m^6A RNA Methylation Is Regulated by MicroRNAs and Promotes Reprogramming to Pluripotency

Highlights

- m^6A modification has gene- and cell-type-specific features
- m^6A modifications are enriched at miRNA targeting sites
- miRNAs regulate m^6A abundance by modulating METTL3 binding to mRNAs
- Increased m^6A formation promotes cell reprogramming to pluripotency

In Brief

Zhou and colleagues show that formation of m^6A on mRNAs is regulated by miRNAs via a sequence pairing mechanism, and that in addition to differential distribution in pluripotent and differentiated cells, m^6A has a positive influence on reprogramming to pluripotency.

Accession Numbers

GSE52125
m^6A RNA Methylation Is Regulated by MicroRNAs and Promotes Reprogramming to Pluripotency

Tong Chen,1,6,7 Ya-Juan Hao,2,6,7 Ying Zhang,3,7 Miao-Miao Li,5,6,7 Meng Wang,1 Weifang Han,3,6 Yongsheng Wu,2 Ying Lv,2,6 Jie Hao,3 Libin Wang,3,6 Ang Li,2,6 Ying Yang,2,6 Kang-Xuan Jin,2,4 Xu Zhao,2,6 Yuhuan Li,3 Xiao-Li Ping,2,6 Wei-Yi Lai,6 Li-Gang Wu,5 Guibin Jiang,4 Hai-Lin Wang,4 Lisi Sang,3,6 Xi-Jie Wang,1,7 Yun-Gui Yang,2,7 and Qi Zhou3,4

1Key Laboratory of Genetic Network Biology, Collaborative Innovation Center of Genetics and Development, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
2Key Laboratory of Genomics and Precision Medicine, Collaborative Innovation Center of Genetics and Development, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China
3State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100085, China
4State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
5State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
6University of Chinese Academy of Sciences, Beijing 100049, China
7Co-first author
*Correspondence: xjwang@genetics.ac.cn (X.-J.W.), ygyang@big.ac.cn (Y.-G.Y.), qzhou@ioz.ac.cn (Q.Z.)

http://dx.doi.org/10.1016/j.stem.2015.01.016

SUMMARY

N^6^-methyladenosine (m^6A) has been recently identified as a conserved epitranscriptomic modification of eukaryotic mRNAs, but its features, regulatory mechanisms, and functions in cell reprogramming are largely unknown. Here, we report m^6A modification profiles in the mRNA transcriptomes of four cell types with different degrees of pluripotency. Comparative analysis reveals several features of m^6A, especially gene- and cell-type-specific m^6A mRNA modifications. We also show that microRNAs (miRNAs) regulate m^6A modification via a sequence pairing mechanism. Manipulation of miRNA expression or sequences alters m^6A modification levels through modulating the binding of METTL3 methyltransferase to mRNAs containing miRNA targeting sites. Increased m^6A abundance promotes the reprogramming of mouse embryonic fibroblasts (MEFs) to pluripotent stem cells; conversely, reduced m^6A levels impede reprogramming. Our results therefore uncover a role for miRNAs in regulating m^6A formation of mRNAs and provide a foundation for future functional studies of m^6A modification in cell reprogramming.

INTRODUCTION

More than 100 types of post-transcriptional modifications have been identified in RNAs so far (Cantara et al., 2011; Globisch et al., 2011; He, 2010), among which N^6^-methyladenosine (m^6A) RNA methylation is one of the most prevalent modifications of messenger RNAs (mRNAs) (Desrosiers et al., 1974; Wei et al., 1975). m^6A accounts for about 50% of total methylated ribonucleotides and is present in 0.1%–0.4% of all adenosines in total cellular RNAs (Desrosiers et al., 1974; Wei et al., 1975). In vivo, the formation of m^6A is catalyzed by a multi-component methyltransferase complex with at least three proteins, namely methyltransferase-like 3 (METTL3), METTL14, and Wilms’ tumor 1-associating protein (WTAP) (Bokar et al., 1997; Finkel and Groner, 1983; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014; Wang et al., 2014b). The m^6A modification can be removed by RNA demethylases, of which the two known ones are fat mass and obesity-associated protein (FTO) and alkylated DNA repair protein alkB homolog 5 (ALKBH5) (Jia et al., 2011; Zheng et al., 2013). So far, two YTH (YT521-B homology)-domain containing proteins, YTHDF2 and YTHDC1, have been identified to specifically recognize m^6A-modified RNAs (Dominghini et al., 2012; Xu et al., 2014; Zhu et al., 2014).

