Research Article

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**Alkbh5** plays indispensable roles in maintaining self-renewal of hematopoietic stem cells

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Abstract: **Alkbh5** is one of the primary demethylases responsible for reversing N6-methyladenosine (m6A) modifications on mRNAs, and it plays a crucial role in many physiological and pathological processes. Previous studies have shown that **Alkbh5** is required for maintaining the function of leukemia stem cells but is dispensable for normal hematopoiesis. In this study, we found that **Alkbh5** deletion led to a moderate increase in the number of multiple progenitor cell populations while compromising the long-term self-renewal capacity of hematopoietic stem cells (HSCs). Here, we used RNA-seq and m6A-seq strategies to explore the underlying molecular mechanism. At the molecular level, **Alkbh5** may regulate hematopoiesis by reducing m6A modification of Cebpa and maintaining gene expression levels. Overall, our study unveiled an essential role for **Alkbh5** in regulating HSC homeostasis and provides a reference for future research in this area.

Keyword: hematopoietic stem cells, self-renewal, demethylation

1 Introduction

In mammals, hematogenesis occurs throughout the lifetime of the animal, primarily in the bone marrow, which is driven by hematopoietic stem cells (HSCs) [1]. HSCs have extensive abilities to regenerate themselves and precipitate all cell lineages of the blood [2-3]. An essential aspect of HSCs is their capacity to remain quiescent at a steady state, which is vital for maintaining their self-renewal capacity, and their ability to initiate hematopoiesis rapidly when required [4-5]. The complex mechanisms involved in maintaining the balance between quiescence and proliferation under stress are tightly regulated [6-7], but they are not yet fully understood. Therefore, increasing knowledge of HSCs homeostasis is essential for the development of therapeutic applications involving these cells.

N6-methyladenosine (m6A) modifications frequently occur in mammalian messenger RNAs and are critical for various cellular processes, including hematopoiesis and leukemia development [8-10]. These reversible methylation events are catalyzed by the m6A methyltransferase complex (Mettl3-Mettl14-Wtap) [11] and two functionally non-redundant demethylases, α-ketoglutarate-dependent dioxygenase AlkB homolog 5 (**Alkbh5**) [12] and FTO α-ketoglutarate dependent dioxygenase (**Fto**) [13]. **Alkbh5** is a ferrous iron/2-oxoglutarate-dependent dioxygenase that can remove m6A methylation and is involved in mammalian RNA metabolism [12,14]. Previous work showed that **Alkbh5** is required for splicing and stabilizing long 3'-UTR mRNAs of male germ cells, which is correlated with spermatogenesis and male fertility [12,15]. There is also evidence of a role for **Alkbh5** in tumor development; **Alkbh5** regulates the self-renewal and proliferation capacity of tumor stem cells of several cancers, including glioblastoma [16], breast cancer [17], and leukemia [18,19].

Despite numerous pieces of evidence supporting **Alkbh5** in HSCs maintenance, **Alkbh5**-deficient mice showed normal...
hematopoiesis [18,19]. However, in our study, we found that Alkbh5 deficiency considerably impaired normal haematogenesis under steady-state and stress conditions. Loss of Alkbh5 led to deficiencies in sustaining HSCs quiescence, resulting in defective self-renewal capacity. Exploration of possible mechanisms underlying these observations showed that Alkbh5 may directly regulate Cebpa stability by impaired m^6^A demethylation in normal hematopoiesis. Our study reveals the indispensable role of Alkbh5 in regulating HSCs homeostasis and provides a reference for future clinical applications.

2 Materials and methods

2.1 Mice

Alkbh5^flfl^/Mx1-Cre mice were generated by crossing Alkbh5^flfl^ mice with Mx1-Cre mice and verified by genotyping tail DNA. Mice aged 4–8 weeks were used for experiments. In vivo ablation of Alkbh5 was accomplished via intraperitoneal injection of poly(I:C) (GE Healthcare, USA) at dosages of 25 μg and 10 μg/g of body weight for partial and complete ablation, respectively.

2.2 Flow cytometry

Single-cell suspensions from the bone marrow, thymus, and spleen were subjected to erythrocyte lysis using red blood lysis buffer (Solarbio, China) and stained with various fluorochrome- or biotin-conjugated antibodies (Table S1) before being analyzed on FACS Canto II flow cytometer (BD Biosciences, USA). For cell-cycle analysis, bone marrow cells were stained with HSCs markers and treated with the Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer. Quantitative analysis was performed using FlowJo software (V10, USA).

