ALKBH5-mediated m⁶A mRNA methylation governs human embryonic stem cell cardiac commitment

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INTRODUCTION

Chemical modifications have a well-defined role in regulating the activity of mammalian messenger RNAs (mRNAs). Among over 100 types of chemical modifications, 1 N6-methyladenosine (m⁶A) represents the most abundant form.2-4 m⁶A methylation is added by the m⁶A methyltransferase complex that consists of METTL3, METTL14, and WTPA, the components of H3K4 modifying enzyme complexes, are identified as downstream targets for ALKBH5 in cardiac-committed hESCs. Loss of function of ALKBH5 alters the expression of KDM5B and RBBP5 through impairing stability of their mRNAs, which in turn promotes the transcription of GATA4 by enhancing histone H3 lys4 trimethylation (H3K4me3) at the promoter region of GATA4. Taken together, we reveal a previously unidentified role of m⁶A demethylase ALKBH5 in determining cardiac lineage commitment of hESCs.

Heart failure (HF) continues to have a high mortality rate due to the lack of effective treatments.21 Generation of cardiomyocytes (CMs) from human pluripotent stem cells (hPSCs) has an unmet need for cell replacement therapy.22,23 Transplantation of hPSC-derived cardiomyocytes has been shown to improve cardiac function of infarcted non-human primate heart.24 In addition, hPSC-derived cardiomyocytes have been extensively used in drug cardiotoxicity screening, disease model establishment, and exploration of human heart development.25,26 However, the molecular mechanism underlying cardiac differentiation remains largely unknown. Cardiogenesis of hPSCs is controlled by temporal expression of transcription factors critical for mesoderm cells (MESs) and cardiac lineage specification.27 Recent studies have suggested that histone epigenetic modifications play a crucial role in cardiomyocyte differentiation.28-30 Nevertheless, whether RNA m⁶A epigenetic modification regulates cardiomyocyte differentiation is currently unknown.
In this study, we first explored the role of m6A modification in cardiac differentiation and found that m6A methylation was significantly up-regulated upon the differentiation of MESs into CMs, which is accompanied by the decrease of m6A demethylase ALKBH5. Moreover, overexpression of ALKBH5 dramatically inhibited the differentiation of MESs into CMs, indicating that m6A methylation caused by the downregulation of ALKBH5 plays a critical role in cardiac differentiation. Mechanistically, m6A methylation targeted mRNAs of lysine demethylase 5B (KDM5B) and retinoblastoma-binding protein 5 (RBBP5), two known H3K4me3-modifying enzymes, and regulated their activity, which in turn promoted the expression of GATA4 by enhancing histone H3 Lys4 trimethylation (H3K4me3) at its promoter region, thereby driving the cardiac differentiation of MESs. Together, our study defined the critical role of ALKBH5-mediated m6A RNA modification in cardiogenesis and revealed the molecular mechanism underlying cardiac lineage commitment of human ESCs (hESCs).

RESULTS

ALKBH5-mediated m6A demethylation was reduced during cardiac differentiation of mesodermal cells

To define the role of m6A methylation in cardiomyocyte commitment of hESCs, we induced the differentiation of hESCs into CMs in vitro according to a previous report (Figure S1A). Beating CMs appeared from day 7 after induction, which are highly expressed with cardiac-specific markers such as cardiac troponin T (cTnT) and α-actinin (Figures S1C and S1D). According to gene-expression patterns, the differentiation of hESCs into CMs was divided into four stages: hESCs (day 0), MESs (day 2), CPCs (cardiac progenitor cells, day 5), and CMs (day 8; Figure S1B). We performed m6A dot blot analysis of mRNAs from all stages of differentiation and found that m6A methylation was not affected from hESC stage to MES stage, whereas it significantly increased during the differentiation of MESs into CMs (Figure 1A). This finding was also supported by m6A-ELISA assay (Figure 1B). Since the cellular level of m6A methylation is determined by the activities of m6A methyltransferases and demethylases, we next investigated the expression of these enzymes. We found that the expression of m6A demethylase ALKBH5 was significantly decreased during the differentiation of MESs into CMs, while m6A methyltransferase METTL3, METTL14, WTAP, and demethylase FTO did not alter in this stage (Figures 1C and 1D). Together, these data raise the possibility that ALKBH5-mediated m6A modification may play a regulatory role in the differentiation of MESs into CMs.