In general, m^6A modification can be detected in the mRNAs of over 7,000 genes in mammalian cells, and it tends to occur at the consensus RRACH motif (R = G or A; H = A, C, or U) (Bodi et al., 2010; Dominghini et al., 2012; Harper et al., 1990; Meyer et al., 2012; Wei and Moss, 1977). On average, the frequency of m^6A modification is about one peak per 2,000 nucleotides (nts), but there are also some regions with clustered m^6A peaks (Dominghini et al., 2012; Kane and Beemon, 1985; Meyer et al., 2012). Strong enrichment of m^6A modification has been found near the stop codons of mRNAs (Dominghini et al., 2012; Meyer et al., 2012).

Although the existence of m^6A does not change the coding capacity or base pairing of adenine with uracil or thymine, it may block the nonstandard A:G base pairing and influence RNA structures (Dai et al., 2007). The presence of m^6A may also affect the expression level, translation efficiency, nuclear retention, splicing, and stability of mRNAs (Camper et al., 1984; Finkel and Groner, 1983; Fustin et al., 2013; He, 2010; Hess et al., 2013; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2013; Tuck et al., 1999; Wang et al., 2014a, 2014b; Zhao et al., 2014; Zheng et al., 2013). Deficiency of m^6A
formation has been proven to affect circadian rhythm, cell meiosis, and embryonic stem cell (ESC) proliferation, and thus it is implicated in obesity, cancer, and other human diseases (Batista et al., 2014; Dominissini et al., 2012; Geula et al., 2015; He, 2010; Liu and Jia, 2014; Liu et al., 2013; Machnicka et al., 2013; Meyer et al., 2012; Niu et al., 2013; Sibbritt et al., 2013). However, the regulatory mechanisms of m6A formation and the function of m6A in regulating cell reprogramming are still largely unknown.

Here we examined the transcriptome-wide distribution of m6A modification in mouse ESCs, induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), and testicular sertoli cells (SCs). Our results identified the difference in m6A distribution between pluripotent and differentiated cell types. We discovered that the m6A formation of mRNAs is regulated by microRNAs (miRNAs) via a sequence pairing mechanism, and we revealed m6A as a positive regulator for cell reprogramming to pluripotency.

RESULTS

General Features of m6A Distribution in Mouse Pluripotent and Differentiated Cell Lines

To investigate the features and distribution dynamics of mRNA m6A modification in different cell types, we performed m6A-seq using mouse ESCs, iPSCs, NSCs, and SCs. In total, 33,000–43,000 m6A-enriched regions, also known as m6A peaks, were identified on mRNAs of 7,000–8,000 expressed genes in each cell type. Using m6A-qRT-PCR, 13 out of 15 randomly selected m6A peaks were verified in all cell types (Figures S1A and S1B), implying a high authenticity of our data. Genes encoding transcripts with m6A modifications involved in many essential biological processes, including transcription regulation, chromatin modification, cell cycle control, apoptosis, etc., among which transcripts encoding proteins for DNA binding activity were identified as the most significantly enriched group (counted for over 10% of m6A-modified genes) (Figure S1C, Table S1, and Table S2).

Similar to previous reports (Dominissini et al., 2012; Meyer et al., 2012), we also observed a tendency toward m6A distribution in the coding sequence (CDS) region of mRNAs, with a strong enrichment around the translation termination sites (TsTS) in all four examined cell types (Figure S1D). Transcripts of majority genes (ESCs, 77%; iPSCs, 72%; NSCs, 63%; SCs, 74%) each harbored fewer than five m6A peaks, yet there were transcripts of some genes (ESCs, 9%; iPSCs, 4%; NSCs, 12%; SCs, 6%) with over 50% of their lengths covered by m6A peaks (Figure S1E); we thus named these “m6A high-coverage transcripts.” The length of these m6A high-coverage transcripts did not differ significantly from that of the overall transcripts, and some of these transcripts encoded proteins involved in the regulation of processes essential for the maintenance of cell-type specific features, such as neuron differentiation and development in NSCs (Table S3).

Common and Cell-Type-Specific m6A Modification

Using the Shannon-entropy-based method (Kie et al., 2013), we identified a total of 8,558 genes with stable expression in all examined cell types (Table S4). Among them, only transcripts of 3,880 genes had m6A modifications in all samples and were enriched in essential biological processes (Figure 1A and Table S4). On the other hand, transcripts of 1,087 stably expressed genes had no m6A modification in any examined cell type, and the functions of these genes tended to relate to the synthesis and functional establishment of proteins (Figure 1B and Table S4).