2.3 Cell isolation

Bone marrow cells were sorted by FACS AriaII flow cytometer (BD Biosciences) into distinct cell populations based on specific marker expression. The following markers were employed: Lineage negative (Lin^−^; Gr-1^−^TER119^−^B220^−^CD19^−^TgM^−^IL-7R^−^CD3^−^); hematopoietic progenitor cell (HPC) (Lin^−^Sca-1^+t^c-Kit^+) murine primitive hematopoietic stem and progenitor cells (LSK) (Lin^−^Sca-1^−^c-Kit^+^); multipotent progenitor (MPP) (Lin^−^Sca-1^−^c-Kit^+^Flk2^−^CD34^+^); HSC (Lin^−^Sca-1^−^c-Kit^+^CD48^−^CD150^+^); short-term HSC (ST-HSC) (Lin^−^Sca-1^−^c-Kit^+^Flk2^−^CD34^+^); common myeloid progenitor (CMP) (Lin^−^Sca-1^−^c-Kit^+^CD16/32^−^CD34^+^); granulocyte-monocyte progenitor (GMP) (Lin^−^Sca-1^−^c-Kit^+^CD16/32^−^CD34^+^); megakaryocyte-erythroid progenitor (MEP) (Lin^−^Sca-1^−^c-Kit^+^CD16/32^−^CD34^+^); and lymphoid-primed multipotential progenitor (LMPP) (Lin^−^Sca-1^−^c-Kit^+^Flk2^−^). Myeloid cells (MAC^−^), B cells (B220^+^), and T cells (CD3^+^) were sorted from the peripheral blood (PB).

2.4 Competitive repopulation assay

Recipient mice (CD45.1^+) were irradiated and subsequently transplanted with bone marrow cells from donor mice (CD45.2^+^, donor types carried either Alkbh5^flfl^ or Alkbh5^+/^Mx1-Cre) and competitor mice (CD45.1^+^CD45.2^+^) at a 1:1 ratio. Six weeks post-transplantation, Alkbh5 in the recipients was knocked out by injecting poly(I:C). Six months after poly(I:C) injection, an equivalent number of bone marrow cells from the first cohort of transplanted mice were transferred into a new cohort of irradiated mice. Donor- and competitor-derived PB cells were assessed on a monthly basis after transplantation using flow cytometry.

2.5 5-Fluorouracil (5-FU) treatment

5-FU (Sigma-Aldrich, USA) was injected intraperitoneally at a dose of 150 mg/kg body weight once a week for 3 weeks, with daily monitoring of survival. The data were analyzed with the GraphPad Prism 6.0 software (GraphPad Software, USA).

2.6 Homing assay

Recipient mice underwent whole-body irradiation 24 h prior to receiving bone marrow cells (2 × 10^6^) from Alkbh5^flfl^ or Alkbh5^−/^mice that had been stained with carboxyfluorescin diacetate succinimidyl ester (CFSE) (5 μmol/L). Six hours after cell injection, bone marrow cells were extracted from recipient mice and stained with antibodies against lineage markers and Sca-1. The percentage of CFSE^+^ cells within the Lin^−^Sca-1^−^ population was evaluated by flow cytometry.

2.7 RNA extraction and quantitative real-time PCR (qRT-PCR)

Bone marrow cells were sorted into different populations for total RNA extraction with RNAiso Plus (TaKaRa, China).
The PrimeScript RT Reagent Kit (TaKaRa) was used for reverse transcription followed by qRT-PCR reactions with SYBR Green Master Mix (Qiagen, Germany) on a BioRad thermocycler using specific primers for Alkbh5 and Actin gene: Alkbh5 forward: 5′-CGCGGTCAACGACTACC-3′; Alkbh5 reverse: 5′-ATGGGCTGAAGCTGAATTG-3′; Actin forward: 5′-ACCTCTACAATGAGCTGCG-3′; Actin reverse: 5′-CTGGATGCTACGTACATGG-3′.

### 2.8 Western blot (WB)

WB analysis was performed using the standard protocols. Mouse antibody against Alkbh5 (Sigma, USA) was used at a 1:1,000 dilution, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Proteintech, USA) was used at a 1:1,000 dilution. All experiments were performed three times with GAPDH as the internal control.