Overexpression of ALKBH5 inhibited cardiac differentiation from MESs

We next examined the effect of ALKBH5-mediated m6A demethylation on the lineage commitment of CMs by overexpressing this enzyme in MESs. As shown in Figures 2A–2D, transfection of ALKBH5-expressing plasmid at day 2 of differentiation dramatically elevated the RNA and protein levels of ALKBH5, which significantly decreased the global m6A levels in differentiating hESCs.
Moreover, the exogenous expression of ALKBH5 significantly reduced the percentage of cTnT+ and α-actinin+ cells and spontaneously contracting colonies derived from hESCs (Figures 2E and 2F). Consistent with this finding, cells expressing exogenous ALKBH5 produced much fewer cardiac genes and ion channel genes on indicated days of differentiation (Figures 2G and 2H). Together, these data suggest that ALKBH5-mediated m6A demethylation inhibits cardiac differentiation of MESs.

Loss of ALKB5 promoted cardiac differentiation of MESs
To further verify the role of ALKB5 in cardiac differentiation, we inhibited its expression in MESs by using small interfering RNA (siRNA)-mediated knockdown. As shown in Figures 3A and 3B, transfections of two siRNAs targeting different regions of ALKB5 significantly reduced both mRNA and protein levels of ALKB5 in cells. ALKB5 knockdown strongly elevated the level of global m6A methylation in MESs (Figures 3C and 3D) and upregulated the expression of cardiac genes and ion channel genes in differentiated cells (Figures 3F and 3H). Moreover, MESs with less ALKB5 had a higher propensity to generate cTnT+ and α-actinin+ cells and spontaneously contracting CMs (Figures 3E and 3G). Together, these data indicate that the blockage of ALKB5-mediated m6A demethylation promotes the differentiation of MESs into CMs.

KDM5B and RBBP5 as downstream targets of ALKB5 that regulate the cardiac differentiation of MESs
To understand how ALKB5-mediated m6A demethylation regulates the cardiac commitment of MESs, we next performed m6A transcriptomic microarray analysis to detect m6A methylation level on which genes have changed in ALKB5-OE differentiating hESCs compared to control. The gene ontology (GO) analysis revealed that targets of m6A methylation were enriched in calcium signaling pathway and transcription coexpression pathway (Figure 4A). Given the critical role of transcription factors such as NKX2-5, MESP1, and GATA4 in cardiac lineage specification,26 hereby we focused on those targets that regulate transcription coexpression activity. Among them, we confirmed that the mRNA level of histone demethylase KDM5B was significantly elevated upon overexpression of ALKB5 (Figure 4B). We next determined whether other components of histone methylation complexes were also altered by ALKB5-mediated m6A demethylation and found that the mRNA level of RBBP5 but not MLL1, ASH2L, and WDR5 was dramatically downregulated upon ALKB5 overexpression and upregulated upon ALKB5 knockdown in contrast to that of H3K9me3, H3K27me3, or H3K36me3 (Figures 5C and 5D). Immunostaining analysis also confirmed that H3K4me3 level was decreased by overexpression of ALKB5 (Figure S2A). Together, these data suggest that ALKB5-mediated m6A demethylation reduced H3K4me3 methylation through altering expression of KDM5B and RBBP5.

The inhibitory role of ALKB5 in cardiac differentiation was rescued by RBBP5 overexpression or KDM5B specific inhibitor AS8351
To confirm that RBBP5 and KDM5B are two key downstream effectors of m6A in cardiac differentiation, we first knocked down RBBP5 by using siRNAs at day 2 of differentiation and found that it significantly decreased the methylation level of H3K4me3 (Figures 6A and 6B) and reduced the percentage of beating colonies and cTnT+ and α-actinin+ cells at day 10 of cardiac differentiation (Figures 6C and 6D).

