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Original Research Article

ALKBH5 regulates arginase 1 expression in MDSCs and their immunosuppressive activity in tumor-bearing host

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) are closely related to the occurrence and development of many cancers, but the specific mechanism is not fully understood. It has been found that N6-methyladenosine (m^6A) plays a key role in RNA metabolism, but its function in MDSCs has yet to be revealed. In this study, we found that MDSCs in mice with colorectal cancer (CRC) have significantly elevated levels of m^6A, while ALKBH5 expression is decreased. Overexpression of ALKBH5 can reduce the immunosuppressive function of MDSCs in vivo and in vitro, and attenuates the protumorigenic ability of MDSCs. Mechanism study found that the overexpression of ALKBH5 in MDSCs reduced the m^6A modification level of Arg-1 mRNA, and then weakened the stability of Arg-1 mRNA and protein expression. These data suggest that the decreased expression of ALKBH5 in CRC tumor mice may promote the expression of Arg-1, enhance the immunosuppressor function of MDSCs, and promote tumor growth. These findings highlight that ALKBH5 may regulate the function of MDSCs in tumor-bearing mice and may be a new target for immunotherapy. This research provides a new perspective for our understanding of the role of MDSCs in cancer development, and also brings new hope for cancer treatment.

1. Introduction

Tumor growth and metastasis cannot be achieved without the support of tumor microenvironment (TME).Myeloid-derived suppressor cells (MDSCs) play a vital role in this process [1]. Under normal circumstances, immature myeloid cells (IMCs) with a similar phenotype to MDSCs but without immunosuppressive activity are continuously generated in the bone marrow. These IMCs are mainly distributed in the bone marrow, and then differentiate into dendritic cells (DCs), granulocytes, or macrophages and enter the peripheral circulation, a process that remains homeostasis. However, under pathological conditions such as infection, tissue injury and tumor growth, the normal growth and differentiation of bone marrow cells are affected. In these abnormally persistent pathological conditions, such as chronic inflammation, autoimmune diseases, and cancer, the differentiation of IMCs is blocked, resulting in their continued accumulation and lack of lineage differentiation. These activated IMCs further generate MDSCs with inhibitory functions. On the one hand, MDSCs can inhibit T cell activity through the secretion of arginase-1 (Arg-1), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), interleukin-10 (IL-10) and other effector molecules, thus promoting tumor progression [2]. On the other hand, MDSCs can also induce the generation of tumor-associated macrophages (TAMs) and regulatory T cells (Tregs), establish an immunosuppressive microenvironment, and further promote tumor progression [3]. Studies have proven that excessive amounts of circulating MDSCs are closely related to poor prognosis, tumor angiogenesis, and tumor evasion immunity [4]. This reveals a new regulatory mechanism of tumor growth and provides a new research direction for tumor therapy [5].

N6-methyladenosine (m^6A) is a dynamic modification that is co-regulated by methyltransferase, demethylase and m^6A binding protein. These proteins are called "writers," "erasers," and "readers." [6]. A

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large number of studies have shown that m^{6}A regulators are abnormally expressed in various tumor types and can be used as both tumor suppressor and carcinogenic factors [7]. The m^{6}A methylase complex, also known as the "writer" complex, has a core subunit of methyltransferase-like protein 3/14 (METTL3/14) and a WT1-associated protein (WTAP) [8]. In addition, RNA binding protein 15/15 B (RBM15/15 B), zinc-containing finger CCCH type 13 (ZC3H13) and virus-like m^{6}A methyltransferase-associated protein (VIRMA/-KIAA1429) were also involved in the formation of the complex [9–11].

Fat mass and obesity-associated protein (FTO) and α-ketoglutarate-dependent dioxygenase alkB homology 5 (ALKBH5) are selective demethylases. Gene expression and cell fate are regulated by oxidative removal of methylation modifications on m^{6}A substrates [12,13]. In addition, Alkb Homolog 1 (ALKBH1) can remove methylcytidine (mC) modifications on mammalian mRNAs [14], while Alkb Homolog 3 (ALKBH3) catalyzes the removal of 1-meA and 3-meC from endogenous methylated RNAs [15].