To study the m6A modification profiles across cell types, we further divided each transcript into TcSS (transcription start sites), 5′ UTR, CDS, TsTS (translation termination sites), and 3′ UTR regions and compared the m6A distribution profile within each region. m6A modifications in CDS and TsTS regions were more conserved across cell types than those in other regions, with about 50% transcripts having m6A modifications in the CDS and TsTS regions in all examined cell types; yet, only less than 5% of m6A modifications in the TcSS and 5′ UTR regions were conserved across cell types (Figures 1C and 1D and Table S4). At the transcript level, only 437 (11% of 3,880) transcripts had consistent m6A distribution profiles in all examined samples (Figure 1C), and the rest of the transcripts (3,443; 89% of 3,880) had variable m6A peaks in at least two cell types (Figure 1D).

A total of 1,695 genes were identified as cell-type-specifically expressed, of which 998 genes had transcripts with m6A modifications (Figure 1E and Table S5). In addition, among the 8,558 genes with stable expression in all cell types, the transcripts of 877 genes had cell-type-specific m6A modifications. Gene ontology analysis revealed transcripts with cell-type-specific m6A modifications involved in many cell-type-specific biological processes, such as stem cell maintenance and developmental regulation in ESCs and iPSCs, as well as neuron differentiation and forebrain development regulation in NSCs (Table S5). As expected, many known cell-type specific markers were among these genes, including key transcription factors essential for specific features of each cell type, such as Oct4, Nanog, and DPPA2 for ESCs and iPSCs; POU3F2 and ROBO2 for NSCs; and DHH and Sox8 for SCs (Figures 1E and S1F).

m6A Peaks Are Enriched at miRNA Target Sites

To investigate the sequence features of m6A methylation sites, we performed motif search among m6A regions of all cell types. More than 87% of identified m6A peaks contained the previously reported RRACH motif, with GGACU as the most frequent motif in all examined cell types (Figure 2A). The enrichment of the RRACH motif among m6A peaks was significantly higher than that among the control peaks (p < 2.2e−16, Fisher’s exact test). In addition, we also identified a few other motifs (ESCs: 15; iPSCs: 9; NSCs: 8; SCs: 12) within 87%–99% of m6A peak regions (Figures S2A–S2D). Intriguingly, we found that the RRACH motif and over two-thirds (67%–89%, depending on the cell type) of the identified motifs were reversely complementary to the seed sequences (5′-2-8 nucleotides) of one or more miRNAs with at most 1 nt mismatch, indicating that the m6A peak regions may be targeted by miRNAs (Figures 2B and 2C and S2A–S2D). Further analysis revealed that 92%–96% of the m6A peaks could pair with miRNAs with relatively strict alignment criteria. In particular, the RRACH motif region of m6A peaks could potentially pair with 482 miRNAs. The enrichment of miRNA binding sites
Figure 1. Dynamic m^6^A Modification among Cell Types
(A and B) Representative Gene Ontology (GO) terms of the biological process category enriched by transcripts stably expressed in all cell types with (A) or without (B) m^6^A modifications.

(C and D) Distribution of m^6^A peaks along cell-type-consistently expressed transcripts with identical (C) or variable (D) modification profiles among cell types. Each horizontal line represents one transcript. Blue lines represent m^6^A peaks within each sequence region. TcSS, transcription start sites; 5' UTR, 5' untranslated region; CDS, coding sequence; TsTS, translation termination sites; 3' UTR, 3' untranslated region.

(E) Expression profile of cell-type-specifically expressed transcripts (left) and the distribution of m^6^A peaks on each transcript (right). Blue lines represent m^6^A peaks within each sequence region. Names of selected cell-type-specific genes are listed.

See also Figure S1, Table S1, Table S2, Table S3, Table S4, Table S5, and Table S6.
among m6A enriched motifs was remarkably higher than those of the randomly simulated motifs (Figure 2D).

To investigate whether these m6A-targeting miRNAs were indeed expressed in corresponding cells, we quantified miRNA expression using small RNA-seq in ESCs. Of the 1,866 m6A-targeting miRNAs, 818 were detected to be expressed in ESCs. These expressed miRNAs had a significant tendency to target m6A peaks as compared to control peaks (71% versus 39%, p < 2.2e-16, Fisher’s exact test). The consistency between small RNA-seq data and cellular miRNA abundance was validated by qRT-PCR on 12 randomly selected miRNAs (including 2 cell-type-specific ones) (Figures S2E and S2F).

Using the same criteria, 75% of m6A peaks were identified as potential targets of expressed miRNAs in HeLa cells using the published m6A data (Wang et al., 2014a), indicating the conservation of miRNA regulation on m6A between human and mouse.

