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N6-methyladenosine regulates PEDV replication and host gene expression

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ABSTRACT

Methylation of the N6 position of adenosine (m6A) is a widespread RNA modification that is critical for various physiological and pathological processes. Although this modification was also found in the RNA of several viruses almost 40 years ago, its biological functions during viral infection have been elucidated recently. Here, we investigated the effects of viral and host RNA methylation during porcine epidemic diarrhea virus (PEDV) infection. The results demonstrated that the m6A modification was abundant in the PEDV genome and the host methyltransferases METTL3 and METTL14 and demethylase FTO were involved in the regulation of viral replication. The knockdown of the methyltransferases increased PEDV replication while silencing the demethylase decreased PEDV output. Moreover, the proteins of the YTHDF family regulated the PEDV replication by affecting the stability of m6A-modified viral RNA. In particular, PEDV infection could trigger an increase of m6A in host RNA and decrease the expression of FTO. The m6A modification sites in mRNAs and target genes were also altered during PEDV infection. Additionally, part of the host responses to PEDV infection was controlled by m6A modification, which could be reversed by the expression of FTO. Taken together, our results identified the role of m6A modification in PEDV replication and interactions with the host.

1. Introduction

Epigenetic regulation refers to heritable changes in gene regulation that do not result from changes in the DNA/RNA sequence. Epigenetic modifications include DNA methylation, histone modification, genomic imprinting, maternal effects, RNA methylation, etc. Among these modifications, N6-methyladenosine (m6A) methylation is characterized by its dynamic regulation and abundance in mRNA. m6A was first discovered in the 1970s in a purified poly (A) RNA fraction (Desrosiers et al., 1974). Later, it was found to play critical roles in various processes of mRNA metabolism, including transcription, decay, degradation, and translation (Zhao et al., 2017). In 2010, antibody-based immune precipitation followed by high-throughput sequencing combined with the identification of the m6A complex facilitated the studies of m6A modification (Dominissini et al., 2012; Jia et al., 2011; Zheng et al., 2013). Since then, novel biological functions of m6A have been widely explored.

M6A modification is added by methyltransferases (writers) or removed by demethylases (erasers) respectively, and exerts its function by m6A binding proteins (readers). In mammalian cells, methyltransferases are a complex mainly consisting of methyltransferase-like protein 3 (METTL3) and METTL14 (Bokar et al., 1994, 1997; Liu et al., 2014; Ping et al., 2014). Other associated proteins have also been identified, such as Wilms Tumor 1 associated protein (WTAP) (Ping et al., 2014), Virilizer (KIAA1429) (Schwartz et al., 2014; Horiiuchi et al., 2013), RNA binding motif protein 15 (RBM15) (Patil et al., 2016), and zinc finger CCCH domain-containing protein 13 (ZC3H13) (Knuckles et al., 2018; Wen et al., 2018). However, The METTL3, a catalytic subunit, and METTL14, an RNA-binding platform, are recognized as key factors (Liu et al., 2014). Two demethylases, fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5), are responsible for the removal of m6A. The m6A demethylase activity of FTO was first discovered in 2011 (Jia et al., 2011). It can oxidize m6A to Adenine (A) and generate N6-hydroxymethyladenosine (hm6A) as an intermediate product and N6-formyladenosine (f6A) as a further oxidized product (Gerken et al., 2007; Jia et al., 2008). The second demethylase, ALKBH5, was identified in 2013 (Zheng et al., 2013). However, ALKBH5 may function in a specific sequence or structural context. In addition, the function of m6A modification on target mRNAs is mediated by specific reader proteins. Members of the YTH family have been shown to specifically bind to m6A-containing precursor RNAs. The main m6A readers, YTHDF1, YTHDF2, and YTHDF3, are located in the cytoplasm. YTHDF1 has been demonstrated to interact with methylated mRNA near the stop codon and enhances its translational efficiency (Wang et al., 2015). YTHDF2 can reduce the stability of m6A modified RNA (Wang et al., 2014; Du et al., 2016). YTHDF3 has
been shown to cooperate with YTHDF1 and YTHDF2 (Li et al., 2017). The combination of methyltransferases, demethylases and m6A-binding proteins contributed to the dynamic regulation of this modification. Its flexibility highly correlates with the metabolism of RNA and plays critical roles in transcription, decay, degeneration and translation. Almost all biological processes related to RNAs have been identified to be affected by m6A modification.

The replication of all the viruses requires RNA except the Prioms. The involvement of m6A in viral infection was discovered in the 1970s. M6A was found to be present on viral RNAs, for instance, the influenza virus and even several DNA viruses (Krug et al., 1976; Sommer et al., 1976; Moss et al., 1977; Furuichi et al., 1975). However, the specific roles of m6A in viral replication remain unclear until 2016, when transcriptome-wide profiling of m6A was made possible through antibody-based immunoprecipitation followed by high-throughput sequencing. The replication of human immunodeficiency virus 1 (HIV-1), RNA viruses within the family Flaviviridae, and influenza virus were first reported to be affected by m6A (Tirumuru et al., 2016; Kennedy et al., 2016; Gokhale et al., 2016; Lichinchi et al., 2016a, 2016b). However, there is still much unknown regarding the functions of m6A during virus infection, e.g. whether m6A is present in the RNA of other viruses, and the dynamic m6A modification of host RNA during viral infection.