On the other hand, overexpression of RBBP5 was able to promote the generation of cTnT+, α-actinin+, and spontaneously beating CMs,
Figure 3. Lack of ALKBH5 promotes cardiac differentiation from MESs

(A) Western blot assay of ALKBH5 on day 3 in control and ALKBH5 knockdown cells. β-actin was used as a loading control. (B) qRT-PCR analysis of ALKBH5 in control and ALKBH5 KD cells at day 3 and day 5 of CM differentiation. ***p < 0.005 (mean ± SEM; n = 4). (C) m6A-ELISA assay of control and ALKBH5 knockdown cells at day 3 of CM differentiation.

(legend continued on next page)
even with ALKBH5 overexpression (Figures 6E–6H). We then used the specific inhibitor of KDM5B, AS8351, to verify its role in m6A methylation-driven cardiac differentiation and found that AS8351 treatment significantly increased global H3K4me3 methylation in differentiating hESCs (Figure 6I) and recovered the cardiac differentiation propensity of hESCs with low m6A methylation, as well as the expression of cardiac genes in differentiated cells (Figure 6J; Figure S3). These results suggest that ALKBH5-mediated m6A demethylation inhibits cardiac differentiation through blocking RBBP5- and KDM5B-mediated methylation of H3K4me3.

ALKBH5 decreased the H3K4me3 methylation at GATA4 promoter regions

We next addressed how m6A methylation drove H3K4me3 methylation in cardiac differentiation. Cardiomyocyte lineage commitment is mainly modulated by temporal expression of transcription factors.27 We thus checked whether KDM5B and RBBP5 were recruited to the promoter regions of these key factors and thereby regulated their expression by adding or erasing active H3K4me3 marks. We performed chromatin immunoprecipitation (ChIP)-qPCR with specific primers targeting promoter regions of key transcription factors (EOMES (Eomesodermin), MESP1 (mesoderm posterior BHLH transcription factor 1), GATA4, NKX2-5 (NK2 homeobox 5), and ISL-1 (islet-1), and found that overexpression of ALKBH5 caused a noticeable downregulation of H3K4me3 methylation at the promoter region of GATA4 but not that of other factors. (Figure 7A). The co-immunoprecipitation (coIP) assays also revealed that both KDM5B and RBBP5 were associated with the GATA4 in differentiating hESCs (Figures S4A and S4B). As expected, the expression of GATA4 was dramatically decreased in cells overexpressing ALKBH5 (Figure 7B; Figure S4C). In addition, the introduction of GATA4 recovered their propensity of cardiac differentiation (Figures 7C and 7D). Altogether, these results indicate that ALKBH5-mediated...
m6A demethylation impairs the H3K4me3 methylation at GATA4 promoter regions and thereby inhibits its expression.

**DISCUSSION**

Recent studies have revealed that m6A methylation is linked to pluripotency regulation of ESCs/induced PSCs (iPSCs). Batista et al. showed that METTL3 impaired ESC exit from self-renewal toward differentiation through prolonged Nanog expression. Geula and colleagues revealed that METTL3 knockout preimplantation epiblasts and naive ESCs fail to terminate their naive pluripotent state. In addition, dysregulation of m6A modification could impair hematopoietic stem cell differentiation and affect embryonic neural stem cell self-renewal. Moreover, m6A modification was also involved in spermatogonial differentiation and meiosis initiation. However, the role of m6A modification in cardiac differentiation of stem cells remains unknown. Our study indicated that ALKBH5-mediated m6A modification was significantly upregulated during the differentiation of MESSs into CMs, and forced expression of ALKBH5 results in markedly inhibition of cardiac differentiation from MESSs. Furthermore, ALKBH5 loss-of-function enhances the capacity of differentiation. Thus, our data demonstrate for the first time, to the best of our knowledge, that m6A RNA modification plays a key role in human stem cell cardiogenesis.

