N6-Methyladenosine modification of lincRNA 1281 is critically required for mESC differentiation potential

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ABSTRACT

Previous studies have revealed the critical roles of N6-methyladenosine (m6A) modification of mRNA in embryonic stem cells (ESCs), but the biological function of m6A in large intergenic noncoding RNA (lincRNA) is unknown. Here, we showed that the internal m6A modification of linc1281 mediates a competing endogenous RNA (ceRNA) model to regulate mouse ESC (mESC) differentiation. We demonstrated that loss of linc1281 compromises mESC differentiation and that m6A is highly enriched within linc1281 transcripts. Linc1281 with RRACU m6A sequence motifs, but not an m6A-deficient mutant, restored the phenotype in linc1281-depleted mESCs. Mechanistic analyses revealed that linc1281 ensures mESC identity by sequestering pluripotency-related let-7 family microRNAs (miRNAs), and this RNA-RNA interaction is m6A dependent. Collectively, these findings elucidated the functional roles of linc1281 and its m6A modification in mESCs and identified a novel RNA regulatory mechanism, providing a basis for further exploration of broad RNA epigenetic regulatory patterns.

INTRODUCTION

Embryonic stem cells (ESCs) have two defining features: the ability of self-renewal and the capacity to give rise to most cell types. These unique features of ESCs have made them a valuable tool for clinical therapies and enabled the in vitro study of early mammalian development. Multiple genes, including transcription factors, chromatin-remodeling enzymes and signaling molecules, are precisely coordinated to control ESC pluripotency (1,2). In addition to protein-coding genes, large intergenic noncoding RNAs (lincRNAs) are a novel class of gene regulators that function as signals, scaffolds, molecular decoys and mediators of long-range chromatin interactions (3). ESC-specific lincRNAs have been identified using the chromatin signature of actively transcribed genes (4,5). Researchers recently demonstrated that the vast majority of these lincRNAs are required for the maintenance of ESC pluripotency by performing systematic loss-of-function experiments, which indicated that lincRNAs are involved in the molecular circuitry of ESCs (6). However, relatively little is known about the functional role of individual lincRNAs in ESCs, and it remains unclear how lincRNAs epigenetically modulate ESC self-renewal and cell-fate decisions. Characterization of lincRNA pathways and their underlying molecular mechanisms is therefore important for understanding ESC pluripotency.

Analysis of the role of RNA modification in the regulation of gene expression has led to the development of the new field of ‘RNA epigenetics’ (7). N6-methyladenosine (m6A) is the most abundant internal RNA modification in mammalian systems (8) and plays a diverse role in epigenetic regulation of RNA metabolism, including mRNA stability (9,10), alternative splicing (11,12), cap-independent protein translation (13) and microRNA (miRNA) biogenesis (14). m6A biology is crucial for cell status regulation, sex determination and development (12,15–23). Dysregulation of this modification is clearly linked to human diseases such as obesity and cancer (24). Recent studies have suggested the involvement of m6A in mouse ESC (mESC) self-renewal and cell-fate decisions (10,25,26). Genetic inactivation or deletion of Mettl3 (an RNA methyltransferase complex component) in mESCs, and hence m6A, blocked differentiation and resulted in early embryonic lethality. To elucidate the function and regulatory mechanism of m6A in mESCs, researchers identified a large number of core pluripotency regulators in mESCs, such as Nanog, Klf4, Med1 and Eed, showing m6A modifications. The m6A mark impairs the stability of methylated transcripts in mESCs.

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by increasing mRNA degradation rates. In the absence of m6A modification, mESCs show prolonged Nanog expression upon differentiation, which inhibits the exit of mESCs from self-renewal. All studies performed on this topic to date have addressed the critical role of the m6A modification of mRNA in the RNA methylation-mediated control of stemness. However, no reports have addressed the biological function of m6A in lincRNAs, despite the fact that some mESC-expressed lincRNAs are m6A modified (26), and lincRNAs are key players in the mESC pluripotency regulatory circuit. We therefore hypothesize that m6A in lincRNAs may mediate the relationship between lincRNAs and transcriptional networks in mESCs.

As key posttranscriptional modifiers, miRNAs have been shown to be involved in maintaining mESC pluripotency. mESCs with genetic deletion of the miRNA processing enzyme Dicer or DGCR8 exhibit differentiation-defective phenotypes (27–29). A number of studies have identified mESC-specific miRNAs and have shown that miRNAs are key regulators of mESC self-renewal and cell-fate decisions (30–33). MiRNAs modulate the pluripotent state by fine tuning the expression of target genes, including mESC-specific transcription factors and key pathway molecules. However, although miRNA-mediated pathways in mESCs have been extensively characterized, the regulatory networks modulating miRNA function have not been fully elucidated. Recently, several authors have proposed a lincRNA-mediated regulatory model in which lincRNA functions as competing endogenous RNA (ceRNA) to modulate the activities and biological functions of miRNAs. These reports have indicated that linc-MD1 acts as a sponge of miR-133 and miR-135 to protect the mRNA of the muscle-specific genes MAML1 and MEF2C (34). Human pluripotency-associated transcript 5 (HPAT5) binds directly with the let-7 miRNA family to modulate reprogramming and human ESC (hESC) differentiation (35). Linc-RoR has also been identified as a ceRNA that sequesters differentiation-related miRNAs in hESCs and regulates the expression of the core transcription factors OCT4, SOX2 and NANOG (36).

Here, we first elucidated the functional role of m6A modification of lincRNA in the regulation of mESC pluripotency. We found that the mESC-specific linc1281 is necessary for mESC differentiation, and its essential functional role relies on m6A enrichment within the last exon of linc1281.