M^{6}A "readers" are a class of m^{6}A binding proteins that specifically recognize m^{6}A modified RNA transcripts and mediate their degradation, splicing and translation [16], mainly including YT521-B homologous (YTH) domain family proteins (YTHDC1/2, YTHDF1/2/3) [17–21]. In addition, insulin-like growth factor 2 mRNA binding protein (IGF2BP) family proteins and heterogeneous ribonucleoprotein (HNRNP) family proteins also belong to m^{6}A "readers" [22,23].

Dysregulation of ALKBH5 has been demonstrated in pancreatic cancer [24] and gastric cancer [25], and it can regulate various tumor processes, such as proliferation, migration, invasion and metastasis. Thus, it is particularly necessary to study ALKBH5. In colorectal cancer (CRC), ALKBH5 can significantly inhibit tumorprogression [26]. Another study found that ALKBH5 promoted the progression of colon cancer by reducing the methylation level of the lncRNA NEAT1 [27]. However, the role of ALKBH5 in MDSCs of colon cancer origin has not been investigated. In this study, the biological role of m^{6}A demethylase ALKBH5 in MDSCs was investigated. The data showed that ALKBH5 can affect the immunosuppressive function of MDSCs by modulating the demethylation of Arg-1, thereby delaying the progression of CRC in tumor-bearing mice. These findings provide a new basis for the treatment of MDSCs and broaden relevant research topics.

2. Materials and methods

2.1. Cell lines and cell culture

CT26 is an undifferentiated mouse colon cancer cell line, which has high research value. This cell line, provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). During the experiment, in order to ensure cell growth and reproduction, CT26 cells were cultured in RPMI-1640 medium (Gibco, USA). In addition, 10% fetal bovine serum (Gibco) and 1% penicillin are added to provide nutrients needed for cell growth and prevent bacterial infection.

3. Mice

BALB/c mice are a commonly used experimental animal model. These mice (female, 6–8 weeks old) were purchased from the Laboratory Animal Center of Jiangsu University and raised in specific sterile facilities to ensure the accuracy of experimental results. To ensure animal welfare and ethics, all animal care and handling procedures are approved by the Animal Ethics Committee of Jiangsu University.

3.1. Xenograft model

Mouse colon cancer CT26 cells were suspended with PBS to every 100 μL containing 1×10^6 cells, and 200 μL (2×10^6 cells) was injected subcutaneously into each mouse. When the tumor grew to 1–2 cm^3, MDSCs in spleen were separated for follow-up experiments.

In vivo model construction: BALB/c mice of 6–8 weeks were randomly divided into two groups, three in each group, and the grouping design was shown in the following table. MDSCs$^{si-ALKBH5}$ or MDSCs$^{LV-ALKBH5}$ cells and mouse colon cancer CT26 cells were respectively suspended with PBS to every 100 μL containing 1×10^6 cells, the above two were mixed 1:1, and 200 μL cells mixture was injected subcutaneously into each mouse, the specific setting is as follows.

Group	Cells mixture
Si-NC	MDSCs^{si-NC} (1×10^6/100 μL) + CT26 cells (1×10^6/100 μL)
Si-ALKBH5	MDSCs$^{si-ALKBH5}$ (1×10^6/100 μL) + CT26 cells (1×10^6/100 μL)
LV-NC	MDSCs^{LV-NC}(1×10^6/100 μL) + CT26 cells (1×10^6/100 μL)
LV-ALKBH5	MDSCs$^{LV-ALKBH5}$(1×10^6/100 μL) + CT26 cells (1×10^6/100 μL)

During the experiment, the mice were removed at the injection site for follow-up observation. After tumor nodules appeared, vernier calipers were used to measure them once every two days, respectively denoted as long diameter (a) and short diameter (b), and the volume was calculated according to $V = 1/2 \times a \times b$ [2]. The mice were killed on the 23rd day, and the tumor tissues were separated, weighed and photographed.