Accumulating evidence has shown that epigenetic modifications play a critical role in cardiogenesis. However, little is known regarding the contribution of histone-modifying enzymes in cardiac differentiation. Previous studies have shown that KDM5B and RBBP5 were widely associated with stem cell stemness maintenance and differentiation. However, the function of KDM5B or RBBP5 in cardiac lineage commitment was unknown. Our study revealed the integral role of KDM5B and RBBP5 in cardiogenesis. coIP combined with ChIP analysis indicated that during cardiac differentiation, KDM5B and RBBP5 inhibit the transcription of GATA4 by removing active H3K4me3 marks at its promoter regions. And RBBP5 overexpression and KDM5B inhibitor AS8351 treatment ameliorated the decreased property of cardiac differentiation arising from m6A reduction.

Transcription factor GATA4 is a known master regulator of cardiogenesis. Our study identified ALKBH5-mediated m6A modification as a regulator of GATA4 and thus uncovered the key crosstalk between m6A and GATA4. This outside-in regulatory axis of ALKBH5-m6A methylation-H3K4me3-GATA4 provides a novel insight into an epigenetic mechanism that controls the fate of hESC cardiac differentiation.

A recent study reported that m6A methyltransferase METTL14 modulates embryonic neural stem cell self-renewal through histone H3K27 modifications. Interestingly, our study indicated that m6A demethylase ALKBH5 regulates hESC cardiac differentiation via H3K4 modifications. It is well documented that similar to histone modifications, DNA methylation also functions as a major epigenetic factor influencing gene activities. DNA methylation is catalyzed by a family of methyltransferases (Dnmts). Future studies may reveal that m6A regulates DNA methylation levels via altering the expression of Dnmts.

Our findings clarify how ALKBH5-mediated m6A modification regulates the differentiation of MESSs into CMs. However, the functions of m6A in endoderm or ectoderm differentiation still remain undefined. Further study of the roles of m6A modifying enzymes in endoderm or ectoderm differentiation could largely broaden our understanding of the stem cell field. Moreover, it would be interesting to determine whether there exist different mechanisms involved in ALKBH5 induced inhibition of differentiation.

In sum, our findings demonstrate the role of ALKBH5 in hESC cardiac differentiation, highlighting the importance of m6A RNA modification in heart development. These findings provide new insights into the mechanisms by which epigenetic modifications...
Figure 6. The inhibitory role of ALKBH5 on cardiac differentiation was rescued by RBBP5 overexpression or KDM5B-specific inhibitor AS8351
(A) qRT-PCR analysis of RBBP5 in control and RBBP5 KD cells at day 3 of CM differentiation. *p < 0.05, **p < 0.01, and ***p < 0.005 (mean ± SEM; n = 4). (B) Western blot analysis of H3K4me3 and RBBP5 in control and RBBP5 KD cells at day 3 of CM differentiation. β-actin and total H3 were used as loading controls. (C) Immunostaining of H3K4me3 and RBBP5 in control and RBBP5 KD cells at day 3 of CM differentiation.
regulates cardiac lineage commitment, which can be used to develop regenerative medicine solutions for congenital heart disease.

MATERIALS AND METHODS

Cell culture
hESCs were cultured as described in our previous work.\(^4^4\) Cells were maintained in Essential 8 medium (05990; STEMCELL Technologies) on 1:100 basement membrane matrix-coated plates (CA3003007; Cellapy) and passaged using ethylenediaminetetraacetic acid (EDTA; Cellapy) every 3 days. Rock inhibitor Y-27632 (HY-10319, MCE) was added for 24 h after passage.

Cell transfection
We used Lipofectamine RNAiMAX for gene interference, and ViaFect Transfection Reagent was used for plasmid transfection. The protocol of transfecting siRNA with Lipofectamine RNAiMAX Reagent is as follows: mix 6 μL of RNAiMAX in 100 μL Opti and 2 μL of siRNA in 100 μL Opti together were incubated for 5 min at room temperature in a 12-well plate when cells are 60%–80% confluent. All target sequences of siRNAs were listed in Table S4.