**MATERIALS AND METHODS**

**Cell culture and differentiation**

Sox1-GFP mouse ES cells (37) (46C; a gift from A. Smith) were maintained on feeders under mESC culture conditions. For embryoid body (EB) differentiation, mESCs were plated at a density of 5 × 10⁴ cells/ml in ultra-low-attachment plates in mESC medium without LIF. The medium was changed every two days, and on day 4, the EBs were seeded onto a gelatin-coated 48-well plate. For neural differentiation, the mESCs were grown in differentiation medium (G-MEM supplemented with 8% Knockout Serum Replacement, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 0.1 mM 2-mercaptoethanol with or without 5% FBS). For quantification of the percentage of Sox1⁺ cells, mESCs were dissociated into single cells in 0.25% trypsin-EDTA, and FACS analysis was immediately performed.

**CRISPR-mediated linc1281 knockout**

Plasmids for guide RNA (designed with the CRISPR design tool Zinc Finger Targeter (38,39)), a Cas9 expression plasmid, and a donor plasmid containing the PGK-Puro insert were cotransfected into mESCs using an electroporation method. Cells were plated at low density and treated with puromycin two days after transfection. Single colonies were selected and tested for loss of linc1281.

**Lentiviral vectors**

For the generation of shlinc1281 or shMettl3, short hairpin RNAs targeting linc1281 or Mettl3-specific regions (shlinc1281-1, ACAGGATACAAAGCCTTTG; shlinc1281-2, GGTCTCTTTTTGCCTTATA; shMettl3-1, GCACACTGATGAAATCTTTA; shMettl3-2, CGTCAGATCTTGGCCAAATT) were designed and cloned into the pLKO.1-TRC cloning vector. The full-length linc1281 (NC_000079.6) was synthesized and cloned into the FUGW vector to generate an overexpression vector. Mutations were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies).

**qRT-PCR analysis**

Total RNA was extracted using the RNAiso Reagent (TaKaRa) according to the manufacturer’s instructions. The synthesis of cDNA was performed using the PrimeScript RT reagent kit (TaKaRa) according to the manufacturer’s instructions. MiRNA levels were measured using the Bulge-Loop™ miRNA qPCR Primer Set (RiboBio) according to the manufacturer’s instructions.

**Immunofluorescence staining**

For immunofluorescence staining, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 for 8 min. The cells were then washed twice with PBS, blocked in 10% FBS for 45 min and incubated overnight with primary antibodies at 4°C. Next, the cells were stained with fluorescent secondary antibodies for 2 h and with Hoechst 33342 dye for 20 min. Images were acquired using a fluorescence microscope. The primary antibodies used in this work included anti-cTnT (Abcam), anti-N-cad (BD) and anti-GFP (Abcam).
Western blotting
Cells were collected, washed twice with ice-cold PBS and incubated on ice for 30 min with RIPA lysis buffer (Beyotime). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo). Primary antibodies including anti-Lin28a (Cell Signaling Technology), anti-Lin28b (Cell Signaling Technology), anti-Mettl3 (Abcam) and anti-GAPDH (Bioworld Technology) were used in these assays.

RNA FISH
For the detection of linc1281, RNA FISH was conducted using a Fluorescent in Situ Hybridization Kit (RiboBio) in accordance with the manufacturer’s directions. The probe mix used for linc1281 was linc1100439 (RiboBio).

Cytoplasmic and nuclear RNA fractionation
Nuclear and cytoplasmic RNA was isolated as previously described (40). mESCs were collected, washed twice with ice-cold PBS and centrifuged at 1000 rpm for 10 min. Cell pellets were gently resuspended and incubated on ice for 5 min in 200 μl of lysis buffer A [Tris (10 mM, pH 8.0), NaCl (140 mM), MgCl2 (1.5 mM) 0.5% Nonidet P-40] and then centrifuged at 1000 × g for 3 min at 4°C. The supernatant, containing the cytoplasmic fraction, was added to 1 mL of RNAiso Reagent. Nuclear pellets were washed with lysis buffer A twice, followed by washing with lysis buffer A containing 1% Tween-40 and 0.5% deoxycholic acid, and then resuspended in 1 mL of RNAlater Reagent. Purification and analysis of cytoplasmic and nuclear RNA were subsequently performed via qRT-PCR assays.

RIP assay
The MS2bp-MS2bs-based RIP assay was performed as described previously (40). HEK293FT cells were cotransfected with 8 μg of the MS2bs-linc1281 vector or the blank control vector MS2bs- Renilla, 8 μg of the MS2bp-YFP plasmid and miRNA mimics (50 nM), using 100 μl of FuGENE HD reagent (Roche). After 48 h, the cells were collected and subsequently lysed with lysis buffer [KCl (100 mM), MgCl2 (5 mM), HEPES (10 mM, pH 7.0), NP-40 (0.5%v/v), DTT (1 mM), PI (100×), RNaseOut (0.1 U/μl), PMSF (1 mM)] for 30 min on ice. Immunoprecipitation was performed using anti-YFP (Abcam) or control rabbit IgG (Cell Signaling Technology). The RNA complexes were isolated through phenol-chloroform extraction and analyzed via qRT-PCR assays.

Biotin-RNA pull-down and LC–MS
To identify linc1281-interacting proteins in mESCs, biotin-RNA pull down and subsequent liquid chromatography-mass spectrometry were performed. Briefly, biotin-labeled RNAs were synthesized using RNA Labeling Mix (Roche, 1163597910) and T3/T7 RNA polymerase (Roche, 10881767001/11031171001). Linc1281 or antisense RNAs were heated to 90°C for 2 min, held on ice for 2 min and then incubated in room temperature for 20 min in RNA structure buffer (10 mM Tris–HCl pH 7.0, 0.1 M KCl and 10 mM MgCl2) to form proper secondary structure. mESCs (1 × 10⁶ cells) were lysed with RIP lysis buffer and Immunoprecipitation was performed by using streptavidin beads coated with 3 μg of linc1281 or antisense RNAs. The RNA-binding proteins were sequenced and identified by LC–MS as described previously (41). The mass spectra were searched using the Mascot Daemon software (Version 2.3.0, Matrix Science, London, UK) based on the Mascot algorithm. The database used to search was the Mouse UniProtKB database.

