenvironment for oocyte growth and help sustain meiotic arrest [89, 90]. MeRIP-seq identified changes in the abundance and distribution of m⁶A modifications in granulosa cells during follicular development [91].

Mettl3 mutation reduced m⁶A levels in zebrafish ovaries. These Mettl3-defective females had smaller gonads than wild-type fish, and a large fraction of follicles failed to fully develop [92]. KIAA1429 was found to be expressed in the oocyte nucleus and granulosa cells from follicles in different stages. Increased numbers of primary follicles, decreased secondary, preantral and antral follicles, as well as multiple oocyte follicles were found in *Kiaa1429* ZP3-cKO mice, indicating that *Kiaa1429* deficiency led to arrest and disorganization of follicle developmental. Moreover, *Kiaa1429* deficiency inhibited the proliferation and promoted the apoptosis of granulosa cells. All of these findings suggested that KIAA1429 is required for normal folliculogenesis in mice [93].

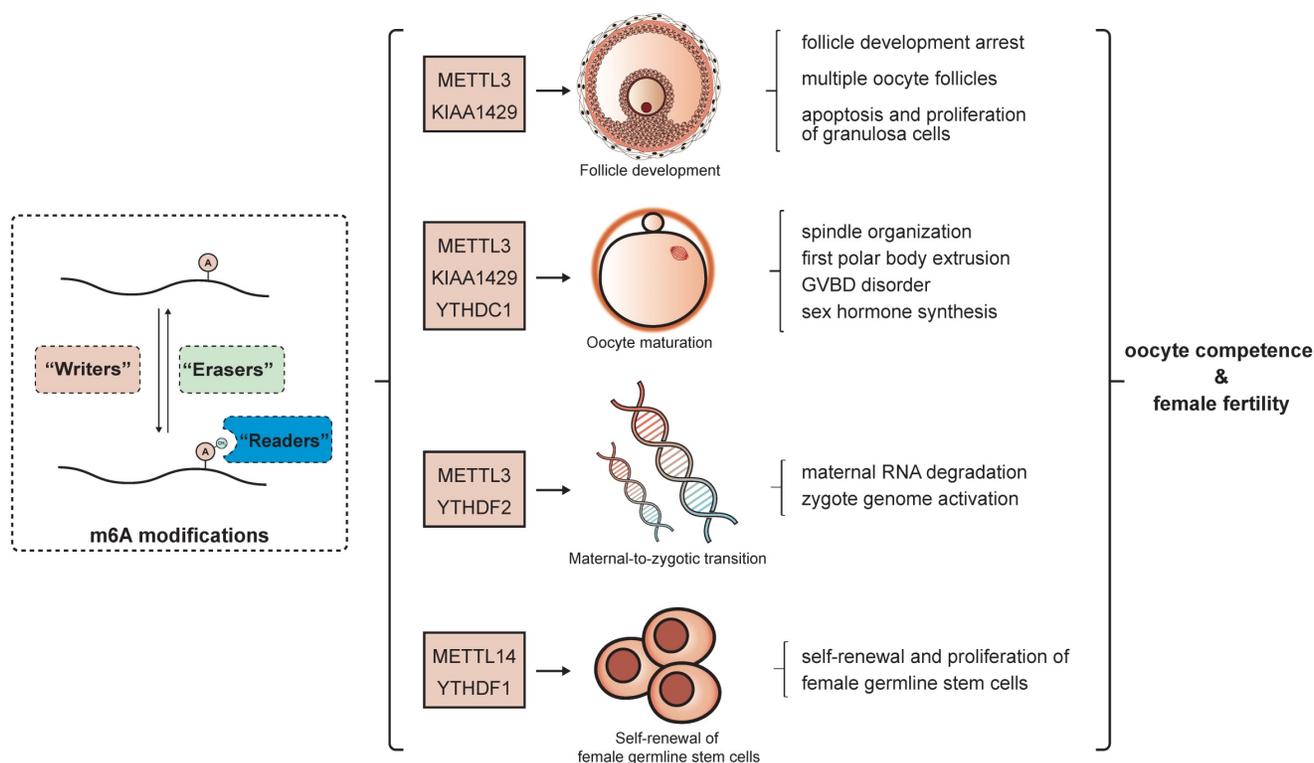


Figure 2. Role of m⁶A modification in gonad development. The m⁶A modification regulates oogenesis in terms of follicle development, oocyte maturation, maternal-to-zygotic maturation and self-renewal of female germline stem cells, which partially determine oocyte competence and female fertility.