### 2.9 RNA sequencing

mRNA libraries were constructed from approximately 8,000 HSCs using Smart-Seq V4 Ultra Low Input RNA Kit (TaKaRa). Fragmented DNA was barcoded with dual indexes and PCR amplified. Purification and selection of DNA fragment sizes were performed using AMPure beads. Sequencing was performed on the Illumina HiSeq 2000.

### 2.10 m6A sequencing

Lin− cells from Alkbh5fl/fl and Alkbh5fl/flMx1-Cre mice were sorted using FACS AriaII flow cytometer and resuspended in Trizol (RNAiso Reagent; TaKaRa). All samples were processed and sequenced at Lianchuan Biotechnology Corporation, Hangzhou, China.

### 2.11 Statistical analysis

Data processing and statistical analyses with unpaired Student’s t-tests were performed using GraphPad Prism v.7. Kaplan–Meier plots were analyzed with the log-rank test. P-values below 0.05 are considered statistically significant, and the levels of significance indicated as *P < 0.05, **P < 0.01, and ***P < 0.001 are compared with the control.

**Ethics statement:** All animal experiments were carried out in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. Animal research facilities were provided by the Third Military Medical University, China, and all animal experiments were approved by this institution (Permit Number SYXK-2017-0011).

### 3 Results

#### 3.1 Generation of Alkbh5 conditional knockout mice

To investigate the role of Alkbh5 in hematopoiesis, we employed qRT-PCR to characterize Alkbh5 expression in subgroups of primitive and mature cells within the total bone marrow. We found that Alkbh5 was highly expressed in hematopoietic progenitor cells, including HSCs, compared to mature cells, indicating a potential role for Alkbh5 in hematopoietic development (Figure 1a). Germline knockout of Alkbh5 did not alter the number of hematopoietic stem and progenitor cells in 6- to 10-week-old mice [18]. Therefore, we generated a conditional knockout model (Alkbh5fl/fl) in which the 5′ UTR and exon 1 of Alkbh5 are flanked with loxP sites. The Alkbh5fl/fl mice were crossed with Mx1-Cre mice to generate control Alkbh5fl/fl mice and Alkbh5fl/flMx1-Cre mice (Figure 1b). Cre recombinase controlled by the Mx1 promoter can be activated by synthetic double-stranded RNA (poly(I:C)) to trigger conditional deletion of the Alkbh5 gene in Alkbh5fl/flMx1-Cre mice (Alkbh5CKO). Accordingly, Alkbh5 deficiency in bone marrow cells was confirmed through analysis of transcript and protein levels via WB and qRT-PCR, respectively (Figure 1c and d).

#### 3.2 Alkbh5 deletion considerably accelerates hematopoietic stem and progenitor cell (HPSC) expansion in the steady state

To investigate the role of Alkbh5 in multilineage hematopoietic development, we conducted a comprehensive analysis of the PB from Alkbh5fl/fl control and Alkbh5-CKO mice 2 months after Alkbh5 ablation. We observed no significant differences in differentiated cell lineages between the two groups (Figure 2a and b), nor in the body weight of the mice (Figure 2c). Furthermore, we observed no significant changes in total bone marrow cellularity or certain subpopulations (such as LMPPs and HPCs, which contain CMPs, GMPs, and MEPs) due to Alkbh5 deletion (Figure 2d–f and i–k). Interestingly, we found that Alkbh5 deficiency led to a considerable increase in the total cell number of LT-HSCs,
ST-HSCs, and MPPs. In addition, two separate phenotypic characterizations demonstrated that the number of CD34−LSK cells and signaling lymphocyte activation molecule-hematopoietic stem cells were significantly increased in Alkbh5-CKO mice (Figure 2d, g and h). However, the frequency of red blood cells, B cells, T cells, and myeloid cells remained unchanged for Alkbh5 ablation (Figure S1).

Collectively, these data indicate that Alkbh5 may play an active role in inhibiting the expansion of multiple hematopoietic stem and progenitor cell populations.

3.3 Loss of Alkbh5 decreases HSCs quiescence

To determine whether Alkbh5 controls HSCs proliferation, the cell cycle status of HSPCs from Alkbh5+/− mice, including bone marrow, Lin−, HSCs, LSK cells, MPPs, HPCs, CMPs, GMPS, MEPs, myeloid cells, B cells, Red cells, CD4+ T cells, and CD8+ T cells.

(b) Scheme for generating Alkbh5 conditional knockout mouse. (c) Immunoblotting for Alkbh5 and GAPDH in Alkbh5+/− and Alkbh5+/−Mx1-Cre mice expressing indicated constructs. (d) qRT-PCR analysis showing the efficiency of Alkbh5 deletion. *P < 0.05; **P < 0.01; ***P < 0.001.