Measurement of m⁶A-modified linc1281 levels
For quantification of m⁶A-modified linc1281 levels, methylated RNA immunoprecipitation was performed. A 1.5 μg aliquot of anti-m⁶A antibody (Synaptic Systems, 202003) or anti-IgG (Cell Signaling Technology) was conjugated to protein A/G magnetic beads overnight at 4°C. A 100 μg aliquot of total RNA was then incubated with the antibody in IP buffer supplemented with RNase inhibitor and protease inhibitor. RNA was eluted with elution buffer, purified through phenol-chloroform extraction and then analyzed via qRT-PCR assays.

Dual-luciferase assay
Luciferase reporters were generated by cloning linc1281 or mutant linc1281 into pGL3-basic vectors. Mutant vectors were obtained using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Then, NIH/3T3 cells (3 × 10⁴) were cotransfected with 100 ng of reporter, 5 ng of the internal control Renilla vector and miRNA mimics (50 mM), with 1 μl of the FuGENE HD reagent. Cell lysates were harvested 48 hr after transfection, and luciferase activities were examined using the Dual-Luciferase Reporter Assay System (Promega).

Teratoma generation
mESCs were trypsinized into single cells, resuspended at a concentration of 1.5 × 10⁶ cells/150 μl and then injected into athymic nude mice (NOD-SCID) obtained from the National Resource Center of Mutant Mouse Model Animal Research Center (NARC), NJU. Tumors were assessed every week and harvested 4 weeks later. All experiments were carried out as approved by the Institutional Animal Care and Use Committee of Tongji University.

Statistical analysis
Statistical significance was analyzed with Student’s t-test (two-tailed). The error bar represents the standard error of the mean (SEM), and all experiments were independently repeated at least three times.

RESULTS
Linc1281 is required for proper mESC differentiation
To explore the functions of linc1281 in mESCs, we analyzed linc1281 levels of mESCs in the presence or absence
of LIF for 4 days via qRT-PCR. **Linc1281** was significantly enriched in mESCs, suggesting its association with pluripotency (Figure 1A). RNA FISH assays and analysis of the cellular distribution of RNAs both showed that **linc1281** transcripts were abundant in the cytoplasm of mESCs (Figure 1B and C). Thus, we performed shRNA-mediated loss-of-function assays, in which two distinct shRNAs resulted in at least 85% knockdown of endogenous **linc1281** in mESCs (Figure 1D). **Linc1281**-depleted cells exhibited normal properties of self-renewal, with no change in the expression levels of mESC-specific genes, including **Oct4**, **Sox2** and **Nanog**, suggesting that **linc1281** is dispensable for mESC self-renewal and may be involved in other aspects of stem cell biology (Figure 1E). We next examined whether the differentiation capacity of mESCs was affected when **linc1281** expression was reduced *in vivo*. We subcubaneously injected **linc1281**-depleted or control cells into immune-deficient mice. Although both cell types formed tumors with morphology consistent with teratomas, the tumors derived from **linc1281**-depleted cells were significantly smaller and lower in weight than control tumors (Figure 1F). Histological analysis of hematoxylin and eosin (H&E)-stained tumor sections revealed that teratomas derived from **linc1281**-deficient cells contained few structures from all three germ layers (Figure 1G). The decreased expression of lineage-specific markers in tumors from **linc1281**-deficient cells further confirmed that differentiation was disrupted in **linc1281**-depleted mESCs (Figure 1H).

Next, we tested the *in vitro* capacity for directed differentiation toward two lineages: mesoderm-derived cardiomyocytes and ectoderm-derived neural lineage cells, which are two ideal *in vitro* differentiation models for analysis of the mechanisms underlying the developmental process. qRT-PCR analysis showed that in the reduction by knockdown of **linc1281**, mESCs displayed significantly reduced expression levels of mesoderm (**Gata4**, **Mesp1** and **Cxcr4**), cardiac progenitor (**Mef2c**, **Is11** and **Tbx5**) and cardiomyocyte marker genes (**Myl2**, **Myb6**, **Myl7**, **Sle8a1**, **MHC** and **cTnT**) (Figure 1I). While 75% (EB Day 10) or 80% (EB Day 12) of EBs generated from control cells could differentiate into spontaneously beating cardiomyocytes, only 40% (EB Day 10) or 50% (EB Day 12) of **linc1281**-deficient EBs developed spontaneously into contracting clusters (Figure 1J). Immunostaining showed that differentiated EBs from **linc1281**-deficient cells had reduced expression of **cTnT** (Figure 1K). Similarly, compared with control cells, **linc1281**-deficient cells showed decreased differentiation into the neural lineage: the cells exhibited a substantially reduced percentage of **Sox1** (a neural-progenitor-specific gene)-positive (**Sox1***) cells (Figure 1L), lower expression levels of neural markers (**Sox1**, **Pax6**, **N-cad**, **Zifp521**, **Map2** and **TuJ1**, Figure 1M) and a decrease in **Sox1**-GFP/N-cad-positive cells (Figure 1N). To further address the functions of **linc1281** in maintaining mESC pluripotency, we targeted **linc1281** via CRISPR-mediated gene editing and generated **linc1281** knockout (KO) mESC lines (Supplementary Figure S1A and B). RNA FISH and qRT-PCR analyses confirmed the absence of **linc1281** (Supplementary Figure S1C-D). Consistent with the results from the shRNA-mediated loss-of-function assays, **linc1281** KO cells showed no significant differences in the expression levels of pluripotency markers (**Oct4**, **Sox2** and **Nanog**) and a reduced capacity for differentiation (Supplementary Figure S1E-M). Furthermore, the forced expression of **linc1281** significantly enhanced mESC differentiation, while showed no effect on the expression levels of mESC-specific genes (Supplementary Figure S2A and B). In EB formation assays, mESCs overexpressing **linc1281** showed increases in the expression levels of lineage markers, the percentages of EBs containing spontaneously beating areas and cTnT-positive cell numbers (Supplementary Figure S2C–G). In neural differentiation assays, the forced expression of **linc1281** enhanced the efficiency of neural generation in nonpermissive cultures containing 5% FBS (Supplementary Figure S2H–J). Furthermore, we precisely evaluated the physiological levels of **linc1281** in mESCs and differentiated cell populations from mESCs and found that **linc1281** showed quite low expression in differentiated cells compared with that in mESCs, which was consistent with the above observation (Supplementary Figure S3). Considering all of these findings together, we concluded that depletion of **linc1281** in mESCs inhibits their capacity for differentiation and that **linc1281** is required for proper differentiation of mESCs.