Porcine epidemic diarrhea (PED) is a devastating enteric disease in pigs that can cause mortality of up to 100% in piglets younger than one week old. Since its re-emergence in 2010, this disease has rapidly spread worldwide and caused substantial economic losses to the global swine industry (Jung and Saif, 2015; Davies, 2015; Wang et al., 2016). PEDV, a member of the genus Alphacoronavirus within the family Coronaviridae family. The genome of PEDV is approximately 28 kb in size and contains four ORFs. Cells were seeded into 10-cm dishes and inoculated with PEDV at a MOI = 0.1. The supernatant was collected 36 h post-infection (hpi) and then centrifuged at 2000 × g for 15 min to remove cellular debris. After centrifugation at 10000 × g for 2 h at 4 °C, the pellets were resuspended in PBS and purified by sucrose density gradient centrifugation. The sucrose cushion was 20%, 40%, and 50%. PEDV particles were between 40% and 50% sucrose cushions.

2. Materials and methods

2.1. Cell culture and virus infection

Vero cells and LLC-PK1 cells were cultured in DMEM (Sigma, Germany) and MEM-199 (Sigma) supplemented with 10% fetal bovine serum (Gemini, USA) and 100 units/ml penicillin (Sigma) in an incubator (5% CO2, at 37 °C). The PEDV LJX01/GS/2014 strain was isolated and stored in our laboratory.

2.2. PEDV titration

Vero cells were seeded into 96-well plates (approximately 5 × 10^4 cells per well) for PEDV titration with the collected supernatant. The collected supernatant was first serially diluted 10-fold and then 100 μl of each dilution was inoculated into four wells of the 96-well plate. The plates were incubated at 37 °C in 5% CO2 until 2 days post infection. Titters were calculated from the supernatant dilution that caused cytopathologic effects in half of the cultures (TCID50/mL) based on the Reed-Muench method.

2.3. PEDV purification

Cells were seeded into 10-cm dishes and inoculated with PEDV at a MOI = 0.1. The supernatant was collected 36 h post-infection (hpi) and then centrifuged at 2000 × g for 15 min to remove cellular debris. After centrifugation at 100000 × g for 2 h at 4 °C, the pellets were resuspended in PBS and purified by sucrose density gradient centrifugation. The sucrose cushion was 20%, 40%, and 50%. PEDV particles were between 40% and 50% sucrose cushions.

2.4. Electron microscopy

Purified PEDV particles were diluted with PBS and spotted onto Formvar-coated grids. Ten minutes later, the PBS was removed. The grids were directly negatively stained with phosphotungstic acid for 10 min at room temperature. A Hitachi electron microscope (HT7700) was used at 80 kV for viral particle observation.

2.5. m6A-seq

High-throughput sequencing of PEDV methylation was carried out by m6A-seq following a previously described protocol (Dominissini et al., 2013). In brief, rRNA was removed from the total RNA by the RiboMinus Eukaryote System v2 (Thermo, USA). Then, the RNA was fragmented using Ambion RNA Fragmentation Reagents (Thermo, USA) and mixed with 25 μg of affinity purified anti-m6A polyclonal antibody (Synaptic Systems, Germany) at 4 °C for 2 h. Sequencing libraries were prepared with eluted RNA, as well as input RNA, using the TruSeq RNA sequencing (RNA-seq) kit (Illumina, USA). Sequencing was carried out...
on Illumina HiSeq 2000 according to the manufacturer’s instructions and was performed by Novel Bioinformatics Company.

2.6. Quantification of RNA m^6^A level

For the quantification of the host RNA m^6^A levels, the cells were harvested at the indicated time points and prepared for RNA extraction. The genomic RNA of PEDV was extracted from purified viral particles by the RNasy Mini Kit (Qiagen, Germany). The EpiQuick m^6^A RNA Methylation Quantification Kit (EpiGentek, USA) was used in the following quantification. Briefly, RNA was bound to strip wells using an RNA high-binding solution. Specific capture N6-methyladenosine antibody and detection antibody were then incubated with bound RNAs. The detection signal was enhanced and detected at a wavelength of 450 nm. The amount of m^6^A was proportional to the OD intensity measured. Three replicate samples were used to ensure that the generated signal was validated. Finally, the percentage of m^6^A was calculated according to the formula provided.

2.7. RNA interference

For the knockdown of genes involved in the process of methylation, siRNAs (200 nM) were transfected into Vero cells or LLC-PK1 cells using X-tremeGENE siRNA Transfection reagent (Roche, Switzerland) according to the manufacturer's instructions. Cells were harvested for western blot analysis. All siRNAs were synthesized by RiboBio Company. The target sequences of the siRNAs are listed in Table 1.

2.8. Immunoprecipitation and RNA

Cells were transfected with plasmids carrying MYC-YTHDF1-3 or an empty vector (EV). The cells were then infected with PEDV at MOI = 0.1 24 h post-transfection. When CPE appeared, the cells were treated (Sigma, USA) and eluted with RNase-free water. Equal volumes of RNA were used as a template for first-strand cDNA synthesis, according to the manufacturer's instructions.