Collectively, these findings suggest that Alkbh5 is necessary for maintaining HSCs in a quiescence state.

3.4 Alkbh5 deficiency compromises the self-renewal capacity of HSCs

To investigate the effect of Alkbh5 ablation on the repopulation capacity of HSCs, we performed serial competitive transplantation assays and monitored engraftment in PB over time (Figure 4a). Our results showed that the percentage of chimeric bone marrow cells from Alkbh5-CKO mice...
Figure 2: Alkbh5 deletion considerably accelerates HSPC expansion in the steady state. (a) Experimental schematic for induction assay. (b) PB complete blood cell counts of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice at 2 months after poly(I:C) injection. WBC, white blood cells; LY, lymphocytes; NE, neutrophils; EO, eosinophils; BA, basophils; MO, monocytes; PLT, platelets; RBC, red blood cells; and Hb, hemoglobin. (c) Weight analysis of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice before and 2 months after poly(I:C) injection. (d) FACS analysis of HPCs, LSK cells, HSCs, MPPs, CD34<sup>−</sup>LSK cells, ST-HSCs, and LT-HSCs in bone marrow cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice at 2 months after poly(I:C) injection. (e) Bone marrow cells count in Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice at 2 months after poly(I:C) injection. (f–h) Count of HPCs, LSK cells, HSCs, MPPs, CD34<sup>−</sup>LSK cells, ST-HSCs, and LT-HSCs in bone marrow cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO at 2 months after poly(I:C) injection. (i) FACS analysis of CMPs, GMPs, HSCs, MEPs, and LMPPs in bone marrow cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice at 2 months after poly(I:C) injection. (j and k) Counts of CMPs, GMPs, HSCs, MEPs, and LMPPs in bone marrow cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice at 2 months after poly(I:C) injection.
Figure 3: Loss of Alkbh5 decreases HSCs quiescence. (a) FACS analysis of BrdU incorporation in LSK cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (b) Cell-cycle analysis of LSK cells in Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (c) FACS analysis of BrdU incorporation in HSCs of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (d) Cell-cycle analysis of HSCs in Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (e) FACS analysis of Ki-67 staining in LSK cells of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (f) Cell-cycle analysis of LSK cells in Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (g) FACS analysis of Ki-67 staining in HSCs of Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice. (h) Cell-cycle analysis of LSK cells in Alkbh5<sup>fl/fl</sup> and Alkbh5-CKO mice.
Figure 4: Alkbh5 deficiency compromises the self-renewal capacity of HSCs. (a) Experimental schematic for the competitive transplantation assay.
(b) FACS analysis of PB cells from recipient mice in a competitive transplantation assay. (c–f) The ratio of donor-derived CD45.2+ PB cells to competitor cell–derived CD45.1+CD45.2+ PB cells (donor/competitor) in the recipient mice. (g) The ratio of CD45.2+/CD45.1+CD45.2+ in the Lin−, HPCs, LSK cells, and CD34 LSK cells. (h) Experimental schematic for the homing assay. (i) FACS analysis of CFSE+ cells in bone marrow Lin−Sca-1+ cells of recipient mice. (j) The histogram indicates in vivo homing percentage of Alkbh5fl/fl and Alkbh5-CKO Lin−Sca-1+ cells in recipient mice at 6 h after transplantation. (k) Survival curve of Alkbh5fl/fl and Alkbh5-CKO mice following sequential 5-FU treatment.
continuously decreased, compared to their wild-type counterparts, with each transplant. This indicated a lower reconstitution capacity of Alkbh5-deficient HSCs (Figure 4b–f). Moreover, the percentage of chimeric myeloid, B, and T cells showed a unanimous decrease, with myeloid differentiation being more severely impaired. In addition, 4 months after the second transplant, Lin− cells, HPCs, LSK cells, and CD34− LSK cells derived from Alkbh5-deficient mice exhibited a striking reduction in their ability to repopulate the bone marrow (Figure 4g).

Despite the decreased reconstitution rate, the homing capacity of Alkbh5-CKO HSPCs was unchanged (Figure 4h–j). Furthermore, administering 5-FU treatment, which removes proliferating and mature cells, stimulated HSCs to replenish the hematopoietic system. Weekly injection of 5-FU into Alkbh5fl/fl control and Alkbh5-CKO mice showed that Alkbh5-deficient mice were unable to survive beyond the second injection, while more than 60% of Alkbh5fl/fl mice remained alive and only succumbed after the third injection (Figure 4k).