The m6A modification is enriched in **linc1281**

Recent studies have suggested that the m6A RNA modification can determine the fate of mESCs, and thousands of mESC-specific transcripts, including lincRNAs, are modified by m6A. We investigated the m6A modification of **linc1281** in mESCs because this lincRNA is a critical regulator of differentiation in mESCs and may be modified by m6A according to a previous report (26). We verified the m6A modification of **linc1281** by performing a methylated RNA immunoprecipitation (MeRIP) assay (Figure 2A). To further characterize the m6A methylation of **linc1281**, we performed shRNA-mediated downregulation of Mettl3, an important component of the m6A methylase complex, in mESCs. The **shMettl3** cell lines exhibited 70% knockdown of the endogenous Mettl3 protein and RNA and no change in the expression levels of **linc1281** (Figure 2B and C). Nevertheless, Mettl3 deficiency substantially decreased the m6A level on **linc1281**, indicating that Mettl3 is a major m6A methylase for **linc1281** in mESCs (Figure 2D).

The internal m6A mark is positively involved in the functional role of **linc1281** in mESCs

The **linc1281** RNA molecule contains 1306 nucleotides and is generated from 3 exons. To further define the potential functions of m6A-modified **linc1281**, we analyzed the m6A sites located in **linc1281** and found three RRACU m6A sequence motifs in the last exon, which was previously described as a location for m6A deposition (Figure 2E). Next, we constructed expression vectors harboring **linc1281** point mutants, in which the adenosine residues embedded within the m6A motifs were replaced with guanine (A-G transition mutation) or thymine (A-T transversion mutation), and a deletion mutant, in which the adenosine residues were deleted. The mutants in which with a single (A1096-G) or three (A1096, 1024 and 1054-G) unmethylated adenosine residues within the m6A motifs were replaced with guanine were employed as controls. We transfected HEK293FT cells, which
Figure 1. linc1281 is required for proper mESC differentiation. (A) Expression levels of linc1281 in mESCs in the presence or absence LIF for 4 days. (B) RNA FISH for linc1281 in mESCs. Scale bar represents 100 or 20 μm. (C) qRT-PCR analysis of transcripts derived from the cytoplasmic or nuclear fractions of mESCs. The log10 ratio of cytoplasmic to nuclear transcripts is presented. (D) qRT-PCR analysis of linc1281 knockdown efficiency. pLKO.1-scramble shRNA was used as a negative control. (E) mRNA levels of Oct4, Sox2 and Nanog upon linc1281 depletion determined via qRT-PCR. (F) Weight differences between teratomas generated from shControl and shlinc1281 cells. (G) Representative sections of teratomas stained with H&E. Scale bar represents 25 μm. (H) Lineage-specific marker expression in teratomas. (I) qRT-PCR analysis of mesoderm (left), cardiac progenitor (middle) and cardiomyocyte markers (right) during EB formation. EB, embryoid bodies. (J) Percentage of EBs with beating activity. (K) Representative images of bodies stained for cTnT and Hoechst 33342 (Ho.33342) on day 12 of EB formation. Scale bar represents 100 μm. (L) Percentage of Sox1+ cells analyzed via FACS during neural lineage differentiation. (M) qRT-PCR analysis of neural markers on day 5 of neural differentiation. (N) Representative images of cells stained for Sox1-GFP, N-cad and Ho.33342 on day 5 of neural differentiation. Scale bar represents 100 μm. Error bars show ± SEM (n ≥ 3). ***P < 0.001, **P < 0.01, *P < 0.05 versus shControl.
Figure 2. m^6^A enrichment is positively associated with the role of linc1281 in mESCs. (A) Enrichment of m^6^A-modified linc1281 in mESCs. Unmethylated transcript Stat3 was used as negative control. The percentage of the input is shown. Error bars show ± SEM (n = 4). ***P<0.001 versus anti-IgG. (B) Western blot analysis of Mettl3 knockdown efficiency. GAPDH was used as the loading control. (C) Transcript levels of linc1281 and Mettl3 in shControl and shMettl3 mESCs. Error bars show ± SEM (n = 3). **P < 0.01 versus shControl. (D) Changes in m^6^A-modified levels upon Mettl3 depletion. The percentage of the input is shown. Error bars show ± SEM (n = 3). **P < 0.01 versus shControl. (E) Schematic representation of the position of m^6^A motifs within linc1281. (F) Levels of linc1281 transcripts in HEK293FT cells transfected with linc1281-overexpressing plasmids or mutants. ND, not detected. linc1281, Fuw-linc1281; A-G mutant, mutant with A-G transition mutations; A-T mutant, mutant with A-T transversion mutations; A-Del mutant, mutant with adenine residues deletion; NC mutant, negative control mutant with A916, 1024 and 1054-G transition mutations; A916-G mutant, mutant with A916-G transition mutation. Error bars show ± SEM (n = 3). **P < 0.01 versus linc1281. (G) Changes in m^6^A-modified levels between linc1281 and mutants in HEK293FT cells. The human transcript GAPDH was used as negative control. The percentage of the input is shown. Error bars show ± SEM (n = 3). ***P < 0.001, *P < 0.05 versus linc1281. (H) Levels of linc1281 in shControl and shlinc1281 mESCs in the presence of the indicated overexpression constructs. shCtrl, shControl; sh1, shlinc1281-1; sh2, shlinc1281-2; FlucC, Fuw-luciferase Control. (I-K) Expression of cardiomyocyte markers on day 10 (I), percentage of beating EBs (J) and representative images of bodies stained for cTnT and Ho.33342 (K) on day 12 during EB formation. Scale bar represents 100 μm. Error bars show ± SEM (n = 3). The asterisk (*) denotes a significant difference from 'shCtrl+FlucC', and the hash mark (#) denotes a significant difference from 'sh1+FlucC' or 'sh2+FlucC'. ***, ###P < 0.001, ***, **P < 0.01, *, #P < 0.05. (L-N) Percentage of Sox1+ cells (L), expression of neural markers (M) and representative images of cells stained for Sox1-GFP, N-cad and Ho.33342 (N) on day 5 of neural differentiation. Scale bar represents 100 μm. Error bars show ± SEM (n ≥ 3). The asterisk (*) denotes a significant difference from 'shCtrl+FlucC', and the hash mark (#) denotes a significant difference from 'sh1+FlucC' or 'sh2+FlucC'. ***, ###P < 0.001, ***, **P < 0.01, *, #P < 0.05.
To assess the relationship between m^6^A and the regulatory functions of linc1281 in mESCs, we stably overexpressed linc1281, the m^6^A-deficient A-G mutant or the m^6^A-decorated NC mutant using lentiviral vectors in linc1281-knockdown (sh1281) mESCs and performed in vitro differentiation assays. qRT-PCR analysis showed that the expression of linc1281 was restored in sh1281 mESCs following the introduction of either wild-type linc1281 or mutants, which were designated as follows: ‘sh1281+linc1281 (sh1+linc1281 and sh2+ linc1281)’; ‘sh1281+A-G mutant (sh1+A-G mutant and sh2+A-G mutant)’; and sh1281+NC mutant (sh1+NC mutant and sh2+NC mutant), respectively (Figure 2H). As expected, the ‘sh1281+linc1281’ cell lines displayed a significantly increased EB differentiation efficiency, as evidenced by increases in cardiomyocyte markers (Supplementary Figure S4). In addition, the m^6^A enrichment of wild-type linc1281 verified that the functions of the m^6^A methylase complex are evolutionarily conserved in humans and mice.