Flow cytometry was used to detect Th1 cells and Cytotoxic T (CTL) in mice. The phenotype of Th1 cells in spleen and lymph node cell suspension was CD3e$^+$CD4$^+$IFN-γ$^+$, the phenotype of CTL cells was CD3e$^+$CD8$^+$IFN-γ$^+$, the phenotype of Th1 cells in tumor tissue cell suspension was CD45$^+$CD3e$^+$CD4$^+$IFN-γ$^+$, and the phenotype of CTL cells was CD45$^+$CD3e$^+$CD8$^+$IFN-γ$^+$.

3.2. Isolation of murine MDSCs

MDSCs were isolated from murine spleens when the tumor volume reached 1–2 cm^3. Suspensions of whole splenocytes from mice were obtained and lysed. Then, 10 μL of a biotin anti-mouse/human CD11b antibody was added to the above cells. After incubation on ice for 30 min, the cells were washed, 15 μL of anti-biotin beads was added, and the cells were incubated on ice for 30 min. The final MDSC suspension was obtained by dropwise addition of the suspension to a magnetic separation column. The mouse MDSCs phenotype is CD11b$^+$ Gr-1$^+$. A total of 1×10^6 MDSCs were removed, placed in an Eppendorf (EP) tube, and washed with PBS. Then, 0.5 μL of a PE-labeled anti-mouse Gr-1 antibody and 0.5 μL of a PE/Cy7-labeled anti-mouse CD11b antibody were added to the cells, mixed and incubated in 4 °C dark for 30 min. Finally, we washed the cells with PBS and determined the purity of MDSCs using flow cytometry (FCM).

3.3. Transfection

MDSCs were transfected with small interfering RNAs (siRNAs) with Ribo transfection reagent (Ribo, China) to achieve a knockdown efficiency of 50–70%. Specific operations are as follows: The 10 × CP Buffer was diluted to 1 × CP Buffer with sterile PBS, and 1 × CP Buffer: Reagent Buffer: siRNA = 30 μL: 3 μL: 2 μL. The transfection complex was mixed and reacted at room temperature for 15min, and then added into the cells drop by drop, each well was added with 35 μL, total system was 500 μL, the cells were cultured at 37 °C in 5% CO2, and the medium was changed after 24 h according to cells status, and GM-CSF was supplemented during the period to maintain cell status. We also used lentiviruses ordered from Genechem (Shanghai, China). These viral vectors can overexpress the target gene ALKBH5 in MDSCs. Cells were plated into 24-well plates before transfection. When the cells were adherent and approximately 60% confluent, 20 μL of the lentiviral supernatant was added to the medium. The siRNA sequences used in the experiment are shown in Supplementary Table 1.

3.4. Western blot analysis

In this study, we used RIPA buffer (China) to extract total cellular proteins. (Beyotime, China). For the cells in the cell medium, we first gently sucked the culture supernatant with a pipette gun, gently blew the cells with PBS, collected the supernatant in a 1.5mLEP tube at 500 g and centrifuged for 5 min. After centrifugation, the supernatant was discarded and washed again with PBS and centrifuged, and the total protein was extracted at a ratio of 40 μL RIPA) lysed 2×10^6 MDSCs cells.

The extracted total protein was isolated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, USA). The membrane was sealed with 5% skim milk at room temperature for 2 h to reduce the risk of non-specific binding, and washed the membrane with TBST 3 times to remove excess impurities.

Incubate the primary antibody overnight at 4 °C, then wash with TBST film 3 times.

HRP labeled secondary antibodies (1:10,000 dilution) were incubated for 1 h at room temperature and TBST was used to remove excess secondary antibodies.

Finally, the exposure solution A and B were prepared at 1:1, then dripped onto PVDF membrane, exposed by gel imager, and the results were preserved. The results were compared by grayscale scanning using ImageJ software.

3.5. RNA isolation and RT−qPCR

We used TRIzol (Invitrogen, USA) reagent to extract total RNA from cells, and by measuring the 260 nm UV absorption value, we determined the RNA concentration.