Oocyte maturation

Under the stimulation of LH surge, fully grown oocytes emerge from meiotic arrest and complete subsequent developmental progression up to MII stage prior to ovulation and fertilization. This process, which marks the maturation of oocytes, is largely dependent on precise epigenetic regulation in oocytes [16]. The roles of m⁶A in oocyte maturation encompass multiple aspects, including RNA splicing, degradation, transport and translation.

METTL3 expression shares a similar pattern with m⁶A levels in the processes of oogenesis and early embryonic development. They are both abundant in GV and MII oocytes, then dramatically decrease in the zygote and gradually increase during early embryogenesis. *Mettl3* knockdown at the GV stage hindered spindle organization and first polar body extrusion in MII oocytes, which could be partially attributed to decreased translational efficiency of several meiosis-related genes [94]. In zebrafish, GVBD disorder and oocyte incompetence were observed in *mettl3*-mutant females. The *mettl3* mutation disturbed sex hormone levels by regulating genes involved in sex hormone synthesis and gonadotropin signaling. A decreased GVBD rate could be partially rescued by hCG or 17 α -20 β -DHP treatment [92]. Oocyte-specific conditional knockout (cKO) of *Kiaa1429* led to a significantly reduced GVBD rate and first polar body

extrusion rate. Mechanistically, *Kiaa1429* cKO impaired the correct localization of splicing-related factors SRSF3 and YTHDC1, perturbed the alternative splicing of oogenesis-related transcripts, and led to meiotic arrest [93]. The nuclear m⁶A reader YTHDC1 regulates RNA splicing in oocytes by interacting with pre-mRNA processing factors CPSF6, SRSF3, and SRSF7. Loss of YTHDC1 changed the alternative polyadenylation pattern and led to massive alternative splicing defects in the mouse oocyte nucleus [66]. It is worth mentioning that both *Kiaa1429*-deficient and *Ythdf1*-deficient oocytes showed abnormal cytoplasmic RNA granules. Considering that both KIAA1429 and YTHDF1 are closely related to m⁶A-mediated RNA splicing, this RNA mislocalization may be caused by inappropriate posttranscriptional processing and transport [66, 93]. However, the impact of these RNA granules is still unclear.

Maternal-to-zygotic transition

The maternal-to-zygotic transition (MZT) consists of selective maternal mRNA degradation and zygotic genome activation. The MZT starts at the resumption of oocyte meiosis rather than fertilization [95]. Correct elimination of maternal transcripts lays the foundation for zygotic genome activation and the upcoming embryogenesis [16, 96], and is part of the oocyte competence built up in preceding

developmental processes. It has been demonstrated that MZT is partially controlled m⁶A modification.

Mettl3 knockdown impeded embryonic development from the two-cell stage to four-cell stage in activated and diploidized MII oocytes. The *Mettl3*-morpholino treated group showed remarkably decreased global transcription. RNA-seq assays detected hundreds of upregulated genes, part of which are enriched in GO terms related to RNA metabolic processes and cell cycle, demonstrating that *METTL3* knockdown may affect MZT by impairing mRNA degradation [94]. The clearance of maternal RNAs was disturbed in *ythdf2*-deficient zebrafish. Upregulated maternal transcripts and downregulated zygotic transcripts were observed in both *ythdf2* homozygous mutant oocytes and *ythdf2*-morpholino treated oocytes. The *ythdf2*-deficiency-mediated RNA retention occurred in a m⁶A preferential manner, leading to obvious embryonic developmental delay in zebrafish [97]. In mice, *Ythdf2* knockout did not affect follicle development or oocyte meiosis, but caused serious embryonic developmental defects, resulting in complete female infertility. YTHDF2 deficiency mainly affected gene expression in MII oocytes instead of GV oocytes. This suggested that YTHDF2 did not regulate the maternal transcriptome but was required to maintain the appropriate transcript dosage [98].