Formation of m6A Depends on Dicer, but Not Argonaute
To investigate whether miRNAs were indeed involved in the regulation of m6A, we examined the effects of key miRNA biogenesis proteins on cellular m6A abundance. Knocking down Dicer, the endonuclease responsible for producing mature miRNAs, significantly reduced cellular m6A abundance in both mouse NSCs (Figures 3A and 3B and S3A) and human HeLa cells (Figures 3D and 3E and S3B). Conversely, overexpressing Dicer increased the m6A modification level (Figures 3G, 3H, 3J, 3K, and S3C). In all these experiments, expected Dicer and miRNA expression changes were detected, whereas the protein abundance of neither m6A methyltransferase METTL3 nor demethylases FTO and ALKBH5 were affected (Figures 3C, 3F, 3I, 3L, and S3D), suggesting that Dicer-induced m6A abundance change was not achieved by the alteration of the quantity of m6A methyltransferase or demethylases in cells.

Argonaute (AGO) proteins are the key components of known miRNA functional pathways and mediate the binding of miRNAs to their target mRNAs (Bartel, 2004; Cenik and Zamore, 2011; Meister, 2013; Rand et al., 2005). We further examined whether AGO proteins participate in the regulation of m6A formation. The genomes of human and mouse each encode four types of AGO clade proteins (AGO1–AGO4) with miRNA binding ability (Cenik and Zamore, 2011; Meister, 2013). Unexpectedly, knocking down individual AGO expression in HeLa cells had no effect on m6A abundance (Figures S3E–S3G). To avoid functional redundancy, we further used mixed siRNAs to knock down all four AGO genes in mouse NSCs and human HeLa cells. Neither miRNAs, significantly reduced cellular m6A abundance in both mouse NSCs (Figures 3A and 3B and S3A) and human HeLa cells (Figures 3D and 3E and S3B). Conversely, overexpressing Dicer increased the m6A modification level (Figures 3G, 3H, 3J, 3K, and S3C). In all these experiments, expected Dicer and miRNA expression changes were detected, whereas the protein abundance of neither m6A methyltransferase METTL3 nor demethylases FTO and ALKBH5 were affected (Figures 3C, 3F, 3I, 3L, and S3D), suggesting that Dicer-induced m6A abundance change was not achieved by the alteration of the quantity of m6A methyltransferase or demethylases in cells.

Argonaute (AGO) proteins are the key components of known miRNA functional pathways and mediate the binding of miRNAs to their target mRNAs (Bartel, 2004; Cenik and Zamore, 2011; Meister, 2013; Rand et al., 2005). We further examined whether AGO proteins participate in the regulation of m6A formation. The genomes of human and mouse each encode four types of AGO clade proteins (AGO1–AGO4) with miRNA binding ability (Cenik and Zamore, 2011; Meister, 2013). Unexpectedly, knocking down individual AGO expression in HeLa cells had no effect on m6A abundance (Figures S3E–S3G). To avoid functional redundancy, we further used mixed siRNAs to knock down all four AGO genes in mouse NSCs and human HeLa cells. Neither
miRNAs Affect the Abundance of m^6^A at Corresponding Target Sites

To investigate whether miRNAs indeed function in regulating m^6^A formation, we overexpressed a few randomly selected miRNAs with sequences pairing to m^6^A peak regions in mouse NSCs, and we observed significantly increased m^6^A abundance at the corresponding miRNA target sites (Figure 4A upper panel and Figures S4A and S4C). Conversely, repressing the expression of miRNAs by antagonors significantly reduced m^6^A abundance (Figure 4B left panel and Figures S4E and S4G). The expression of target genes (Figure 4A lower panel and Figure 4B right panel) as well as m^6^A methyltransferase METTL3 and demethylases ALKBH5 and FTO (Figures S4B, S4D, S4F, and S4H) remained unaffected in these experiments, suggesting that the abundance change of m^6^A was not caused by altered expression of target genes or m^6^A regulating enzymes. Consistently, overexpression or knockdown miRNAs (mir-423-3p and mir-1226-3p) also increased or decreased m^6^A abundance in human HeLa cells (Figures 4C and S4I).

To investigate whether miRNAs are capable of mediating the ab initio formation of m^6^A, we mutated three nucleotides in the 5’-2-8 nt region (seed sequence of miRNAs) of four miRNAs, namely miR-330-5p, miR-668-3p, miR-1224-5p, and miR-1981-5p, to make the mutated miRNAs pairing with m^6^A regions originally without m^6^A peaks. Consistent results from six individual loci in mouse NSCs demonstrated that overexpression of the mutated miRNAs indeed caused the formation of m^6^A at the designed target sites, whereas regions not targeted by the mutated miRNAs had no m^6^A abundance change (control: KIF1B and control: SCD2) (Figures 4D and S4J). Due to the mutations, some m^6^A peaks originally targeted by endogenous miRNAs were no longer targeted by the mutated ones, and no m^6^A abundance change was detected at these sites either (i.e., control: SSRP1 was targeted by miR-1224-5p, but not its mutant) (Figures 4D and S4J). These results demonstrated that miRNAs are capable of inducing de novo m^6^A methylation via a sequence-dependent manner.