In summary, our study provides clear evidence that Alkbh5 is required for the long-term self-renewal capacity of HSCs under stress conditions.

### 3.5 Incomplete depletion of Alkbh5 did not impair hematopoiesis in mice

Alkbh5 has been shown to be necessary for maintaining acute myeloid leukemia stem cell function but is expendable in normal hematogenesis [18,19]. However, our data indicate that complete ablation of Alkbh5 affects hematopoiesis. To reconcile these differences, we attempted to replicate the work of Wong et al., where Alkbh5 was conditionally deleted by injecting three doses of poly(I:C) (25 µg) over 6 days [19] (Figure S2a). Comparing the PB from Alkbh5fl/fl and Alkbh5-CKO mice, we found that the differentiated cell lineages remained unchanged (Figure S2b). Similarly, the total number of cells within the bone marrow was unaltered by analyzing Alkbh5fl/fl and Alkbh5-CKO mice (Figure S2d). The same is true for HSPCs populations including HPCs, LSK cells, CD34− LSK cells, HSCs, LT-HSCs, ST-HSCs, MPPs, CMPs, GMPs, MEPs, and LMPPs (Figure S2c and e–j). Furthermore, the percentage of red blood cells, B cells, T cells, and myeloid cells is unaltered between the two groups of mice (Figure S3). However, WB data indicated an incomplete knockout of Alkbh5 in the Alkbh5-CKO mice (Figure S2k).

Therefore, our data indicate that incomplete deletion of Alkbh5 has no apparent effect on multilineage hematopoiesis.

### 3.6 Alkbh5 deficiency inhibits Cebpa signaling

To uncover the mechanism by which Alkbh5 regulates HSCs homeostasis and function, we performed RNA sequencing (RNA-seq) on freshly sorted HSCs and found that 113 were upregulated and 343 genes were downregulated in Alkbh5-CKO HSCs compared to Alkbh5fl/fl HSCs (fold change >1.5 and P value <0.05; Figure 5a). Gene-set enrichment analysis (GSEA) indicated that the downregulated genes were enriched for acute myeloid leukemia and family acute myelogenenous leukemia genes (Figure 5b). As Alkbh5 is a major m6A demethylase, we hypothesized that Alkbh5 may regulate HSCs homeostasis by demethylating target genes. To identify these targets, we sorted Lin− cells and performed m6A sequencing (m6A-seq) and found that m6A enrichment of total mRNA is significantly increased in Alkbh5-CKO mice (Figure 5c). Furthermore, we observed significant changes in 51 hypo-down genes, 136 hypo-up genes, 13 hyper-down genes, and 42 hyper-up genes in Alkbh5-CKO cells (fold change >1.2 and q value <0.05; Table S2) and identified 10 potential target genes through integrative analysis of RNA-seq and m6A-seq (Figure 5d). Of these, we focused on Cebpa, as its m6A level significantly upregulated, while its RNA expression decreased in Alkbh5-CKO. Previous reports showed that Cebpa maintains the HSCs self-renewal and inhibits progenitor population expansion [20]. We detected a significant decrease in the expression of Cebpa in Alkbh5-CKO HSCs, indicating that Alkbh5 directly regulates Cebpa (Figure 5e), and identified 17 potential m6A modification sites on Cebpa using the sequence-based SRAMP m6A modification site predictor (http://www.cuilab.cn/sramp) (Figure 5f and Figure S4). We also found compared to Alkbh5fl/fl Lin− cells, Cebpa mRNA from Alkbh5-CKO Lin− cells have increased m6A modifications (Figure 5g), and m6A-seq showed that the m6A modification on 5′ UTR of Cebpa. Our findings suggest that Alkbh5 deficiency leads to an increased m6A enrichment and reduced RNA expression of Cebpa, ultimately leading to the activation of the programs promoting HSCs proliferation and impaired self-renewal capacity.