2.9. RNA stability assay

Cells were transfected with siRNAs (200 nM) and/or treated with actinomycin D (4 μg/ml) (Solarbio, China) in DMSO. Cells were then infected with PEDV at a MOI = 0.1. The total RNA was collected at the indicated time points and reverse-transcribed into cDNA. The mRNA stability was measured by analysis of the relative expression at 0, 3 h, and 6 h after PEDV infection. The primers for target gene detection were as follows: PEDV-N forward: 5′-GATACCTTTGGCCTCTTGTTGTG-3′, reverse: 5′-CACAACCGAATGCTATTGACG-3′; porcine-IL-8 forward: 5′-GATACTTTGGCCTCTTGTG-3′; reverse: 5′-CACACCAGATGCTATTGACG-3′; probe (5′-FAM-TTCAGCATCCTGCGTATACT-TAMRA-3′) were derived from our previous study (Huang et al., 2019). For the detection of host gene expression levels, real-time qPCR was performed with a unique aptamer qPCR SYBR green master mix (Novogen, China). Briefly, the reactions were incubated at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. All reactions were run in triplicate. The probe and primer sets are listed in Table 3. The ΔΔCt method was used to measure the expression level of target genes.

2.10. Construction of pseudovirus and stable cell lines

To construct stable cell lines with Mettl3/Mettl14 knockdown for PEDV production, duplexes of synthesized oligonucleotides containing target sequences were inserted into the lentivirus vector pLVX-shRNA2. The plasmids (21 μg) were then transfected into HEK293T cells with the packaging plasmids psPAX2 (14 μg) and pMD 2.0g (7 μg). At 48 h post transfection, the culture supernatant was concentrated by Amicon Ultra-15 centrifugal filters. The concentrated pseudovirus was transduced into LLC-PK1 cells for further studies. For the construction of LLC-PK1 cells with FTO over-expressing, the FTO gene was inserted into the pFUGW vector using SfiI to generate the pFUGW-FTO plasmid. The packing and transduction procedures were the same as mentioned above. The target sequences and primers for lentivirus construction were listed in Table 2.

2.11. Real-time PCR analysis

To assess the number of PEDV genomes binding to YTHDF proteins, real-time PCR was used for the quantitation assay. Real-time PCR was performed in a Bio-Rad CFX96 system with TransStart Probe qPCR SuperMix (Transgen, China). Briefly, the reactions were incubated at 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s and 60 °C for 30 s. The primers (forward: 5′-GATACCTTTGGCCTCTTGTTGTG-3′, reverse: 5′-CACAACCGAATGCTATTGACG-3′) were derived from our previous study (Huang et al., 2019). For the detection of host gene expression levels, real-time qPCR was performed with a unique aptamer qPCR SYBR green master mix (Novogen, China). Briefly, the reactions were incubated at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. All reactions were run in triplicate. The probe and primer sets are listed in Table 3. The ΔΔCt method was used to measure the expression level of target genes.

2.12. Statistical analysis

The differences between matched groups were examined for statistical significance using Student’s t-test. An unadjusted P value of less than 0.05 was considered to be significant, and a P value of less than 0.01 was considered to be highly significant. NS means not significant.

3. Results

3.1. The PEDV genome contains m^6^A modifications

Although m^6^A was proven to be present in the RNA of several viruses, there have been no reports about its existence in the genomic RNA of PEDV. To investigate the presence of m^6^A in PEDV RNA, purified PEDV particles were prepared for viral RNA extraction. To do so, the LLC-PK1 cells were infected with the PEDV LJX01/GS/2014 strain. When a significant cytopathic effect (CPE) was observed, the supernatant was collected and then subjected to sucrose density gradient centrifugation for PEDV purification. The purified viral particles were observed with a transmission electron microscope (TEM). The results showed that the collected particles exhibited classical morphology of coronavirus and were of high purity (Fig. 1A). After the extraction of viral RNA, we first quantified the m^6^A level of the PEDV RNA genome with an m^6^A RNA Methylation Quantification Kit. The result demonstrated that the m^6^A ratio of PEDV RNA was approximately 0.05–0.06%.

| Gene name | Species | Target Sequence |
|-----------|---------|-----------------|
| FTO       | Sus scrofa | GCACCTCAGAATGCTGAA |
| METTL3    | Sus scrofa | CGAACCAACAAACTCTA |
| METTL14   | Sus scrofa | AGAGAAGATGAGAACAA |
| YTHDF1    | Sus scrofa | CTCCGGGCAATAAGCATA |
| YTHDF2    | Sus scrofa | CAAAGAAAAAGTGCCAA |
| YTHDF3    | Sus scrofa | GGGAGGAATAAGAACAA |
| FTO       | Chlorocebus sabaeus | TGGAGTGGTGGCAGATGTT |
| METTL3    | Chlorocebus sabaeus | CGACCTCAGAATGCTGAA |
| METTL14   | Chlorocebus sabaeus | GCTTCCTGCAAACTCTAA |
| YTHDF1    | Chlorocebus sabaeus | CTACCTTACTGACATG |
| YTHDF2    | Chlorocebus sabaeus | GCAAATATGGTTATACA |
| YTHDF3    | Chlorocebus sabaeus | GGTCACACTGGATTTA |
The primers used for m6A real-time PCR analysis.

| Gene Name | Sequence          | Note     |
|-----------|-------------------|----------|
| METTL3    | GCATTGTTGATCTAAGGAAATTC | Target   |
| METTL14   | GCATTGGTCGCTTTGTTAAATA | Target   |
| FTO       | F: ACAGGCCCCATTAGGGCCATGAAAGGCGGCTTGCA | Primer   |
|           | R: TACCGGGGCGGGCCCTTAAATTTAAAGATGGGGTTG |         |

To further map the m6A peaks within PEDV RNA, we performed immunoprecipitation with viral RNA. Viral RNA was fragmented into approximately 200 bp fragments and then conserved as input control or immunoprecipitated with m6A-specific antibodies. The RNA was then eluted and analyzed by high-throughput RNA sequencing. The reads were mapped to identify the regions of the PEDV genome enriched in m6A. Seven m6A peaks were identified in PEDV genomic RNA, mainly in the genes that can be translated into nonstructural proteins (Fig. 1C). The specific information is listed in Table 4.