ViaFect Transfection Reagent was used to transfect plasmid as follows: DNA was diluted to 2 μg per 200 μL of Opti, and 8 μL of DNA was diluted to 2 μg per 200 μL Opti and was incubated for 5 min at room temperature in a 12-well plate when cells are 60%–80% confluent. All target sequences of siRNAs were listed in Table S4.

Samples were collected after transfection 24 and 48 h to measure results.

Cardiomyocyte differentiation
At day 0 of cardiac differentiation, hESCs were incubated with basal medium comprising RPMI 1640 (Invitrogen) plus B27 supplement.
minus insulin (A1895601; Thermo Fisher, Waltham, MA, USA). On days 0–1, 6 μM CHIR-99021 (HY-10182; MCE, Monmouth Junction, NJ, USA) was added to the medium. On days 3–5, 2 μM wnt-C59 (HY-15659; MCE) was added to the medium. On day 7 of differentiation, the medium was changed to RPMI 1640 plus B27 supplement (17504044; Thermo Fisher). The medium was changed every 48 h. Beating cardiomyocytes appeared starting on day 7 of differentiation.

RNA extraction and quantitative real-time PCR
RNA extraction and qRT-PCR assay were performed as described in our previous studies.45 Total RNA was isolated using mirNeasy Mini Kit (QIAGEN, 217004) according to the manufacturer’s instructions and quantified by NanoDrop 8000 spectrophotometer (Gene, Shenyang, China). Then using FastStart Universal SYBR Green Master (RoX) and 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was normalized to those of housekeeping genes in each reaction. Poly(A)+ mRNA were isolated by using PolyATtract mRNA Isolation Systems (Promega) in accordance with the instructions. All primer sequences are listed in Table S1.

DNA stability assay
Cells were incubated with actinomycin D (Sigma-Aldrich) at 5 μg/mL. After 0 h and 6 h of incubation, total RNA of cells was isolated for qPCR assay.

ChIP assays
ChIP assay was performed using the ChIP kit (49-2024; Invitrogen, Carlsbad, CA, USA). The methods in detail were described in our previous study.44 The ChIP primers were listed in Table S2.

Western blotting
Cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer (P0013; Beyotime). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Life Sciences, assembled in Mexico). Then membranes were washed with PBS-Tween 20 (PBST) and incubated with primary antibodies overnight at 4°C. Then cells were incubated with secondary antibodies for 1 h. Lastly, membranes were exposed to Odyssey (LI-COR Biosciences, Lincoln, NE, USA). All antibodies and their dilutions are listed in Table S3.

Co-IP
Differentiating hESCs were collected and lysed in RIPA buffer. Then 300 μg of protein was incubated with antibodies of interest conjugated with Protein A/G Magnetic Beads (HY-K0202, MCE) overnight at 4°C. The protein-bead complex was washed with IP elusion buffer (0.15 M Glycine, 0.5% Triton X-100, pH 2.5–3.1) 6 times. After discarding the supernatant, loading buffer was added and samples were heated at 100°C for 7 min for western blotting analysis.

m6A-mRNA and lncRNA epitranscriptomic microarray
Total RNAs were extracted from the control group and the ALKBH5 OE group and quantified using the NanoDrop ND-1000. The RNA samples were incubated with anti-m6A antibody. The m6A modified RNAs were eluted from the magnetic beads as the “IP” while unmodified RNAs were recovered from the supernatant as “Sup.” The “IP” and “Sup” RNAs were labeled with Cy5 and Cy3, respectively, as cRNAs using Arraystar Super RNA Labeling Kit. The cRNAs were hybridized to Arraystar Human mRNA & lncRNA Epitranscriptomic Microarray (8x60K, Arraystar). The arrays were scanned by using Agilent Scanner G2505C.