miRNAs Modulate METTL3 Binding to mRNAs

The ab initio induction of m^6^A methylation by mutated miRNAs drove us to speculate that miRNAs may regulate the interaction between METTL3 and mRNAs. To test this hypothesis, we first examined whether modulating Dicer expression could affect the subcellular localization of METTL3, as it has been shown that METTL3 locates and functions at nuclear speckles (Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). Knocking down Dicer significantly reduced the nuclear staining density of METTL3 in human HeLa cells (Figures 5A and 5B). Further examination using ASF (a nuclear speckle marker) staining revealed that the nuclear speckle localization of METTL3 was indeed disrupted in Dicer knockdown HeLa cells (Figure 5C), whereas the METTL3 abundance in both nucleus and cytoplasm almost remained unchanged (Figures 5D and 5A). Co-immunoprecipitation assay revealed that Dicer did not associate with METTL3 (Figures 5S5B and S5C), ruling out a potential physical interaction between METTL3 and Dicer. Taken together, these results indicated that Dicer regulates the nuclear speckle localization of METTL3.

We next performed Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) to examine the amount of RNA associated with METTL3. Intriguingly, upon Dicer depletion, the amount of RNA crosslinked to Myc-tagged METTL3 was significantly reduced in human HeLa cells (Figures 5E and 5F). To further investigate whether the binding of METTL3 on mRNAs could be altered by individual miRNAs processed by Dicer, we performed an RNA immunoprecipitation (RIP) assay with METTL3 antibody to precipitate endogenous METTL3 and its associated mRNAs from HeLa cells, after the overexpression of miR-423-3p and miR-1226-3p or their antagonors. In concert with the results of Dicer manipulation, overexpression miRNAs significantly increased the amount of mRNAs associated with METTL3, whereas downregulating miRNA abundance by antagonors significantly reduced METTL3 binding on mRNAs (i.e., DGCR2 and TUBB4B, targeted by miR-423-3p and miR-1226-3p, respectively) in HeLa cells (Figures 5G and 5H). Consistently, the amounts of METTL3-crosslinked total RNAs (Figure 5I) and mRNAs (i.e., TCFC4 and RPS13) targeted by designed miRNAs (Figures 5J and S5D) were also altered in mouse NSCs when manipulating Dicer or corresponding miRNAs, respectively. In both the mouse and human experiments, the abundance of METTL3-bound mRNAs not targeted by the designed miRNAs was not altered (i.e. TXNRD1 and CTNNA1 in Figure 5G; EEF1A1 in Figure 5J). Collectively, these results showed that miRNAs regulate the m^6^A methyltransferase activity of METTL3 by modulating its binding to mRNAs.

mi^6^A Actively Promotes Cell Reprogramming Efficiency

To investigate whether m^6^A of mRNAs plays roles in cell fate determination, we resorted to the iPSC technology to examine...
Increased m6A abundance (Figure S6A left panels and Figure S6B) and significantly improved the reprogramming efficiency, with the number of obtained iPSC colonies (Oct4::GFP-positive and AP-positive) in the METTL3 overexpression experiment almost double that of the control (Figures 6A and 6B). Enhanced expression of key pluripotent factors, such as Oct4, Sox2, and Nanog, was also observed in METTL3-overexpressing cells (Figure 6C). Conversely, inhibiting m6A formation by knocking down METTL3 expression using siRNAs during the reprogramming process resulted in reduced iPSC colony numbers as well as decreased pluripotent gene expression (Figures 6D–6F, Figure S6A right panels, and Figure S6C). Decreased m6A abundance and iPSC colony numbers were also observed with the addition of cycloleucine, a competitive inhibitor of methionine adenosyltransferase (Finkel and Groner, 1983), during the reprogramming process (Figures S6D–S6F).

Furthermore, overexpression of human Mcy-METTL3 insensitive to mouse METTL3 siRNAs in mouse METTL3 siRNAs in mouse METTL3 knockdown MEFs successfully rescued the reprogramming efficiency (Figures 6D–6F, Figure S6A right panels, and Figure S6C). These data indicated that m6A is required for MEF reprogramming to pluripotency and can promote the reprogramming efficiency.