Together, the results above confirmed that the genomic RNA of PEDV contained m6A modifications at multiple sites within the viral genome.

3.2. The m6A modification regulates PEDV replication in different cell lines

Since the PEDV genomic RNA was modified by m6A, we next attempted to determine whether this modification could regulate PEDV replication. The siRNAs against porcine FTO, METTL3, and METTL14 were synthesized and transfectected into LLC-PK1 cells. At 48 h post transfection, the cells were collected for western blotting analysis. The results demonstrated that these molecules were efficiently knocked down in the knock down of the corresponding genes (Fig. 2A). The cells were also infected with MOI = 0.01 PEDV at 24 h after transfection. The supernatant was collected at 24, 48, and 72 hpi and used for virus titration and real-time PCR analysis. We found that the METTL3 and METTL14 knockdown facilitated PEDV replication, while the FTO knockdown resulted in a significant decrease of viral titers at 24, 48, and 72 hpi. To further verify whether this effect may reduce the viral RNA replication or impact the infectivity of the virus, viral RNA copy numbers were then measured by real-time RT-qPCR analysis. The results showed that the METTL3/14 knockdown increased the PEDV RNA copy numbers at 48 and 72 hpi (Fig. 2C). The knockdown of FTO also decreased PEDV copies at all three time points. These results were consistent with the viral titers and suggested m6A modification directly reduced viral RNA production. Myc-tagged FTO gene was then overexpressed in LLC-PK1 cells to test whether it had a similar effect on PEDV growth. The viral titers of PEDV were significantly increased by the overexpressed exogenous FTO except at 72 hpi (Fig. 2D). The overexpression of FTO significantly accelerated the PEDV replication and resulted in cell apoptosis and a decrease of viral loads at 72 hpi while the viral titers of the control group remained increase at this time point. These data showed that the m6A modification increased PEDV replication in LLC-PK1 cells.

Vero is another cell line commonly used for PEDV isolation and propagation. This cell line was derived from the African green monkey kidney epithelium. M6A modification can be varied among different cell lines. Therefore, we also used this cell line to test the effect of m6A on PEDV replication. The siRNAs against M6A molecules, including METTL3, METTL14, and M6A synthetase 3 (M6A-3) were synthesized and transfectected into Vero cells. The results showed that the silencing of M6A molecules significantly reduced the PEDV replication in Vero cells (Fig. 2E).

Table 3

The primers used for m6A real-time PCR analysis.

| Gene Name | Gene Description | Sequence          |
|-----------|-------------------|-------------------|
| CDC42     | cell division 42  | F: AAAAGGGGGAAGCGAGGAGTCT |
| CTNN      | cTn                | R: TTGCAGCTTGGTCTGCTT |
| MYH       | A/G-specific adenine | F: CAGGCTTGCGAGGAGAGGAAG |
| NRAS      | proto-oncogene     | R: TGGAGGCGCTTCTGGATTT |
| VYX3      | V-box binding protein 3 | F: CCCCTATAACTATCGGCGGC |
| CDK4      | cyclin dependent kinase 4 | F: GGGCCCGAGATGTCGTCTA |
| CDKN1A    | cyclin dependent kinase inhibitor 1A | F: CACAGGCGACATCTGACAGTT |
| GADD45B   | growth arrest and DNA damage inducible beta | F: GGGGAGGCGGCTTTAGTTG |
| LOC10073797 | tumor necrosis factor receptor superfamily member 10B-like | F: CTAACAGGACTGCGGCTTCT |
| MDM2      | MDM2 proto-oncogene | F: TCCACAGCACCAGTGAAGGAA |
| PMAI1     | phorbol-12-myristate-13-acetate-induced protein | F: CTCCTACTTTGGGCTTCTC |
| RRM2      | ribonucleotide reductase regulatory subunit M2 | F: TTGTCGCCAGAATCGTAAAG |
| ATP2      | activating transcription factor 2 | F: TCTGCCAGGCGGTGGTGAAA |
| DAXX      | death domain associated protein | F: GGAACCTCAGGCGGCGGGAG |
| DUSP9     | dual specificity phosphatase 6 | F: AGTGCACAGCAGTGGCAGGC |
| HSP70     | heat shock protein 70 | F: TGGGATGAGCTTGCAGAGT |
| DUSP10    | dual specificity phosphatase 10 | F: AAGGCAGCACTCGACAGAG |
| MAP2K5    | mitogen-activated protein kinase 5 | F: AGTGCACAGCAGTGGCAGGC |
| MAPK8P3   | mitogen-activated protein kinase 8 | F: TCTGCCAGGCGGTGGTGAAA |
| MAP3K4    | mitogen-activated protein kinase 4 | F: ATGGGACGTGTTTGGGGCAT |
| MYC       | MYC proto-oncogene, bHLH transcription factor | F: AGAAGCCTCTGCTGGCATT |
| PAK       | p21-activated kinase | F: GGAAGACGACAGCAGTGACAG |
| RAC       | AKT serine/threonine kinase 1 | F: TGGTCGGGGGCAAATACAG |
| SIX4      | SIX homeobox 4 | F: ATTCACGGGGCTTGACCACAG |
| SRF       | serum response factor | F: CTCGAGCTTGGTGTCCTCCT |
| TAB2      | TAK1-associated binding protein 2 | F: CTCGCCCAATATCCAGGCC |
| TGFBR3    | transforming growth factor beta 3 | F: GTGTCGCTTGGAGAATACAGT |
| TOK2      | TOK2 potassium channel | F: TTCTCCGTCAGCAGCCCT |

of the total adenosines (Fig. 1B).