Self-renewal of female germline stem cells

The discovery of female germline stem cells (FGSCs) provided a theoretical basis to obtain additional oocytes even after birth. Zou et al. established a neonatal mouse FGSC line and produced offspring using FGSCs retrieved from adult mice, providing a novel idea for research on oogenesis [99].

RNA m⁶A profiling revealed differential m⁶A patterns between FGSCs and somatic STO cells. Compared to STO cells, FGSCs had higher m⁶A abundance and manifested dissimilar mRNA expression levels of m⁶A regulators. Knockdown of the m⁶A reader *YTHDF1* hindered the proliferation of FGSCs, but the specific mechanisms have not yet been explored [100]. *METTL14* participates in the self-renewal of FGSCs by facilitating the m⁶A modification of circGFR α 1, which acts as a sponge of miR-449 and promotes the expression of their common target GFR α 1 to sustain FGSCs proliferation. *METTL14* knockdown resulted in decreased m⁶A modification of circGFR α 1, which did not change its expression level but led to its nuclear retention. Similarly, m⁶A-motif-mutated circGFR α 1 mainly accumulated in the nucleus after its overexpression, suggesting that cytoplasmic transport was dependent

on m⁶A [101]. All these findings support the involvement of m⁶A in postnatal oocyte production from FGSCs.

m⁶A in female reproductive diseases

There is evidence that m⁶A modification takes part in the process of inflammation and immune activity [102, 103]. Considering that inflammatory or immune disorders play a critical role in many female reproductive diseases such as endometriosis and adenomyosis, polycystic ovary syndrome, preeclampsia and spontaneous abortion [104, 105], it stands to reason that m⁶A modification may also contribute to the pathological mechanisms underlying these diseases.

Endometriosis and adenomyosis

Bioinformatic analyses revealed that most of the m⁶A regulators in the eutopic endometrium were aberrantly expressed compared to normal or eutopic endometrium. Among all the regulators, *HNRNPA2B1* and *HNRNPC* were negatively correlated with the severity of endometriosis, and might influence infiltrating immune cells [106]. The m⁶A content was found to be dramatically decreased in eutopic and ectopic samples, which was consistent with decreased *METTL3* and increased *ALKBH5* levels. *METTL3* regulates the migration and invasion of endometrial stromal cells by facilitating DGCR8-mediated maturation of miR126, contributing to endometriosis development [107]. Similarly, the expression pattern of m⁶A regulators in the myometrium was also found to be different from normal tissues. The cluster of m⁶A regulators may potentially affect the immune response and cell adhesion according to data mining, playing roles in the myometrium dysfunction [108].

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is often accompanied by insulin resistance. *FTO* overexpression diminished m⁶A modification in *FLOT2* transcripts and enhanced its expression in granulosa cell line KGN. *FLOT2* is associated with insulin resistance and was found to be upregulated in granulosa cells from PCOS patients. *FTO* was found to regulate cell proliferation, apoptosis and insulin resistance in KGN cells through a *FLOT2*-dependent manner, indicating that *FTO* might participate in regulating insulin resistance in PCOS [109]. Luteinized granulosa cells from non-obese PCOS patients had higher m⁶A levels and exhibited a dissimilar distribution pattern of m⁶A peaks in comparison to the control group. Mechanistically, the *FOXO3* transcript was m⁶A modified and underwent

YTHDF2-mediated decay in controls, which was in contrast to the samples from PCOS patients. Reduced m⁶A modification of the *FOXO3* mRNA in controls enhanced the expression of *FOXO3* after *METTL3* or *METTL14* knockdown, while having the inverse effect after *FTO* knockdown. However, this effect was absent from the PCOS granulosa cells [110]. These findings illustrate a potential mechanism driving insulin resistance in PCOS patients.

Premature ovarian insufficiency

In addition to increased m⁶A modification in naturally aging mouse ovaries [111], the levels of m⁶A modification were also found to be obviously upregulated in either ovarian tissues or human granulosa cells from premature ovarian insufficiency (POI) patients. This elevation was associated with a relative reduction of *FTO* compared to *ALKBH5*. *FTO* knockdown impeded cell proliferation, alleviated apoptosis and disturbed marker expression in human granulosa cells [112]. Furthermore, m⁶A was also increased in a mouse model of cyclophosphamide (CTX)-induced premature ovarian insufficiency in a concentration- and time-dependent manner. CTX treatment upregulated the m⁶A writers *METTL3*, *METTL14*, *ZC3H13* and *KIAA1429*, while downregulating the demethylase *FTO* and several m⁶A readers [112, 113]. These results indicate that m⁶A modification may act as a potential biomarker of ovarian dysfunction [112, 113].