**DISCUSSION**

Increasing lines of evidence have shown that m6A modification may play pivotal physiological functions in regulating RNA metabolism and various biological processes (Sodi et al., 2012; Bokar, 2005; Fustin et al., 2013; Geula et al., 2015; Jia et al., 2011; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2013; Wang et al., 2014a, 2014b; Zhao et al., 2014; Zheng et al., 2013; Zhong et al., 2008). With the advances of m6A-seq technology, the basic features of m6A modification have been characterized in some tissues and cell lines of mouse and human (Batista et al., 2014; Dominissini et al., 2012; Fustin et al., 2013; Meyer et al., 2012; Schwartz et al., 2013; Wang et al., 2014b). Yet the dynamics of m6A among different cell types and its regulatory mechanisms are still largely unknown. Here we reported cross cell-type comparison of m6A profiles using mouse pluripotent and differentiated cell lines. We identified transcripts with cell-type-dependent common or specific m6A modifications and revealed the dynamic changes of m6A across cell types on some consistently expressed transcripts. These results will provide clues for further functional studies of m6A modification.

miRNAs are a group of important post-transcriptional regulators in eukaryotes. Two previous reports discussed that the presence of m6A may affect the binding of miRNAs to target mRNAs (Meyer et al., 2012; Wang et al., 2014b), but whether miRNAs have direct regulatory roles in the formation of m6A has not been explored yet. Here, we showed that the overall cellular m6A abundance and m6A on individual mRNAs can be altered by the modulation of the expression of the miRNA biogenesis enzyme Dicer or miRNAs. In addition, overexpression of miRNA mutants creates m6A methylation ab initio on originally unmethylated mRNA sequences via a sequence-dependent mechanism. We have further found that the function of miRNAs in regulating m6A is achieved by the mediation of the binding of m6A methyltransferase METTL3 to mRNAs. These results reveal the functions of miRNAs in regulating the formation of m6A, and they also partially explain the site selection mechanism of m6A.

As the key effector proteins of the miRNA functional cascade,AGO proteins have been shown to bind to miRNAs and help miRNAs to execute their functions. However, our results showed that in both human and mouse cells, none of the AGO1–AGO4 proteins were involved in miRNA-mediated m6A regulation. It is likely that miRNAs associate with proteins other than AGOs to regulate m6A formation. Given the presence of a large number of RNA binding proteins with unknown functions, finding the miRNA binding proteins involved in m6A modification remains challenging and needs further investigation.

The physiological roles of m6A modification in cell fate determination are still largely unknown so far. By examining the functions of m6A in regulating cell reprogramming using the iPSC technology, we have revealed a positive role of m6A in regulating cell reprogramming. Such effects were accompanied by altered expression of key pluripotent factors, such as Oct4, Sox2, and Nanog. Consistently, two recent studies reported that proper formation of m6A is required for maintaining the ground state of human and mouse ESCs (Batista et al., 2014; Geula et al., 2015), which is in concert with the function of m6A in promoting the iPSC process identified in this work. All these suggested that proper m6A formation is essential for differentiated cells to regain pluripotent property.

In summary, our study provided the m6A profiles in mouse pluripotent and differentiated cell lines and identified the cell-type-specific and several other features of m6A modification. We have demonstrated that miRNAs are involved in the
Figure 5. miRNAs Affect METTL3 Binding to RNAs

(A) Immunofluorescence analysis of FAM-labeled Dicer-siRNA (green), METTL3 (red), and DAPI (blue, cell nuclei) in Dicer knockdown and control HeLa cells. Scale bar, 7.5 μm.

(B) Quantification of fluorescence intensity of METTL3 in (A). n = 101 cells for each sample.

(C) Immunofluorescence analysis of METTL3 (red), ASF (green, nuclear speckles), and DAPI (blue, cell nuclei) in Dicer knockdown and control HeLa cells. Scale bar, 5 μm.

(D) Western blot (left panel) and quantitative analysis (right panel) of nuclear and cytoplasmic fractions of METTL3 in Dicer knockdown and control HeLa cells. PARP-1 and β-Tubulin are used as nuclear and cytoplasmic markers, respectively.

(E and F) Blot (E) and quantitative analysis (F) of RNAs pulled down by Myc-METTL3 in the control and Dicer knockdown HeLa cells.

(legend continued on next page)
regulation of m6A formation in both mouse and human cells by their mediation of the binding of METTL3 on mRNAs. These findings revealed a role of miRNAs in regulating mRNA epitranscriptomic modification in eukaryotes. Our findings on the functions of m6A in cell reprogramming also suggested that modulating m6A may serve as a strategy to regulate cell reprogramming.