The primers used for m6A real-time PCR analysis.
Fig. 2G). The overexpression of exogenous FTO accelerated PEDV replication compared to that in cells transfected with an empty vector (Fig. 2H). Compared with the knockdown results, overexpression of FTO was more efficient in accelerating PEDV replication.

The results above also raised other scientific concerns. What is the m6A level of PEDV genomic RNA without the help of METTL3/14 or FTO? Does m6A modification influences the PEDV production or its infectivity? To answer these questions, different cell lines with METTL3/14 knockdown or FTO over-expression were constructed using a pseudovirus vector delivery system. We used the LLC-PK1 cells as the parent cell lines. The western blotting analysis showed the target proteins were successfully knocked-down or overexpressed (Fig. 2I). PEDV was inoculated to these cell lines and then collected for m6A quantification and titration. Although the production of viral particles differs in different cells, the ELISA result of m6A level exhibited no significant difference in their genomic RNA (Fig. 2J), suggested that there might be other proteins involving in this modification. We then titrated the viruses derived from the above cell lines. The growth curves demonstrated similar patterns between different groups, indicated that the virus m6A might influence the PEDV production but not its infectivity (Fig. 2K).
3.3. YTHDF1-3 proteins bind to PEDV RNA and negatively regulate PEDV replication

The YTHDF proteins are the m6A readers, which play a role in the RNA-binding process. Given that m6A regulates PEDV infection, we next tested whether YTHDF1-3 could bind to the PEDV genome and contribute to PEDV replication. We transfected Myc-tagged porcine YTHDF1/YTHDF2/YTHDF3 or empty vectors into LLC-PK1 cells and then infected the cells with MOI = 0.1 PEDV at 24 h post transfection. The PEDV genome binding activity to YTHDF proteins was analyzed by
Fig. 2. PEDV replication was regulated by m^6^A modification. The siRNAs against porcine METTL3/METTL14/FTO were transfected into different cells and the cells were infected with PEDV at a MOI = 0.1. The supernatants were collected at different time points for viral titration. A) Western blotting analysis of LLC-PK1 cells transfected with siRNAs and scrambled siRNA as the negative control (NC) at 48 hpi. B) The relative fold change of viral titers in LLC-PK1 cells transfected with siRNAs or NC at different time points. C) The relative fold change of viral RNA copies of PEDV in LLC-PK1 cells at different time points. D) The relative fold change of viral titers in LLC-PK1 cells overexpressing FTO at different time points. E) Western blotting analysis of Vero cells transfected with siRNAs or NC at 48 hpi. F) The relative fold change of viral titers in Vero cells transfected with siRNAs or NC at different time points. G) The relative fold change of viral RNA copies of PEDV in Vero cells at different time points. H) The relative fold change of viral titers in Vero cells overexpressing FTO at different time points. I) Western blotting analysis of constructed cell lines with METTL3/METTL14 knockdown and with GFP-FTO overexpressed. J) The m^6^A level of PEDV RNA produced in different cell lines was quantified by ELISA. The synthetic scrambled RNA was taken as a negative control. K) The growth curve of PEDV derived from different cell lines. *P < 0.05, **P < 0.01, ns means not significant.

real-time PCR (Fig. 3A). The results showed that PEDV replicated in all groups. The three YTHDF proteins had a much higher affinity to the PEDV genome than the control. The YTHDF2 exhibited a superior affinity than the other two proteins. YTHDF1/YTHDF2/YTHDF3 of green monkey or empty vectors were also transfected into Vero cells. The results also showed that YTHDF1-3 could bind to PEDV RNA but with different affinities compared to porcine proteins. YTHDF3 had the highest affinity in Vero cells (Fig. 3B). The siRNA molecules against both porcine and green monkey YTHDF proteins were synthesized. Western blot analysis showed that these molecules could efficiently knock down the endogenous genes (Fig. 3C and F). We then determined the virus titers in the cells. The knockdown of YTHDF proteins led to an increase in virus titers at 48 and 72 hpi in LLC-PK1 cells except YTHDF3 (Fig. 3D) and at 24 and 48 hpi in Vero cells (Fig. 3G). The depletion of YTHDF exerted the most significant upregulation of viral production at 24 hpi in Vero cells and at 72 hpi in LLC-PK1 cells. These results suggested that YTHDF proteins might be critical for PEDV replication in Vero cells at early stage. Besides that, YTHDF2 played more important roles in LLC-PK1 cells than in Vero cells, suggested that the function of YTHDF proteins may vary in different cell lines. In addition, the plasmids carrying YTHDF genes were transfected into both cell lines to observe their function on PEDV replication. The results illustrated that the overexpression of YTHDF proteins dramatically delayed the PEDV replication. The viral titers in YTHDF-transfected groups still increased while the EV group dropped at 72 hpi (Fig. 3E and H). Taken together, these data suggested that the YTHDF proteins could regulate PEDV infection.