To test the specificity of let-7 binding to predicted target sites, we constructed mutant luciferase reporters containing a 7-bp antisense mismatch within the target site of linc1281 combined with MS2bp-YFP and let-7 mimics. The vector (MS2bs-linc1281) expressing linc1281 combined with MS2bs elements was co-transfected into HEK293FT cells with the vector encoding MS2bs-YFP and let-7 mimics. The vector (MS2bs- Renilla) expressing Renilla luciferase RNA was used as a negative control. The transcript-specific binding RNA-protein complexes were immunoprecipitated with a YFP antibody and then analyzed via qRT-PCR. Immunoglobulin G (IgG) was used as a negative control. We found that let-7 mimics significantly reduced luciferase activity when cotransfected with let-7 mimics, whereas the mutant reporters were re-fractory to let-7-driven reporter inhibition (Figure 3C).

Next, to further explore the interaction of linc1281 and let-7 miRNAs in a more physiological context, we transfected mESCs with MS2bs-linc1281 or MS2bs-Renilla combined with MS2bp-YFP and performed RIP assays. As expected, let-7 miRNAs were expressed in mESCs and showed significant enrichment in MS2bs-linc1281-binding RNAs compared with the negative control (Figure 3D).
Figure 3. Linc1281 functions as a ceRNA of the let-7 family in mESCs. (A) Summary of let-7 family target sites in linc1281. (B) Relative luciferase activities of reporters containing linc1281 (pGL3-linc1281, B) or mutants (C) in NIH/3T3 cells 48 hr after cotransfection with the indicated miRNAs or the scramble negative control (Ctrl mimics). Firefly luciferase activity was normalized to control Renilla luciferase activity. pA, polyadenylation signal. Error bars show ± SEM (n = 3). **P < 0.01, *P < 0.05 versus Ctrl mimics. (D and E) Enrichment of immunoprecipitated miRNAs in HEK293FT cells (D) or mESCs (E). MS2bs-Renilla luciferase RNA was used as a negative control. The fold enrichment relative to the IgG control is shown. Error bars show ± SEM (n = 3). ***P < 0.001, **P < 0.01, *P < 0.05 versus MS2bs-Renilla. (F and G) Expression levels of mature let-7 miRNAs in mESCs upon linc1281 knockdown (F) or knockout (G). SnoRNA U6 was used as an internal control for normalization. Error bars show ± SEM (n = 3). **P < 0.01, *P < 0.05 versus shControl or WT. (H) Representative western blot analysis of Lin28a and Lin28b in mESCs upon linc1281 depletion. GAPDH was used as the loading control.
NAs can influence miRNA expression (35,36). Thus, we examined miRNA levels and found that mature let-7 miRNAs were substantially increased in linc1281-knockdown and linc1281-knockout mESCs (Figure 3F and G), suggesting that let-7 miRNAs are inversely associated with linc1281 levels. In addition, following an increase in let-7 family abundance, the Lin28 RNA-binding proteins, which are the critical targets of let-7 miRNAs, displayed significantly reduced protein levels (Figure 3H), which confirmed that linc1281 indeed regulates the let-7/Lin28 axis.