**EXPERIMENTAL PROCEDURES**

**Generation of iPSCs and Reprogramming Efficiency Evaluation**

Generation of pluripotent iPSC lines was performed as previously (Wernig et al., 2008). MEFs were isolated from E13.5 embryos heterozygous for the Oct4-GFP transgenic allele, as previously described (Huangfu et al., 2008), and cultured under established iPSC conditions with the four Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) expressed. The efficiency of iPSC formation is estimated according to the number of Oct4-GFP-positive colonies. GFP-positive colonies after 15 days of reprogramming were trypsinized and then analyzed using a FACS Calibur (BD Biosciences). A minimum of 10,000 events were recorded. Detection of alkaline phosphatase, which is an indicator of undifferentiated ESCs, was carried out after 15 days of reprogramming. The number of iPSC colonies per well was counted in triplicates. The expression of key pluripotent factors Oct4, Sox2, and Nanog was detected by qRT-PCR.

m6A-seq Library Generation and Sequencing

m6A immunoprecipitation and library construction procedure were modified from published procedure (Meyer et al., 2012). In brief, fragmented and ethanol precipitated mRNA (3 μg) from different cell lines was incubated with 5 μg of anti-m6A polyclonal antibody (Synaptic Systems, 202003) in IPP buffer (150 mM NaCl, 0.1% NP-40, and 10 mM Tris-HCl [pH 7.4]) for 2 hr at 4°C. The mixture was then immunoprecipitated by incubation with 50 μl protein-A beads (Sigma, PA924) at 4°C for an additional 2 hr. After being washed three times, bound RNA was eluted from the beads with 0.5 mg/ml N7-methyladenosine (BERRY & ASSOCIATES, PR3732) in IPP buffer and then extracted by Trizol. The remaining RNA was re-suspended in H2O and used for library generation with mRNA sequencing kit (Illumina). Sequencing was carried out using the RNA-seq method as described in the Supplemental Procedures.

**Sequencing Data Processing and m6A Peak Calling**

Sequence reads were mapped to the mouse reference genome (mm9) using TopHat (version 2.0.4) with a RefSeq-based transcript index (Kim et al., 2013). For RNA-seq analysis, the expression of transcripts was quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) and estimated by Cufflinks (version 2.0.2) (Trapnell et al., 2012). Cell-type-specific transcripts were identified using the Shannon entropy of each transcript following the previously reported method (Xie et al., 2013). To identify m6A-enriched regions (m6A peaks), the normalized values of Reads Per Kilobase of genome per Million mapped reads (RPKM) of both mapped m6A-seq reads and RNA-seq reads were calculated and used. m6A peaks were identified by the comparison of the read abundance between m6A-seq and RNA-seq samples of the same loci with a method modified from a previous report (Meyer et al., 2012). Briefly, the entire mouse genome was divided into 25 nt bins and the numbers of both m6A-seq reads and RNA-seq reads (used as control) mapped to each bin were counted and compared. Bins with statistically enriched m6A-seq reads as compared to the RNA-seq reads (adjusted p ≤ 0.01, Fisher’s exact test together with Benjamini-Hocberg procedure) were identified and concatenated adjacently. m6A-seq reads enriched regions with lengths no less than 75 nts were kept as m6A peaks. m6A peaks longer than 200 nts were split into 200 nt smaller peaks during the total m6A peak counting process. Using the same criteria, regions statistically enriched for RNA-seq reads were chosen as the control peaks. The m6A peaks of human HeLa cells were identified using the same criteria with data from the GEO database GSE46705.

**Motif Identification among m6A Peaks**

Sequence motifs enriched in m6A peaks were identified by HOMER with m6A peaks as the target sequences and control peaks as the background using default parameters (Henz et al., 2010) and visualized using WebLogo (Crooks et al., 2004). The enriched motifs were randomly shuffled to generate 500 groups of simulated motifs in each cell type and were used for specificity analysis.

**Relationship Analysis of miRNAs with m6A Peaks**

Mouse mature miRNA sequences were downloaded from miRBase (Release 20 with 1,908 mouse miRNA sequences) (Kozomara and Griffiths-Jones, 2011), then compared with the motifs identified by HOMER or randomly simulated motifs using in-house scripts. miRNAs with seed regions (5’-2-8 nts) reverse complementarily pairing (with at most one mismatch) to m6A motifs were selected. To compare miRNA sequences with all m6A peaks, the entire sequences of identified m6A peaks and control peaks were extracted and paired with the miRNA sequences using miRanda software with “–sc 155 –en 20” and other default settings as parameters (Enright et al., 2003). m6A peak sequences and control peak sequences that passed the above criteria were identified as miRNA-targeted peaks.