Preeclampsia

Dysfunction of the placenta in preeclampsia and eclampsia patients usually leads to abnormal fetal growth and serious complications for the pregnant women. Previous studies have identified the role of epigenetics in preeclampsia and fetal growth restriction [114, 115]. The potential involvement of m⁶A modifications in the pathological processes of preeclampsia has been proposed based on several lines of evidence. MeRIP-seq analysis demonstrated a correlation between higher m⁶A levels at the 5'-UTR in placental mRNAs and small-for-date children, whereas decreased m⁶A levels near stop codons were dominant in heavy-for-date placenta samples. Higher *SMPD1* protein levels in preeclampsia placenta samples, in particular, are more likely to result from m⁶A enrichment at 5'-UTR instead of increased mRNA levels [116]. Enhanced *METTL3* expression was found to be responsible for increased m⁶A methylation in placental trophoblasts from preeclampsia patients. These aberrant m⁶A changes lead to the upregulation of *hnRNPC1/C2* expression, which may induce vitamin D deficiency by inducing *VDR* and trophoblast dysfunction [117]. Furthermore, *METTL3* was found to facilitate the maturation of *miR-497-5p/195-5p* by mediating *DGCR8*'s recognition of *pri-miR-497-5p/195-5p* in an m⁶A-dependent manner. Increased *miR-497-5p/195-5p* in the preeclampsia-affected placenta was found to suppress *WWP1* expression, disturbing trophoblast proliferation, migration, and invasion, eventually aggravating preeclampsia progression [118].

Table 1. Role of m⁶A in female reproductive diseases.

diseases	m ⁶ A regulator	aberrant expression	target	effect of m ⁶ A on target RNA	effect of m ⁶ A in diseases	reference
Endometriosis	<i>METTL3</i>	↓	miR-126	Enhanced <i>DGCR8</i> -mediated maturation of <i>pri-miR126</i>	Decreased <i>METTL3</i> and m ⁶ A promoted the migration and invasion of endometrial stromal cells in endometriosis	[107]
Polycystic ovary syndrome	<i>FTO</i>	-	<i>FLOT2</i>	<i>FTO</i> overexpression diminished m ⁶ A modification in <i>FLOT2</i> transcripts and enhanced its expression in granulosa cell line KGN	<i>FTO</i> might regulate insulin resistance process in PCOS by enhancing <i>FLOT2</i> expression	[109]
	<i>YTHDF2</i>	-	<i>FOXO3</i>	Lower m ⁶ A levels inhibited <i>YTHDF2</i> -mediated <i>FOXO3</i> transcript decay	m ⁶ A might regulate function of granulosa cells by controlling <i>FOXO3</i> expression	[110]
Premature ovarian insufficiency	<i>FTO</i>	↓	-	-	<i>FTO</i> knockdown impeded cell proliferation, alleviated cell apoptosis and disturbed expression of cell markers in human granulosa cells	[112]
Preeclampsia	-	-	<i>SMPD1</i>	m ⁶ A modification of <i>SMPD1</i> at the 5'-UTR promoted protein translation at posttranscriptional level	m ⁶ A levels in placenta were related to birth weight	[116]
	<i>METTL3</i>	↑	<i>hnRNPC1/C2</i>	<i>METTL3</i> knockdown significantly reduced <i>hnRNPC1/C2</i> expression in trophoblast cells	-	[117]
	<i>METTL3</i>	-	miR-497-5p/195-5p	<i>METTL3</i> facilitated maturation of <i>miR-497-5p/195-5p</i> by mediating recognition of <i>pri-miR-497-5p/195-5p</i> by <i>DGCR8</i>	<i>METTL3</i> -mediated upregulation of <i>miR-497-5p/195-5p</i> disturbed trophoblast proliferation, migration, and invasion, eventually aggravating preeclampsia progression	[118]
Recurrent miscarriage/spontaneous abortion	<i>METTL14</i>	↑	<i>lncHZ01</i>	<i>METTL14</i> stabilized <i>lncHZ01</i> and formed a positive feedback loop with <i>lncHZ01</i>	<i>METTL14</i> enhanced <i>lncHZ01</i> expression to inhibit trophoblast cell proliferation and induces miscarriage.	[122]
	<i>ALKBH5</i>	↑	<i>CYR61</i>	<i>ALKBH5</i> diminished m ⁶ A modification on <i>CYR61</i> mRNA, impaired its stability and subsequent protein expression	inhibited proliferation and invasion of trophoblast at the maternal-fetal face in early pregnancy	[121]