Collectively, these findings indicated that linc1281 serves as a ceRNA through direct binding with let-7 miRNAs in mESCs.

The m^6^A modification is necessary for the linc1281-mediated ceRNA model

To determine the relationship between the m^6^A modification of linc1281 and linc1281-mediated RNA-miRNA interactions, we first examined the expression levels of let-7 miRNAs in 'sh1281+linc1281', 'sh1281+A-G mutant' and 'sh1281+NC mutant' mESCs. We found that 'sh1281+linc1281' and 'sh1281+NC mutant' cells showed decreased let-7 miRNA levels compared with 'sh1281+FlucC' cells (Figure 4A). However, the 'sh1281+A-G mutant' population still displayed high miRNA levels, despite the fact that we observed no difference in linc1281 transcript levels between these cell types. Coupled with the observation that the A-G mutant contained an insufficient level of the m^6^A modification, we hypothesized that m^6^A on linc1281 may be involved in the direct RNA-RNA interaction. Thus, we established a Mettl3 knockdown NIH/3T3 cell line using shRNA, which exhibited at least 80% reduction of the endogenous Mettl3 protein (Figure 4B). Cells infected with lentivirus containing scrambled shRNA were used as control cells. Then, we performed luciferase reporter assays in shControl and shMettl3 cells after transfection with the linc1281 reporter and miRNA mimics. The significant decrease in luciferase activity observed in control cells suggested direct binding and regulation of linc1281 by miRNAs, which was consistent with the previous results (Figure 4C). However, the effect of let-7 miRNAs on the linc1281 reporter was abolished upon Mettl3 knockdown, as reporter activity showed no change upon cotransfection with let-7 miRNAs in shMettl3 cells (Figure 4D). These results revealed that the m^6^A methyltransferase Mettl3 is critical for the binding of mature let-7 miRNA to linc1281. We overexpressed wild-type Mettl3 (WT) or a catalytically dead mutant of Mettl3 (Mut; residues 395–399: DPPW-APPA) (14,50) in shMettl3 mESCs (Figure 4E) and detected the m^6^A levels of linc1281 in these cell lines. Restoration of the m^6^A modification of linc1281 by wild-type Mettl3, but not the Mettl3 mutant, further confirmed that the m^6^A modification of linc1281 is dependent on the enzymatic activity of Mettl3 (Figure 4F). Then, we introduced Mettl3 and the mutant into Mettl3 knockdown NIH/3T3 cells (Figure 4G) and performed luciferase reporter assays. Despite the equal expression levels of Mettl3 and the mutant in shMettl3 cells, the abolished effect of let-7 miRNAs on the linc1281 reporter in shMettl3 cells was restored by Mettl3, but not by the catalytically dead mutant (Figure 4H-I), which further suggested that the Mettl3-dependent m^6^A modification of linc1281 is necessary for linc1281-miRNA interactions.

To rule out the possibility that this result was caused by a Mettl3 deficiency-mediated decrease in the widespread m^6^A modification in shMettl3 cells, we constructed luciferase reporters containing an m^6^A-deficient mutant in the 3' UTR of the luciferase gene. We then transfected wild-type NIH/3T3 cells with let-7 miRNA mimics combined with wild-type linc1281 or m^6^A mutant reporters and measured the resultant luciferase activities. As expected, let-7 miRNAs decreased luciferase activities in the presence of wild-type linc1281 (Figure 4J), while they showed no effect on the m^6^A-deficient mutant reporters (Supplementary Figure S7A-B). Expectedly, let-7 miRNAs showed a significant effect on the negative control mutant reporters (Supplementary Figure S7C and D). Then, we performed luciferase reporter assays in shControl and shMettl3 cells after transfection with miRNA mimics combined with the NC or A-G mutant reporters and observed that A-G mutation abolished the effect of miRNAs on NC reporter in the presence of Mettl3 (Supplementary Figure S7E), while the luciferase activities showed no difference between NC and A-G mutant reporters in shMettl3 cells (Supplementary Figure S7F). Collectively, these results provided strong evidence that m^6^A enrichment of linc1281 is required for the linc1281-mediated ceRNA model.

The let7/Lin28 pathway fully restores the differentiation capacity in m^6^A-linc1281-deficient mESCs