YTHDF2 reduces the stability of m^6^A modified RNA (Wang et al., 2014; Du et al., 2016). To test whether YTHDF2 decrease PEDV replication by the degradation of viral RNA, we employed Actinomycin D combined with YTHDF2 siRNA for further studies. Actinomycin D is an inhibitor of DNA transcription and replication but doesn’t affect the function of RNA viruses (Koba and Konopa, 2005). PEDV N was selected as the target gene to verify the RNA stability because it was demonstrated that m^6^A could inhibit PEDV infection, we next investigated its effect on host RNAs. Both LLC-PK1 and Vero cells were infected with PEDV and harvested for total RNA and proteins at different time points. Using the m^6^A RNA Methylation Quantification Kit, we first detected the m^6^A ratio of total RNA. It was interesting to find that PEDV infection could trigger a drastic increase in the m^6^A ratio in PEDV-infected cells compared to mock infection (Fig. 4A and B). The initial modification percentages of mock cells were nearly the same in LLC-PK1 and Vero cells at approximately 0.03%. After PEDV infection, the m^6^A ratio immediately climbed to approximately 0.09% in LLC-PK1 cells and over 0.06% in Vero cells at 1 hpi. Although it decreased with the course of viral replication, the m^6^A ratio of infected cells was higher than that of mock cells. At 72 hpi in LLC-PK1 cells or 48 hpi in Vero cells, the PEDV infection had disrupted most of the cells, and the curve of the ratio flattened out thereafter. This enhancement of m^6^A modification occurred as early as 1 hpi, suggested that it might closely correlated with PEDV entry. To prove this, cells were incubated with or without PEDV at 4 °C and then subject to RNA extraction and ELISA analysis. The results showed that there were no significant differences between the infection group and mock group (Fig. 4C), further indicated that PEDV infection enhanced host m^6^A RNA methylation.

We then detected the expression pattern of m^6^A methyltransferases and demethylases in LLC-PK1 cells to determine their relationship with the curve of the m^6^A ratio. The western blotting analysis demonstrated that the FTO expression was easily influenced by different factors. The expression level of FTO was pretty low at 1 hpi due to the sufering of trypsin treatment when cell passaging. However, it was increased in the mock groups at 24, 48 and 72 hpi while remained low expression level at infection groups. In comparison, the expression of the methyltransferases METTL3 and METTL14 was not changed at 24, 48 and 72 hpi (Fig. 4D). In Vero cells, the expression levels of FTO were lower in the infection groups compared to mock groups at 24, 48, and 72 hpi. The expression of the methyltransferases remained stable (Fig. 4E). Both host RNA quantification of and western blotting analysis showed that PEDV infection could enhance the m^6^A ratio in host RNA, which suggested that the host may restrict PEDV replication by m^6^A modification though different mechanisms.

3.5. The topology of the m^6^A RNA methylome during PEDV infection

In addition to the m^6^A quantification of host RNA, we also performed m^6^A-seq to determine the dynamics of host RNA methylation after PEDV infection. The distribution of m^6^A on host transcripts was not significantly changed during viral infection. Of the total transcripts with m^6^A modification, mRNA accounted for approximately 87% in both groups. The percentage of methylated noncoding RNA (ncRNA) was 12% in the mock group and 13% in the PEDV-infected group. The m^6^A modification was rarely identified in rRNA, misc RNA, and pre-cursor miRNA (Fig. 5A). However, the detailed analysis further demonstrated that the distribution of m^6^A on gene structures in PEDV-infected cells was different from that in mock cells. PEDV infection increased the distribution of m^6^A on the untranslated regions (UTRs) and decreased its distribution on the coding sequence (cds). In mock cells, 64% of m^6^A was located in the cds, while in PEDV-infected cells, the percentage dropped to 57%. In contrast, the percentage of m^6^A distribution on the UTR had dropped by approximately 6% due to PEDV infection (Fig. 5B). Next, functional analysis of m^6^A-modified mRNAs was carried out. Gene Ontology (GO) and KEGG databases were used in this process. GO annotation analysis revealed that most m^6^A-modified mRNAs were involved in the process of metabolism. The mRN
processing and RNA splicing were the top two processes belonging to the biological process (BP) ontology of the GO analysis. Molecule function (MF) showed that most transcripts functioned in RNA or protein binding. In addition, the nucleus was the main location of the proteins translated from m6A-modified mRNAs (Fig. 5C). All these results demonstrated that m6A modification was a key player in the
regulation of RNA processes. KEGG pathway analysis showed that the spliceosome and cell cycle were the top two pathways in which m6A-modified genes were involved. Several pathways related to virus infection and host immune response were also identified in this analysis, which suggested that the m6A modification also affected virus infection and host defenses (Fig. 5D).

3.6. Validation of m6A modification to gene expression

The results above showed that many genes had changes in their m6A
Fig. 5. PEDV infection influences RNA methylation of host cell transcripts
A) Distribution of m^6^A peaks in different types of host RNA transcripts. B) Distribution of m^6^A peaks in different structures of host mRNA. The m^6^A-modified mRNAs were searched in the C) GO database for functional significance and in the D) KEGG pathway database for pathway analysis.
modification during PEDV infection. According to our statistical analysis, 647 genes exhibited m^6^A modification, and 67 genes lost this modification with a p-value < 0.05. The differently modified genes were then clustered by the pathways that they were involved in (Fig. 6A). In this network, the MAPK signaling pathway was identified as the hotpot of upregulated genes, while the tight junction pathway was identified as the hotpot of downregulated genes. We then selected 26 genes that were differentially modified during PEDV infection and were involved in the above pathways for further analysis. First, real-time PCR was performed to confirm the dynamic modification of m^6^A in these fragmented RNAs prepared for m^6^A-seq. The primers were designed according to m^6^A-modified sequences obtained from m^6^A-seq (Table 3). As no genes have been found to be stably modified by m^6^A, no internal control was set in the analysis but equal amounts of RNA were used for reverse-transcription. The results showed that all these genes had changed their modification during PEDV infection, except DAXX, SRF, and TOK2 (Fig. 6B).