Recurrent miscarriage/spontaneous abortion

The pathogenesis of recurrent miscarriage (RM) has not been fully elucidated yet. In addition to the known factors including genetic, endocrine, anatomical, and immunological disorders [119], epigenetic abnormalities are also part of the underlying mechanisms [120]. So far, the research on the role of m⁶A in RM has mainly focused on its effects on trophoblast function. Higher ALKBH5 expression and lower m⁶A levels were found in chorionic villi from RM patients. ALKBH5 diminished m⁶A modification of *CYR61* mRNA, reducing its stability and subsequent protein expression. These changes inhibited the proliferation and invasion of trophoblast cell at the maternal-fetal interface in early pregnancy, suggesting that ALKBH5 may be a potential key factor in the pathogenesis of RM [121]. The m⁶A modification was also found to be involved in BPDE-related RM. BPDE, a metabolite of environmental benzo(a)pyrene, was found to upregulate *lncHZ01* in trophoblast cells, and *lncHZ01*-mediated *MXD1* upregulation promoted *METTL14* transcription, which increased m⁶A modification and enhanced the stability of *lncHZ01*, forming a positive feedback loop that eventually inhibited trophoblast cell proliferation and induced miscarriage [122]. Higher m⁶A levels and several aberrantly expressed m⁶A regulators were found in spontaneous abortion (SA) patients. FTO, in particular, was significantly downregulated in the chorionic villi and trophoblasts of SA patients. Lower FTO changed the m⁶A patterns of several genes involved in immunotolerance, immune cell infiltration and angiogenesis, demonstrating a possible role of m⁶A in SA progression [123].

Conclusions

The research on m⁶A RNA modification has expanded broad horizons for researchers and uncovered several new epigenetic mechanisms underlying a number of pathophysiological processes. This reversible modification is controlled by three kinds of regulators and influences various RNA-related processes such as RNA degradation, splicing, transport and translation, regulating gene expression from multiple aspects at the post-transcriptional level. The involvement of m⁶A modifications has been observed in various bioprocesses including cell differentiation, development, metabolism, stress response and other cellular activities [2, 13-15], serving as an important epigenetic regulatory mechanism underlying both physiological and pathological processes.

In the female reproductive system, m⁶A modifications regulate multiple stages of oogenesis,

including follicle development, oocyte maturation, and the maternal-to-zygotic transition, as well as the self-renewal of female germline stem cells artificially established *in vitro*. Furthermore, m⁶A participates in the pathogenesis of a variety of female reproductive diseases including endometriosis and adenomyosis, polycystic ovary syndrome, premature ovarian insufficiency, preeclampsia, and recurrent miscarriage, all of which underscore the impact of m⁶A on female fertility.

Recently several researches have revealed the clinical potential of m⁶A-targeting strategies. *METTL3* inhibitors exhibited the promising anti-cancer effect against acute myeloid leukemia [124, 125]. Pharmacological inhibition of FTO significantly suppressed leukemia stem/initiating cell self-renewal and sensitized leukemia cells to T cell cytotoxicity [126]. However, m⁶A was only considered as a prospective therapeutic target of cancer treatment in current evidences [127]. Considering its crucial participation in female gonad development and reproductive diseases, m⁶A-targeting interventions should also be taken into account as promising approaches in treatment of infertility and reproductive disorders.

Nevertheless, our understanding of the full impact of m⁶A is far from complete. For example, how different m⁶A readers recognize m⁶A-modified transcripts and lead to different transcript outcomes is still unclear. More importantly, translational studies aiming to utilize m⁶A as a novel target for therapy are in their infancy. All these unsolved questions merit further studies in the future.

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Competing Interests

The authors have declared that no competing interest exists.

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