Next, we use a reverse transcription system (Vazyme, China) to use RNA for first strand cDNA synthesis according to the manufacturer's protocol. Using SYBR Green PCR Master Mix (Vazyme, China) according to the manufacturer's protocol, we performed RT-qPCR analysis on the synthesized cDNA. During the experiment, we used β-Actin as the internal parameter and calculated the relative expression of RNA by comparing Ct. This process helps us understand the expression levels of target genes in cells. Finally, we list the primer sequence (Supplementary Table 2).

3.6. Colorimetric measurement of m^6A levels

We extracted total RNA from MDSCs, and the corresponding reagents were added according to the manufacturer's instructions for the m^6A detection kit (EpiGentek, USA). Finally, changes in the OD value in each well were detected by an enzymatic labeling system at a wavelength of 450 nm within 2–15 min of reagent addition. The following formula was used:

$$M^6A\% = [(Sample_{OD}\text{-}NC_{OD}) + S]/[(PC_{OD}\text{-}NC_{OD}) + P] * 100\%$$

S: The total amount of sample RNA added (ng)

P: Total amount of positive control RNA added (ng)

3.7. RNA-seq analysis

During the experiment, the culture supernatant was first discarded. Subsequently, we added 1 mL of TRIzol to the sediment. This step helped to extract total RNA from the cells, then we collected the cells in RNase-free EP tubes, a step that helped to reduce RNA degradation. Next, we divided the samples into si-NC groups and si-ALKBH5 groups, each with repeated pores. This step helps to compare the differences between the two groups of cells and improves the reliability of the experimental results. Finally, we sent the samples to Shanghai Ouyi Biotechnology Co., Ltd. (Shanghai China) for transcriptome sequencing (Contract No. HT2021-24911).

3.8. Methylated RNA immunoprecipitation (MeRIP)-qPCR

First, the MeRIP kit (EpiGentek, USA) was placed at room temperature to equilibrate, total RNA was isolated, and the required mix was prepared according to the instructions. The final RNA obtained was analyzed by RT−qPCR.

3.9. RNA immunoprecipitation (RIP)

In this study, the RIP assay was conducted using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Sigma, USA) and *anti*-m^6A antibodies (Abcam, USA).

We first lysed the cells (1×10^7) in a cold RIP lysis buffer. This process helps release RNA-protein complexes inside the cell. Cell lysates were collected and stored at −80 °C to ensure the stability of the experiment. Subsequently, we prepared magnetic bead-antibody complexes using 5 μg target antibodies or control IgG and 50 μL protein A/G magnetic beads. The magnetic beads-antibody complex was incubated overnight with an equal volume of cell lysate at 4 °C so that the antibody could fully contact and bind to the protein.10 μL cell lysate as input (control). To extract and purify RNA, we used a prepared protease K buffer. Finally, RT-PCR was used to analyze the extracted RNA.

3.10. RNA stability assay

In this study, we first focused on the stability of RNA in si-ALKBH5 and LV-ALKBH5 cells. This is achieved by incubating the cells using 5 μg/mL actinomycin D (Act-D, Sigma, USA). We first collected the cells at the specified time. This step helps us study the stability of RNA at different points in time. Subsequently, we extracted RNA from the collected cells and performed RT-QPCR analysis. This process helps us to quantitatively analyze the expression levels of RNA and thus assess its stability at different points in time.

3.11. Statistical analysis

In this study, statistical analysis was used to assess the significance of the differences between the experimental and control groups. This is achieved by using Student's *t*-test and ANOVA. In order to ensure the accuracy of the experimental results, we conducted at least three independent experiments. Experimental data are reported as mean ± SD (mean ± SD) for easy comparison and analysis. During the statistical analysis, we used a *P*-value less than or equal to 0.05 as the criterion for determining statistically significant differences.