### 4 Discussion

Previous evidence has suggested that Alkbh5 plays a crucial role in leukemogenesis but is dispensable for hematopoiesis [18,19]. In this study, we provided alternative evidence that challenges these assumptions and supports the notion that Alkbh5 sustains HSCs self-renewal and inhibits the expansion of multiple HSPC populations. However, incomplete ablation of Alkbh5 had minimal effect on
Figure 5: Alkbh5 deficiency inhibits Cebpa signaling. (a) Representative scatters of upregulated genes or downregulated genes by 1.5-fold or more in Alkbh5-CKO HSCs compared with Alkbh5fl/fl HSCs. (b) GSEA of the selected gene sets. (c) m6A-seq shows the total m6A modification in Lin− cells of Alkbh5fl/fl and Alkbh5-CKO mice. (d) Integrative analysis of RNA-seq and m6A-seq to identify transcriptome-wide potential targets of Alkbh5 in HSCs. (e) RNA-seq showed the expression level of Cebpa transcript in HSCs of Alkbh5fl/fl and Alkbh5-CKO mice. (f) 17 Potential m6A sites of Cebpa mRNA predicted by SRAMP program. (g) m6A-seq shows changes in Cebpa transcript m6A modification in Lin− cells from Alkbh5fl/fl and Alkbh5-CKO mice.
normal hematopoiesis, suggesting that even a very low level of Alkbh5 expression is sufficient for maintaining HSCs quiescence. Mechanically, Alkbh5, as a significant m^6^A demethylase, may regulate these homeostatic functions in the hematopoietic system by decreasing the m^6^A modification of Cebpa, thereby maintaining the expression of this gene.

Differences in experimental models may account for the discrepancy between our results and previous works [18,19] regarding the role of Alkbh5 in normal hematopoiesis. Shen et al. generated germline knockout of Alkbh5 using CRISPR-Cas9, resulting in a whole-body deletion of 19 amino acids from the protein [18]. In contrast, we specifically knocked out Alkbh5 in the hematopoietic system of adult mice using a Cre-loxP system. Germline deletion of the protein may permit compensatory mechanisms to mask the role of Alkbh5 in hematopoiesis. Wang et al. used a similar conditional knockout system to ours, wherein poly(I:C) was used to stimulate the excision of exon 1 of Alkbh5 [19]. Although they validated the knockout efficiency of Alkbh5 by qRT-PCR, the protein level was not verified [19]. In our model, poly(I:C)-induced exon 1 knockout of Alkbh5 causes a frame-shift mutation that completely deletes Alkbh5 at the protein level. When we used the same poly(I:C) dose reported by Wang et al., the knockdown of Alkbh5 protein was incomplete, and the phenotype of these mice was consistent with the results of Wang et al. These findings suggest that the timing and efficiency of Alkbh5 knockout are important in determining the effect of this gene on hematopoiesis and highlight the need for further research in this area.

In our study, we have identified 17 potential m^6^A modification sites on Cebpa. According to Su et al., an increase in m^6^A modification on Cebpa mRNA can be detected by Ythdf2, which leads to a decrease in the stability and expression level of Cebpa [21]. The m^6^A reader is responsible for the fate of RNA, with Ythdf2 mainly promoting the degradation of m^6^A-tagged mRNAs [22]. Given the presence of m^6^A modification on the 5′ UTR of Cebpa, we hypothesize that increased m^6^A modification at site 5′ UTR of Cebpa mRNA may be recognized by Ythdf2, leading to a reduction in stability and expression of Cebpa. However, further experiments are required to validate this hypothesis.

Wang et al. and Shen et al. have identified two signaling pathways involving Alkbh5/m^6^A/TACC3 and the Kdm4c/Alkbh5 in acute myeloid leukemia development [18,19]. We have shown that Alkbh5 may play an important role in regulating HSCs self-renewal and the maintenance of HSPC populations by acting on Cebpa as a potential target. There are likely to be many other signaling pathways through which Alkbh5 mediates its functions.

m^6^A modification is a dynamic process facilitated by m^6^A-binding or “reader” proteins, such as Ythdf1/2/3 and Igf2bp1/2/3, which can lead to the regulation of mRNA stability [23,24]. Inactivation of Ythdf2 has been shown to increase HSCs expansion [25,26], while a lack of Ythdf2 could reduce the population of quiescent HSCs [27]. Alkbh5 may also regulate normal hematopoiesis through these signaling pathways, although further research is needed to confirm this.

In summary, our finding provides evidence that Alkbh5 plays a crucial role in maintaining the inherent self-renewal capacity of HSCs and prevents the expansion of multiple hematopoietic stem and progenitor cell populations. Alkbh5 may regulate normal hematopoiesis directly by maintaining the expression of Cebpa through the reduction of m^6^A modification.

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Conflict of interest: The authors declare that they have no competing interests.

Data availability statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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