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The *N*⁶-methyladenosine RNA modification in pluripotency and reprogramming

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Summary

Chemical modifications of RNA provide a direct and rapid way to manipulate the existing transcriptome, allowing rapid responses to the changing environment further enriching the regulatory capacity of RNA. *N*⁶-methyladenosine (*m*⁶A) has been identified as the most abundant internal modification of messenger RNA in eukaryotes, linking external stimuli to an intricate network of transcriptional, post-transcriptional and translational processes. *M*⁶A modification affects a broad spectrum of cellular functions, including maintenance of the pluripotency of embryonic stem cells (ESCs) and the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). In this review, we summarize the most recent findings on *m*⁶A modification with special focus on the different studies describing how *m*⁶A is implicated in ESC self-renewal, cell fate specification and iPSC generation.

Keywords

METTL3; *N*⁶-methyladenosine; RNA methylation; stem cell; iPSC

Introduction

Massive transcriptome sequencing through high-throughput platforms has revealed that the vast majority of the mammalian genome undergoes transcription into RNA, but many RNA transcripts are never translated into protein [1]. RNA is not only an essential intermediate in

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the flux of genetic information from DNA to proteins, but rather is a molecule that plays crucial roles in the regulation of fundamental cellular processes, being the dysregulation of certain RNAs implicated in a number of important pathological processes, including cancer [2, 3]. The coding and non-coding transcriptome is extensively and dynamically regulated by chemical modification adding a new layer of complexity and functionality to the emerging roles of RNAs in health and disease [4]. Such covalent modifications provide a direct and rapid way to manipulate the existing transcriptome, allowing fast responses to the changing environment [5]. To date, at least 100 chemically distinct RNA modifications have been identified [6-8], among which *N*⁶-methyladenosine (m⁶A) is the most abundant in messenger RNA (mRNA) and long non-coding RNA (lncRNA) [9]. Although m⁶A was detected in poly(A)⁺ RNA in the 1970s [10-13], it is only recently that transcriptome-wide profiling studies have revealed that m⁶A is pervasive throughout the transcriptome and it is distributed within the R(m⁶A)CH consensus motif (where R = G or A; H = A, C, or U) [14-17]. Despite the strong consensus, only a small fraction of RACH sites is detectably methylated in vivo, arguing that the sequence motif is not sufficient to determine the distribution of m⁶A.

The m⁶A landscape is dynamically regulated by a complex interplay between various families of m⁶A-specific proteins, termed ‘writers’, ‘erasers’ and ‘readers’ which add, remove and decipher the m⁶A mark, respectively [18]. The core mammalian m⁶A methyltransferase complex (‘writers’) includes the methyltransferase-like 3 (METTL3, also known as MT-A70) [19] and the methyltransferase-like 14 (METTL14) [20, 21]. The complex associates with Wilms’ tumor 1 associating protein (WTAP), which is necessary for catalytic activity of the m⁶A methyltransferase complex in vivo [20, 22]. The presence of m⁶A erasers suggests that the effects of m⁶A can potentially be reversed, and that cellular demethylation pathways might be a mechanism of dynamic m⁶A regulation within mRNAs. Fat mass and obesity-associated protein (FTO) and α -ketoglutarate-dependent dioxygenase alkB homologue 5 (ALKBH5) are the two m⁶A demethylases (‘erasers’) identified to date [23-25]. The discovery of the ‘readers’ has been a milestone in elucidating the impact of such modification in RNA. Different ‘readers’ with different cellular localization, influence almost all aspects of RNA metabolism. Proteins with YTH-domain located in the cytoplasm (YTHDF1, YTHDF2 and YTHDF3) and in the nuclei (YTHDC1 and YTHDF2) directly recognize the m⁶A mark [15, 26]. YTHDF1 promotes cap-dependent translation [27], and YTHDF2 regulates mRNA stability by localizing m⁶A-containing mRNA to cellular RNA decay sites [26]. Interestingly, upon heat shock stress, YTHDF2 localizes to the nucleus where preserves 5’UTR methylation and promotes cap-independent translation initiation [28], and YTHDC1 directly regulates splicing events through recruitment of pre-mRNA splicing factors to the targeted RNA [29]. Also in the nuclei, the RNA-binding protein heterogeneous nuclear ribonucleoprotein A2B1 (HNRNPA2B1) recognizes m⁶A sites and promotes microRNA (miRNA) biogenesis and alternative splicing [30], and the binding of pre-mRNA splicing factor heterogeneous nuclear ribonucleoprotein C (HNRNPC) to the introns of its targeted mRNAs after ‘m⁶A switch’ affects their processing [31]. In the cytoplasm, a single m⁶A in the 5’ UTR is sufficient to boost cap-independent translation through interaction with the eukaryotic translation initiation factor eIF3 and subsequent ribosome recruitment [32]. Therefore, m⁶A modification operates in a cascade of cellular

processes, including circadian rhythm [33], cell meiosis [34], sperm development [24], X chromosome inactivation [35], embryonic stem cell (ESC) self-renewal and differentiation [36–38], and the reprogramming of somatic cells into induced pluripotency stem cell (iPSC) [36, 38, 39] among others, and thus alteration of m⁶A homeostasis is implicated in obesity [40], cancer [41–43], and many other human disorders [44–46] (Figure 1).