Next, we asked whether the ceRNA model is responsible for the linc1281-mediated regulation of mESC differentiation. We constructed let-7 miRNA sponges, which served as decoy targets for miRNAs, as described in a previous study (51), and then established let-7 family sponge-expressing mESCs, in which linc1281 was also knocked down. Examination of miRNA levels via qRT-PCR showed that the sponges effectively inhibited let-7 family expression, which was increased by linc1281 deficiency (Supplementary Figure S8A). Through phenotypic analysis during mESC differentiation, we found that concomitant expression of the let-7 sponge was sufficient to rescue the impaired mESC differentiation capacity after linc1281 knockdown, as shown by upregulation of cardiomyocyte markers (Figure 5A), an increased percentage of beating EBs (Figure 5B) and enhanced cTnT-positive cell numbers (Figure 5C) in EB assays, together with increases in Sox1^+^ cells (Figure 5D), levels of neural genes (Figure 5E) and Sox1-GFP/N-cad-positive cells (Figure 5F) in neural differentiation. These results confirmed that let-7 miRNAs were indeed functional targets of linc1281. Moreover, since Lin28 proteins, which are the direct targets of the let-7 family and critical pluripotency factors in mESCs, showed decreased levels upon linc1281 knockdown (Figure 3H), we also tested whether Lin28 contributed to the underlying mechanism of the linc1281-let-7 model. For this purpose, we overexpressed Lin28a or Lin28b in linc1281 knockdown mESCs (Supplementary Figure S8B) and examined the differentiation of these cell lines, revealing that overexpression of Lin28 re-
Figure 4. The m^6^A mark is critical for the functional role of linc1281 as a natural decoy. (A) Expression levels of mature let-7 miRNAs in shControl and shlinc1281 mESCs in the presence of the indicated overexpression constructs. SnoRNA U6 was used as an internal control for normalization. Error bars show ± SEM (n = 3). The asterisk (*) denotes a significant difference from ‘shCtrl+FlucC’, and the hash mark (#) denotes a significant difference from ‘sh1+FlucC’ or ‘sh2+FlucC’. **P < 0.01, *P < 0.05. (B) Western blot analysis of Mettl3 knockdown efficiency in shMettl3 NIH/3T3 cells. GAPDH was used as the loading control. (C and D) The relative luciferase activities of reporters containing linc1281 in shControl (C) and shMettl3 (D) NIH/3T3 cells 48 hr after cotransfection with the indicated miRNAs or Ctrl mimics. Firefly luciferase activity was normalized to control Renilla luciferase activity. Error bars show ± SEM (n = 4). ***P < 0.001, **P < 0.01, *P < 0.05 versus Ctrl mimics. (E) Western blot for Mettl3 in shControl and shMettl3 mESCs in the presence of the indicated overexpression constructs. WT, wild-type Mettl3; Mut, catalytically inactive mutant of Mettl3. GAPDH was used as the loading control. (F) Wild-type Mettl3, but not the mutant, restored the m^6^A modification of linc1281 in Mettl3 knockdown mESCs in MeRIP assays. The percentage of the input is shown. Error bars show ± SEM (n = 4). *P < 0.05 versus ‘shCtrl+FlucC’; **P < 0.01 versus ‘shMettl3+FlucC’; ns, non-significant against ‘shMettl3+FlucC’. (G) Western blotting of Mettl3 in the indicated NIH/3T3 cells. GAPDH was used as the loading control. (H–I) pGL3-linc1281 reporter assays in ‘shMettl3+WT’ (H) and ‘shMettl3+Mut’ (I) NIH/3T3 cells 48 h after cotransfection with the indicated miRNAs or Ctrl mimics. Firefly luciferase activity was normalized to control Renilla luciferase activity. Error bars show ± SEM (n = 3). **P < 0.01, *P < 0.05 versus Ctrl mimics. (J–K) Relative luciferase levels of pGL3-linc1281 and A-G mutant reporters 48 hr after cotransfection with the indicated miRNAs or Ctrl mimics. Firefly luciferase activity was normalized to control Renilla luciferase activity. Error bars show ± SEM (n = 3). **P < 0.01, *P < 0.05 versus Ctrl mimics.
Figure 5. The let-7/Lin28 pathway restores the impaired capacity of mESCs in the absence of m^6^-linc1281. (A) qRT-PCR analysis of cardiomyocyte markers on day 10 of EB differentiation in shControl and shlinc1281 mESCs in the presence of FlucC or the let-7 miRNA sponge. Sponge, the construct expresses a sponge RNA that contains three tandemly repeated complementary binding sites to each of the 5 miRNAs. (B) Percentage of beating EBs. (C) Representative images of cTnT and Ho.33342 stained on day 12 of EB formation. (D–F) FACS isolation of Sox1^+ cells (D), qRT-PCR analysis of neural markers (E) and representative images of cells stained for Sox1-GFP, N-cad and Ho.33342 on day 5 of neural differentiation (F). (G–I) The spontaneous differentiation capacity of shControl and shlinc1281 mESCs overexpressing FlucC, Lin28a or Lin28b, based on EB formation. (J–L) Examination of the neural lineage differentiation capacity of the indicated mESC lines. Scale bar represents 100 μm. Error bars show ± SEM (n ≥ 3). The asterisk (*) denotes a significant difference from ‘shCtrl+FlucC’, and the hash mark (#) denotes a significant difference from ‘sh1+FlucC’ or ‘sh2+FlucC’. *** p < 0.001, ** p < 0.01, * p < 0.05.
stored the phenotype caused by linc1281 deficiency (Figure 5G-L).

Collectively, our results showed that linc1281 is required for proper differentiation of mESCs and that this critical function relies upon sufficient enrichment of the m6A modification on linc1281 via an m6A-A-dependent model in which m6A-modified linc1281 attenuates let-7 miRNAs and then regulates the let-7/Lin28 pathway (Supplementary Figure S8C).

DISCUSSION

The requirement of lincRNAs for the maintenance of mESC pluripotency has been demonstrated through systematic loss-of-function experiments (6), but relatively little is known about the functional role of individual lincRNAs in mESCs, and it is unclear how lincRNAs epigenetically modulate mESC self-renewal and cell-fate decisions. The identification of specific lincRNAs has shown the importance of lincRNAs in developmental biology and promotes our understanding of the posttranscriptional regulatory network maintaining mESC identities. In this study, we characterized the role of linc1281 in controlling mESC pluripotency. We found that linc1281 serves as a mESC-specific RNA and is required for proper mESC differentiation.