**m6A Manipulation during the iPSC Process**

Under the iPSC induction condition as described above, the following experiments were carried out from the first day of reprogramming. In the METTL3 overexpression experiments, 5 μg of plasmids expressing pCMV-Myc-METTL3 and 5 μg pCMV-Myc-control plasmids were transfected into MEFs using Lipofectamine 2000 kit (Invitrogen) three times every 3 days. In the METTL3 knockdown experiment, 75 nM siRNAs targeting METTL3 and 75 nM control siRNAs were transfected into MEFs using Lipofectamine RNAiMAX (Invitrogen) four times every 3 days. In the rescue experiment, 5 μg of plasmids expressing pCMV-Myc-hMETTL3 (Ping et al., 2014), which does not contain the target site of mouse METTL3 siRNAs, and 5 μg of control plasmids were transfected into the METTL3 knockdown MEFs three times every 3 days. In the chemical m6A inhibition experiments, 20 mM cycloleucine was added to the culture medium of MEFs once per day for 10 days.

**Statistical Analysis**

Student’s t test was used for all statistical analyses for experimental results (unless stated otherwise).

See Supplemental Experimental Procedures for a full description of the methods.

**ACCESSION NUMBERS**

Sequencing data generated by this work have been deposited into the Gene Expression Omnibus (GEO; accession number GSE52125).

(G) METTL3-RIP-qRT-PCR showing the changes of METTL3 binding at predicted target sites (DGCR2 and TUBB4B) of overexpressed (OE-miR) and repressed (anti-miR) miRNAs. Non-target regions (TXNRD1 and CTNNA1) of the operated miRNAs were used as controls.

(H) Western blot analysis showing equal amounts of METTL3 in control cells, miRNA-overexpression HeLa cells, and miRNA-knockdown HeLa cells (left panel) and comparable METTL3 immunoprecipitation efficiency (right panel).

(I) Blot analysis of RNAs pulled down by Myc-METTL3 in the control and Dicer knockdown mouse NSC cells. β-Tubulin is used as a loading control.

(J) METTL3-RIP-qRT-PCR showing the changes of METTL3 binding at predicted target sites (TCF4 and RPS13) of overexpressed (OE-miR) and repressed (anti-miR) miRNAs. A non-target region of miRNAs (EEF1A1) is used as a control.
Figure 6. Modulating m^6A Abundance by METTL3 Regulates Cell Reprogramming

(A) Morphology (left panel) and quantitative (right panel) analysis of Oct4-GFP-positive clones among reprogrammed MEFs with control vector (OE-Control) and mouse METTL3 overexpression (OE-METTL3).

(B) AP-positive clones among reprogrammed MEFs with control vector (OE-Control) and mouse METTL3 overexpression (OE-METTL3).

(C) The expression levels (detected by qRT-PCR) of endogenous Oct4, Sox2, and Nanog in cells during the reprogramming process of MEFs with the control vector (OE-Control) and mouse METTL3 overexpression (OE-METTL3).

(D) Morphology (left panel) and quantitative (right panel) analysis of Oct4-GFP-positive clones among reprogrammed MEFs with control siRNAs (si-Control), siRNAs for mouse METTL3 (si-mETTL3), and human Myc-METTL3 rescue (si-mETTL3+OE-hMETTL3).

(E) AP-positive clones among reprogrammed MEFs with control siRNAs (si-Control), siRNAs for mouse METTL3 (si-mETTL3), and human Myc-METTL3 rescue (si-mETTL3+OE-hMETTL3).

(F) The expression levels (detected by qRT-PCR) of endogenous Oct4, Sox2, and Nanog in cells during the reprogramming process of MEFs with control siRNAs (si-Control), siRNAs for mouse METTL3 (si-mETTL3), and human Myc-METTL3 rescue (si-mETTL3+OE-hMETTL3).

Values and error bars in all plots represent the mean and SD of three independent experiments. *p < 0.05 by Student’s t test. See also Figure S6 and Table S6.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.01.016.

AUTHOR CONTRIBUTIONS

Q.Z., Y.Y., and X.W. conceived this project, supervised the experiments, analyzed data, and wrote the manuscript. T.C. and X.W. performed bioinformatics analysis, prediction, experimental candidates selection, and
ACKNOWLEDGMENTS

This work was supported by China 973 programs (2011CBA01101 to Q.Z. and X.-J.W. and 2014CB964900 to X.-J.W.); CAS Strategic Priority Research Program Grants XDA01020101 (to Q.Z.), XDA01020105 (to X.-J.W.), and XDB14030300 (to Y.-G.Y.); and the National Natural Science Foundation of China (91313308 to Q.Z. and 31430022 and 3137096 to Y.-G.Y.). We thank Big Sequencing core facility for sequencing.

REFERENCES