Next, we attempted to determine how these candidate genes respond to PEDV infection and whether demethylase can reverse their influence. LLC-PK1 cells were divided into three groups: untreated cells, cells infected with PEDV, cells overexpressing FTO, and infected with PEDV. The gene expression level in cells infected with PEDV was compared with that in the other two groups. The real-time PCR analysis showed that most genes had changed expression levels during PEDV infection. However, the expression pattern of these genes was reversed after the FTO transfection. For example, DAXX was upregulated during PEDV infection, indicating that the expression of DAXX was downregulated by FTO transfection. The expression of SRF and TOK2 was upregulated during PEDV infection, indicating that the expression of SRF and TOK2 was downregulated by FTO transfection. The expression of DUSP6 was upregulated during PEDV infection, indicating that the expression of DUSP6 was downregulated by FTO transfection. The expression of MAPK5 was upregulated during PEDV infection, indicating that the expression of MAPK5 was downregulated by FTO transfection. The expression of MAPK5 was upregulated during PEDV infection, indicating that the expression of MAPK5 was downregulated by FTO transfection.

**Fig. 6.** M^6^A modification regulates the host response to PEDV infection
A) The pathway network analysis of different signaling pathways in which the m^6^A-modified genes were involved. B) Real-time PCR verification of m^6^A-modified genes involved in the tight junction and MAPK/p53 signaling pathway. C) Real-time PCR analysis of selected m^6^A-modified genes in LLC-PK1 cells. The expression levels of all genes were normalized to GAPDH levels (internal control). The 2^−ΔΔCt^ method was used to calculate the relative gene expression data. All experiments were performed in triplicate.
PEDV infection, while the expression of FTO decreased its transcription (Fig. 6C). Other genes had similar expression patterns. These results indicated that PEDV infection could change the distribution of m^6^A in mRNA. The distribution in most transcripts was upregulated, and de-methylase could reverse the expression level of these genes. This result correlated with the previous hypothesis (Hoermes et al., 2016). The dynamic methylation changes in host RNA implied that the enhancement of m^6^A modification during PEDV infection might be a regulation driven by host immunity.

4. Discussion

M^6^A modification has been shown to regulate the replication of several viruses. However, the specific role and mechanism still need further exploration. In this study, m^6^A modification was identified to be present in PEDV genomic RNA. We then quantified and mapped seven internal m^6^A peaks in PEDV RNA. Further studies showed that m^6^A modification reduced PEDV replication in different cell lines. The knockdown of METTL3 and METTL14 accelerated PEDV replication, while knockdown of FTO suppressed PEDV replication. The replication of viral RNA was also influenced. Subsequently, we investigated the role of m^6^A in host response during PEDV infection in LLC-PK1 and Vero cells. The ratio of m^6^A modification increased while the expression level of FTO decreased after PEDV infection. High-throughput sequencing revealed that there were many genes differently modified by m^6^A during PEDV infection. Real-time PCR analysis also proved these results. The overexpression of FTO could reverse the host response. Our findings demonstrated that m^6^A modification represents an important component in host regulation of virus replication.

Modifications in biological molecules have been recognized as an important regulatory measure for various life processes. The modification of RNAs can alter their biochemical structure and cell biology function. To date, 163 posttranscriptional modifications of RNA have been discovered to introduce functional diversity (Ioscaletto et al., 2018). The m^6^A modification is the most abundant internal modification in eukaryotic RNAs. After its discovery in the 1970s (Desrosiers et al., 1974), researchers attempted to determine whether this modification also existed within viral RNA. With the development of the isotope-labeling method and other detection methods, m^6^A was found in the RNA of several viruses, including RNA viruses, DNA viruses, and retroviruses (Krug et al., 1976; Sommer et al., 1976; Moss et al., 1977; Furuichi et al., 1975). However, sequencing techniques and poor understanding of the regulation process have restricted further exploration. In 2011, 2013, the discovery of demethylases combined with the previous identification of a methyltransferase complex and RNA-binding proteins highly accelerated the studies of m^6^A (Jia et al., 2011; Zheng et al., 2013; Bokar et al., 1994; Liu et al., 2014). In 2012, antibody-based immunoprecipitation followed by high-throughput sequencing enabled the transcriptome-wide profiling of m^6^A (Dominissini et al., 2012). These developments promoted the studies on m^6^A in viral replication.

HIV-1 was the first virus with m^6^A peaks mapped within its genomic RNA. In 2016, three groups investigated the role of m^6^A during HIV-1 infection (Tirumuru et al., 2016; Kennedy et al., 2016; Lichinchi et al., 2016a). Two studies showed that m^6^A promoted the replication of HIV, while the other reported the opposite result. Later, the repetitions of the Zika virus, hepatitis C virus, and members of the Flaviviridae family were found to be negatively regulated by m^6^A (Gokhale et al., 2016; Lichinchi et al., 2016a). For the influenza virus and enterovirus 71, it was demonstrated that m^6^A contributed to their replication (Hao et al., 2019; Courtney et al., 2017). In our study, m^6^A inhibited and overexpression of FTO facilitated PEDV growth. All of these studies suggest that m^6^A modification could play different roles in the regulation of viral replication. Various factors may account for this. First, m^6^A contributes not only to the regulation of viral RNA but also to host RNAs. Both viral and host factors may influence the replication of viruses (Gokhale and Horner, 2017). The output of viral replication is determined by the balance of interaction effects between viral and host factors. Therefore, it is reasonable that the final effects of m^6^A are different among different viruses. Second, viruses have hypervariable regions among their RNAs, which also exhibit different characteristics (Prentoe and Bukh, 2018; Smith, 1999). The presence of m^6^A may also be divergent for different strains, which may have different effects. Third, different cell lines usually are originated from different species and organisms and have different features and regulate viral growth by different pathways. Finally, the reversible process of m^6^A may have different effects on viral replication. The time points selected for analysis may also affect the results.