m⁶-methyladenosine decorates RNAs in mammalian embryonic stem cells

The role of m⁶A modification in embryonic stem cells (ESCs) has been recently investigated with discrepant results among different studies. The first proposed model describes that m⁶A methylation is required to keep ESCs in a pluripotent state [21] whereas a more recent emerging model postulates that m⁶A is not required for ESC maintenance but for transition of ESCs to differentiated lineages [37, 38]. Supporting the first model, knockdown of either *Mettl3* or *Mettl14* in mouse ESCs resulted in reduced levels of m⁶A modification, decreased expression of pluripotency genes, increased expression of developmental regulators, and reduced self-renewal capability [21]. The same study showed that m⁶A modification selectively targeted lineage specific markers rather than pluripotency-related genes. The presence of m⁶A methylation on mRNA encoding developmental regulators promoted their destabilization by preventing Hu-antigen R (HuR; also known as ELAVL1) binding, which blocked microRNA targeting [47, 48], thereby maintained the mouse ESC in a pluripotent state [21] (Figure 2A). However, posterior studies stated that METTL3 operates to control stem cell differentiation and showed that mRNA encoding the core pluripotency regulators, including *Nanog*, *Sox2*, and *Myc*, were modified with m⁶A [37, 38]. Genetic ablation of *Mettl3* retained the high pluripotent capability of ESCs which were unable to differentiate towards cardiomyocytes and the neural lineage. Moreover, teratomas derived from *Mettl3* KO ESCs were poorly differentiated and were highly stained with NANOG and the proliferation marker KI67 [37]. A different study showed that depletion of *Mettl3* in both naïve mouse ESCs and primed (epiblast stem cell, EpiSC) states resulted in upregulation of pluripotent and developmental regulators, respectively [38]. This seemingly contradictory effect was explained by the fact that METTL3 targeted the dominating transcripts in either state, increasing the abundance and dominance of already-expressed genes. Therefore, in naïve ESCs, where transcript levels of pluripotency genes are highly abundant, depletion of *Mettl3* resulted in higher half-life of the pluripotent genes, leading to the so-called hyper-naïve pluripotent state. By contrast, in primed EpiSC where the lineage commitment makers become abundantly expressed, loss of *Mettl3* led to fast differentiation, ultimately disrupting the stability of the primed state and resulting in cell death [49]. In vivo, *Mettl3* knockout epiblasts and naïve ESCs were viable at the pre-implantation stage. However, they failed to adequately repress pluripotent genes, being unable to exit the naïve state at the post-implantation period, and leading to embryonic lethality [38]. More recently, another study showed that Zinc finger protein 217 (ZFP217) had crucial regulatory functions in m⁶A deposition, adding a layer of complexity to our understanding of the molecular events regulating RNA methylation in ESCs [36]. ZFP217 enhanced the expression of the core pluripotency factors *Nanog*, *Sox2* and *Myc*, and protected such transcripts from rapid degradation. To achieve this dual function ZFP217 interacted with epigenetic modifiers and bound at the regulatory regions of such pluripotency-associated genes to enhance mRNA

transcription, and at the same time, ZFP217 sequestered METTL3, diminishing METTL3 binding to RNAs to restrain m⁶A deposition (Figure 2B). Interestingly, ZFP217 did not interact with METTL4 strongly suggesting that METTL3-ZFP217 was held in an inactive complex. ZFP217 occupancy at promoters of genes encoding ZFP217-bound transcripts and m⁶A-methylated RNAs was higher than at unbound and unmodified transcripts, indicating that epigenetic mechanisms may impose the composition of the ESC epitranscriptome [36].