Array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Raw intensities of “IP” and “Sup” were normalized with average of log2-scaled spike-in RNA intensities. After spike-in normalization, the probe signals having present (P) or marginal (M) QC flags in at least 1 out of 2 samples were retained as “All Targets Value” in the Excel sheet for further “m6A methylation level” and “m6A quantity” analyses. “m6A methylation level” was calculated for the percentage of modification based on the IP (Cy5-labeled) and Sup (Cy3-labeled) normalized intensities. “m6A quantity” was calculated for the m6A methylation amount based on the IP (Cy5-labeled) normalized intensities. Differentially m6A-methylated RNAs between two groups were identified by filtering with the fold change and statistical significance (p value) thresholds. Hierarchical clustering was performed to show the distinguishable m6A-methylation pattern among samples.

MeRIP-qPCR
The level of m6A modifications on genes was measured using Magna MeRIP Kit (Millipore, cat. 17-700) according to the manufacturer’s instructions. Cells were harvested and lysed in lysis buffer and centrifuged at 1,500 rpm for 5 min. 5 μg m6A or normal rabbit immunoglobulin G (IgG) antibody were incubated with magnetic beads and rotated for 30 min at room temperature. Beads were washed two times and then mixed with cell lysates after the lysates were thawed and centrifuged at 14,000 rpm for 10 min at 4°C. After the beads were rotated overnight at 4°C, they were washed with high salt buffer to wash off unbound materials, followed by extraction with RIP wash buffer. Each sample was analyzed by qRT-PCR.

m6A dot blot assay
Cellular RNA was isolated using miRNeasy Mini Kit (QIAGEN, 217004) and quantified by NanoDrop 8000 spectrophotometer (Gene, Shenyang, China). The m6A dot blot assay was performed following a previously published protocol.46 Briefly, the RNA samples were loaded to the N+ nylon membranes (RPN303B, BIOSHARP, China) then UV crosslinked using crosslinker (CL-1000, UVP). The membranes were blocked with 5% nonfat dry milk for 2 h at room temperature before they were incubated with anti-m6A antibody (1:1,000 dilution, Synaptic Systems, 202003) overnight at 4°C. Then the membranes were incubated with secondary antibody for 1 h at room temperature before exposure to Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

m6A quantification
The quantification of m6A RNA methylation level in differentiating hESCs was detected using m6A RNA Methylation Quantification
Kit (Abcam, ab185912) as described by the manufacturer. Briefly, total RNA or mRNA was isolated from cells and bound to strip well for 90 min. Each well was washed and capture antibody, detection antibody, and enhancer antibody were added. Then color developing solution was added and absorbance was measured in 450 nm. Finally, to determine the relative m^6^A RNA methylation status, the result calculation for the percentage of m^6^A in several stages RNA was carried out using the following formula:

$$m^6^A\% = \frac{(SampleOD - NCOD)}{(PCOD - NCOD)} \times 100$$

S is the amount of input sample RNA in ng.

P is the amount of input positive control in ng.

**Immunofluorescence assays**

Cells were fixed with 4% paraformaldehyde (PFA) before permeabilization with 0.3% Triton X-100 for 10 min. After they were blocked overnight at 4°C and incubated with secondary antibodies for 1 h at room temperature. Images were captured using a live cell imaging system (Olympus Fluoview 10i). All antibodies and their dilutions are listed in Table S3.

**Statistics**

Error bars represent the standard error of the mean. Data were analyzed using Prism 7 (GraphPad). The significance of the differences was analyzed using one-way ANOVA and presented as *p < 0.05, **p < 0.01, and ***p < 0.005.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.omtn.2021.05.019](https://doi.org/10.1016/j.omtn.2021.05.019).

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**AUTHOR CONTRIBUTIONS**

B.C. and Z.H. designed the project and wrote the manuscript. Z.H., Z.X. and Y.Y. performed collection and/or assembly of data, data analysis, and interpretation. Z.H., Y.Y., Z.B., X.G., D.Y., G.Y., R.G., J.X., L.Z., W.M., X.W., and F.Y., H.L., B.D., Y.L., D.L and C.L. performed collection and/or assembly of data. Z.H., Y.T., S.H., Y.Z. and N.W. reviewed, discussed, and edited the manuscript. Z.P., B.Y., and B.C. gave final approval of manuscript and financial support.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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