The presence of m^6^A also varies among different kinds of viruses. Although the number of identified m^6^A peaks varied among the studies of HIV, the studies agreed on the presence of m^6^A at the 3’ end of HIV-1 genomic RNA (Tirumuru et al., 2016; Kennedy et al., 2016; Lichinchi et al., 2016a). N5B and NS3 were the main regions in which m^6^A peaks were present among viruses within the Flaviviridae family (Gokhale et al., 2016; Lichinchi et al., 2016a). For influenza virus and enterovirus 71, m^6^A were peaked are located in the genes encoding structural proteins (Hao et al., 2019; Courtney et al., 2017), while our results showed that the m^6^A peaks were mainly presented in ORF1b, which encodes non-structural proteins. As m^6^A peaks are present in different regions of viral genomic RNA, the specific mechanism of m^6^A in regulating viral replication may vary between different viruses. For HCV, mutation of m^6^A sites within the E1 region can increase HCV RNA binding to the Core protein (Gokhale et al., 2016). The expression of HIV-1 gag and p24 was reduced by the knockdown of METTL3 and METTL14, which suggested that m^6^A might facilitate the translation of viral proteins (Tirumuru et al., 2016). Although m^6^A has been found to affect most posttranscriptional steps in gene expression, such as mRNA stability, splicing, translational efficiency and pri-microRNA processing (Zheng et al., 2013; Wang et al., 2015; Alarcon et al., 2015; Ozkurede et al., 2019), the regulatory mechanism has not been clearly elucidated and needs to be explored further.

With a better understanding of the effects of m^6^A on viral replication, the identification of specific sites modified by m^6^A requires additional research. The M^6^A-seq technique employed in the above studies was based on captured nucleotides of 100–200 bp length. Therefore, the clusters of m^6^A within 200 nucleotides cannot be precisely located by sequencing. The identification of Tth DNA polymerase I solved this problem. This DNA polymerase was previously shown to act as reverse transcriptase in the presence of Mn^2^+. Later, it was found that Tth DNA polymerase I could discriminate m^6^A from adenosine (A) in RNA. It was selective by up to 18-fold for the incorporation of thymi dine opposite unmodified A over m^6^A (Harcourt et al., 2013). The identification of two m^6^A modification sites in the EV71 genome benefited from the use of this polymerase. The adenines at 3055 and 4555 in the EV71 genome were proven to be m^6^A-modified. Mutations at these two sites resulted in decreased virus replication (Hao et al., 2019). However, this method needs to incorporate radiolabeled probes which have limited its use, such as for our study. The locations of m^6^A modification in RNAs at nucleotide resolution will no doubt improve the understanding of their function.

It was of great interest to us to find that PEDV infection increases the m^6^A ratio in host RNAs. KEGG analysis also showed that there were more genes with increased m^6^A modification. Most genes were upregulated during PEDV infection, while the overexpression of FTO downregulated their expression. These results suggested that m^6^A also functioned in the host response to PEDV infection. This finding correlated with previous studies showing that reversible m^6^A could regulate gene expression (Fu et al., 2014). This phenotype was also reported recently in HIV and VSV (Lichinchi et al., 2016b; Tirumuru and Wu, 2019; Liu et al., 2019). The infection of both viruses can enhance m^6^A modification in host RNA. It is interesting to further identify whether virus infection always enhances m^6^A modification and potential
reasons. Besides, we found that the expression of FTO was altered by virus infection. FTO, as the key component of demethylases, was decreased during PEDV infection. It is not clear whether the infection of other viruses can drive similar changes. These results suggested that m\textsuperscript{6}A modification could be an important mechanism in regulating gene expression, especially for the host response to viral infection.

To our knowledge, this is the first evidence to report the presence of m\textsuperscript{6}A in coronaviruses and its regulation of both virus and host responses. We presented global m\textsuperscript{6}A profiling of PEDV and found that this modification can inhibit PEDV infection. Furthermore, we found that the m\textsuperscript{6}A modification in host RNAs was also drastically changed during PEDV infection. Our study suggests that m\textsuperscript{6}A modification may represent a novel and conserved target for antiviral mechanisms. We believe that the results from this study will facilitate the understanding of the role of m\textsuperscript{6}A and broaden our knowledge on virus-host interactions at the RNA level.

Author contribution

GL and JC conceived the project, designed the experiments, analyzed the data, wrote and edited the manuscript. JC, LJ, ZW, LW, QC, YC performed the experiments. All authors approved the final manuscript.

CRediT authorship contribution statement

Jianing Chen: Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. Li Jin: Investigation. Zemei Wang: Investigation. Liyuan Wang: Investigation. Qingbo Chen: Investigation. Yaru Cui: Investigation. Guangliang Liang: Conceptualization, Methodology, Validation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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