The let-7/Lin28 pathway has been identified as an essential regulator of early embryonic development and mESC pluripotency (46,52). Let-7 miRNAs repress Lin28 mRNA translation, which in turn inhibits miRNA biogenesis (53,54). Transcription factors and miRNAs regulate the expression level and function of let-7 by directly targeting Lin28 (46,52). While most studies have focused on the regulation of miRNA transcription and processing, here, we showed that mature let-7 levels were modulated by linc1281, which contributes to the complexity and stability of the regulatory network involved in the let-7/Lin28 pathway. In addition, precise control of mature let-7 miRNAs enables a rapid response to differentiation cues compared with primary regulation at the level of the transcription and processing of pri-miRNAs or pre-miRNAs. Thus, we propose that the lincRNA-mediated ceRNA model is a flexible and effective modulator in mESCs. Coincidently, linc1281 was very recently identified as a regulator of naïve ESC transition by indirectly targeting Lin28a, although the underlying mechanism remains unclear (55). In our model, linc1281 functions as a posttranscriptional modulator by directly interacting with let-7 miRNAs and subsequently regulates the miRNA target Lin28. We hypothesize that linc1281-miRNA interaction might also contribute to naïve ESC transition and that linc1281 regulates the pluripotency and differentiation potential of ESCs by acting as a multi-layered control machinery.

The m6A modification is widespread throughout the transcriptome, accounting for 0.1–0.4% of total adenosine residues in native cellular RNAs. As one of the most common RNA modifications, m6A is found on almost all types of RNAs and has been implicated in a variety of cellular processes, including miRNA metabolism (9–13,15,16), miRNA biogenesis (14) and m6A-derived circular RNA translation (56). However, the functions of m6A in lincRNAs remain undefined. Here, we first proposed an m6A-A-dependent model of lincRNA/miRNA interaction in mESCs, in which m6A modification of linc1281 is required for the direct binding of let-7 to linc1281. The lincRNA-mediated ceRNA pathway plays a key role in regulating gene activities and has been documented in several studies. Since the crosstalk between lincRNAs and miRNAs is always mediated via a simple sequence pairing mechanism, whether modulators regulate this interaction has not yet been explored. Our study identified m6A as a positive mediator in the ceRNA model, which is supported by the observation that the m6A-deficient A-G mutant abolished the interaction between linc1281 and let-7 miRNAs. Furthermore, in the absence of Mettl3, linc1281 exhibited decreased m6A levels and lacked the ability to bind let-7 miRNAs, suggesting that Mettl3 is predominantly responsible for m6A lincRNA methylation and the m6A-mediated ceRNA model. These observations were supported by the fact that wild-type Mettl3, but not an enzymatically inactive mutant, restored the m6A modification of linc1281 and the RNA-RNA interaction. The identification of individual m6A-marked lincRNAs has additional value in clarifying the functions of Mettl3-dependent RNA modification in the field of RNA epigenetics. Intriguingly, previous research revealed that 67% of mRNA 3’UTRs that contain m6A peaks also contain miRNA binding sites, and the overall distribution of these two groups shows an anti-correlated relationship (57,58), suggesting a possible mechanism by which m6A might cooperate or compete with miRNAs to regulate target miRNAs. Here, we propose a model in which lincRNAs contain both m6A peaks and miRNA binding sites, where m6A cooperates with the miRNA pathway to operate the lincRNA-mediated regulation of pluripotency targets and control the differentiation of mESCs, adding a layer of complexity to our knowledge of the molecular events modulating RNA-miRNA interactions and providing insights into the lincRNA modification-mediated regulatory network in mESCs. Since m6A could alter the local structure of RNA (59) and thus, enhance the RNA bindings of hnRNPs, we identified linc1281-interacting proteins by performing biotinylated linc1281 RNA pull down and LC/MS experiments (Supplementary Table S1) and further explored whether linc1281 binding hnRNPs affect the m6A-dependent RNA-miRNA interactions. However, let-7 mirRNAs still significantly decreased the luciferase activities of linc1281 reporters in the absence of defined hnRNPs (Supplementary Figure S9). To detect the possibly undiscovered RBPs in the large number of proteins bound to linc1281 in the future is useful for the elucidation of the detailed mechanisms of the m6A-mediated linc1281-miRNA interaction in mESC behaviors.

m6A has emerged as a critical fate determinant in mESCs, and genetic inactivation or depletion of the m6A methylase Mettl3, and hence m6A, disrupts normal lineage priming and differentiation (10,26). While all studies performed to date suggest that the m6A-decorated mRNAs of mESC pluripotency factors, such as Sox2 and Nanog, play an essential role in the m6A-mediated control of mESC features, the function of m6A in mESC-specific lincRNAs, which are also integral components of the mESC regulatory network, is unclear. In the present work, we showed that m6A
is enriched on linc1281, and this modification is responsible for the linc1281-mediated control of mESC identities, as impairment of the mESC differentiation capacity upon linc1281 loss can be rescued by wild-type linc1281, but not by the m6A modification-deficient A-G mutant. The necessity of m6A for the linc1281-mediated maintenance of mESC differentiation further stresses the requirement of the m6A modification for mESC pluripotency. Several reports have proposed that the decreased stability of methylated core mESC transcripts contributes to Mettl3-deficient mESC phenotypes (10,26,60). Therefore, in undifferentiated mESCs, pluripotency-related transcripts tend to exhibit relatively low m6A levels. This hypothesis was supported by the identification of ZFP217, which functions to sequester Mettl3 and prevents the methylation of core mESC transcripts (25). Nevertheless, it remains unclear whether m6A plays a positive role in modulating the pluripotent regulatory circuitry, as the methyltransferase Mettl3 shows high levels and activity in undifferentiated mESCs (25,26,61). Conceptually, we propose that m6A in linc1281 is required for the role of linc1281 in mESC pluripotency regulatory networks and is positively involved in the linc1281-mediated maintenance of the mESC differentiation capacity. This model suggests that the m6A modification of lincRNA is an important component of the complex regulatory network driven by RNA methylation and reveals how m6A regulates the physiological functions of lincRNAs. Since the study of the biological role of RNA methylation is still in its infancy, additional discoveries of regulatory patterns mediated by m6A will help elucidate the overall function of RNA methylation.

In summary, we have demonstrated that m6A-linc1281 is critical for proper differentiation of mESCs and identified a novel role of m6A as a necessary flexibility factor in maintaining mESC identities.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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