The role of m⁶ methyadenosine RNA modification in reprogramming

Although a function of m⁶A in regulating cell reprogramming by using iPSC technology has been described in several reports, there is a controversy whether m⁶A can enhance or block the reprogramming process. In one study, combinatorial overexpression of METTL3 with the Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC [OSKM]) [50, 51] in mouse embryonic fibroblasts (MEFs) promoted the accumulation of m⁶A, enhanced the expression of key pluripotency factors and improved the reprogramming efficiency. Conversely, the number of iPSC colonies was reduced after knock down of *Mettl3* and with the addition of cycloleucine, a competitive inhibitor of methionine adenosyltransferase [52], during the reprogramming process [39]. Interestingly, the authors revealed a role of microRNAs (miRNAs) in inducing the novo m⁶A methylation by altering the binding of METTL3 to mRNAs containing miRNA targeting sites, which actively promoted cell reprogramming efficiency. On the contrary, another study attributed the reduced numbers of iPSC colonies in reprogramming MEFs transduced with *Mettl3* siRNA to reduced proliferation capacity of such cells at early stages of reprogramming. Thus, loss of *Mettl3* in MEFs resulted in a severe block in somatic replication compared to MEFs transduced with siRNA control. However, depletion of *Mettl3* through reprogramming after at least 3 days of induced OSKM expression did not compromise the reprogramming efficiency [38]. In agreement with the later study, somatic cell reprogramming was impaired after depletion of *Zfp217* that led to an increase in global m⁶A levels in reprogramming MEFs. Conversely, concomitant depletion of *Mettl3* partially rescued iPSC reprogramming in *Zfp217*-depleted cells, suggesting that increased m⁶A levels occurring upon *Zfp217* knockdown was a barrier for efficient somatic cell reprogramming [36].

Concluding remarks

An increasing body of research indicates that m⁶A modification is part of an ancient, yet just recently explored mechanism to modulate both pluripotency and cellular differentiation through distinct biological activities. Future investigations in this area are necessary in order to understand the existing discrepancies on the role of m⁶A modification in ESCs. One model proposes that m⁶A methylation on developmental regulators destabilizes such transcripts thereby maintaining pluripotency [21], and that increased m⁶A abundance promotes the reprogramming of MEFs to pluripotent stem cells [39]. On the contrary, other studies postulate that m⁶A is not required for ESC maintenance but for transition of ESCs to differentiated lineages [37, 38], and that increased levels of m⁶A after depletion of ZFP217 causes a dramatic reduction in reprogramming efficiency [36]. Such differences could be explained by variations in the media conditions in which the cells are cultured that would shift the balance between pluripotent stages [38]. Thus, similar to the first described model,

depletion of *Mettl3* in primed cells would reduce stem cell self-renewal and push the cells toward differentiation. By contrast, and as proposed by posterior studies, depletion of *Mettl3* in the ground naïve state would promote stem cell maintenance and block differentiation. A common characteristic of both models is the focus of just one of the many functions of m⁶A modification: decrease of the RNA stability. How do other aspects of RNA metabolism mediated by m⁶A methylation affect the equilibrium between stemness and differentiation? Significant advances will be gained after the identification of new ‘readers’ and further elucidation of their interplay to dictate the downstream function of the m⁶A mark. Another important goal to achieve is the identification of additional factors that control m⁶A deposition during differentiation of ESCs towards different lineages or during somatic cell reprogramming. Such is the case of ZFP217, which restricts the methyltransferase activity of METTL3 in ESCs to prevent aberrant methylation of the pluripotency factors [36]. Other fundamental questions remain unknown and need careful consideration for future studies: (i) What additional factors that remain to be determined control the specificity of such methylated sites? (ii) Is the m⁶A methylation dictated by epigenetic regulation? (iii) How do other RNA post-transcriptional modification pathways converge to functionally regulate stem cell fate decisions? (iiii) Could some of the processes be targeted for therapeutic applications in regenerative medicine? Further investigation will definitively answer some of these questions and expand our current understanding of the underlying mechanisms for stem cell self-renewal and iPSC generation associated with the stem cell epitranscriptome in this fast-growing field.

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- m⁶A methylation is the most abundant modification in mRNA and lncRNA
- m⁶A landscape is dynamically regulated by ‘writers’, ‘erasers’ and ‘readers’
- The role of m⁶A modification in ESCs and iPSC generation is controversial
- m⁶A modification modulates both pluripotency and cellular differentiation

Figure 1. Scheme of m⁶A reversible methylation and mediated functions

Dynamic m⁶A modification is regulated by writers (METTL3, METTL14 and WTAP) and erasers (FTO and ALKBH5). Recognition of m⁶A by different readers in the nucleus or the cytoplasm mediates divergent functions, including splicing, pri-miRNA processing, translation, and RNA stability. Unbalanced m⁶A regulation will cause defects in adipogenesis, spermatogenesis, development, carcinogenesis, stem cell self-renewal and differentiation, and X-chromosome inactivation among others.

Figure 2. m⁶A RNA methylation regulates embryonic stem cell fate

(A) In naïve ESCs, depletion of *Mettl3* results in a decrease of global m⁶A levels on developmental regulators which in turn promote ESC differentiation. (B) In naïve ESCs, ablation of *Mettl3* results in a decrease of m⁶A at pluripotency RNAs. As a result, cells remain in a ‘hypernaïve’ pluripotent state and fail to differentiate. On the contrary, depletion of *Zfp217* increases m⁶A deposition at pluripotency RNAs, promoting cell differentiation and/or cell death. In EpiSC, ablation of *Mettl3* decreases m⁶A levels on developmental regulators, pushing cells towards differentiation and/or cell death. A and B reflect different models currently existing in the field.