4. Results

4.1. In MDSCs of CT26 tumor-bearing mice, the level of ALKBH5 was decreased

To evaluate the m^6A level in MDSCs, we first isolated MDSCs from the spleens of CT26 tumor-carrying mice and compared the m^6A levels of these cells with those of $CD11b^+Gr1^+$ cells in the spleen of wild-type mice (WT-MDSCs). The m^6A level in TU-MDSCs was markedly higher than that in WT-MDSCs (Fig. 1a). The m^6A level is dynamically regulated mainly by methyltransferases and demethylases [6]. Among these enzymes, the level of ALKBH5 in MDSCs gradually decreased as the tumor progressed (Fig. 1b and c), while the level of another demethylase, FTO, did not change significantly (Fig. 1d and e). Next, tumor cell-conditioned medium (TCCM) from CT26 cells was collected and added to WT-MDSCs, and the expression of ALKBH5 in MDSCs was found to be lower than that in MDSCs cultured in control RPMI 1640 medium (Fig. 1f and g). These data indicated that ALKBH5 expression was decreased in MDSCs, this suggests that ALKBH5 may play an important role in tumor development.

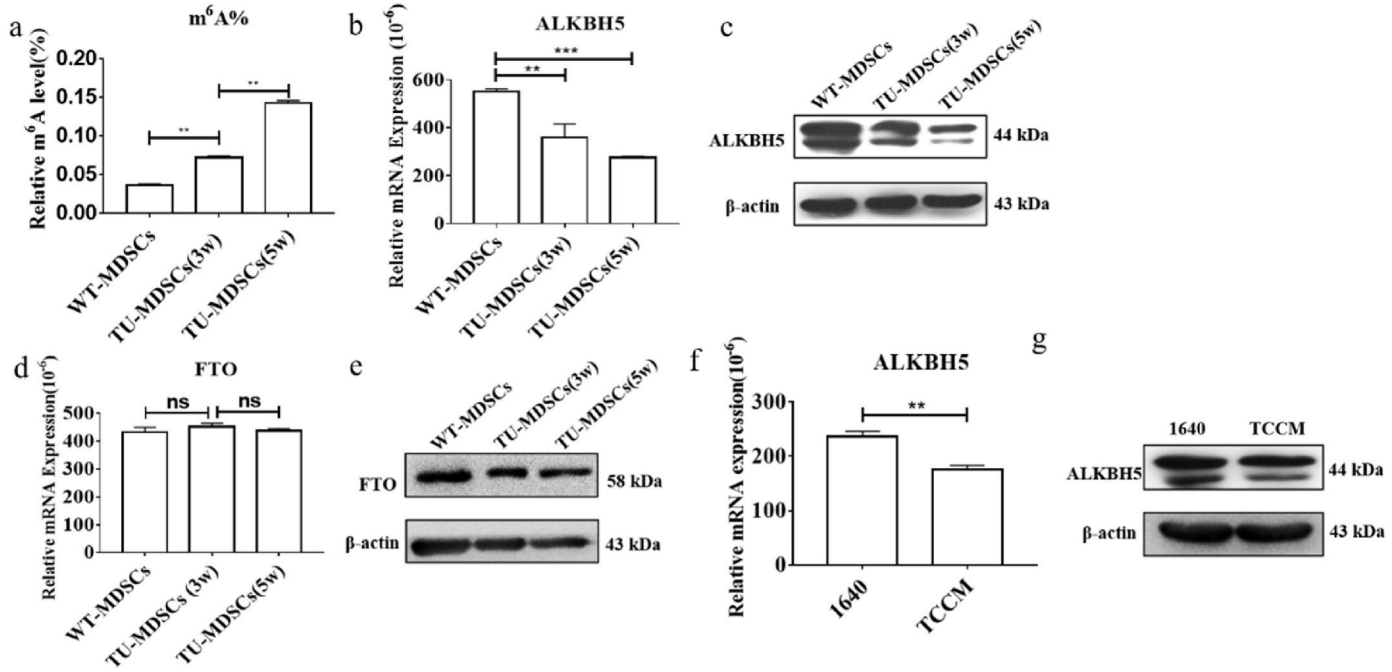

Fig. 1. ALKBH5 expression was decreased in MDSCs of CT26 tumor-bearing mice. a The m^{6}A levels in MDSCs isolated from mice at different times (weeks) after tumor cell implantation and in WT-MDSCs were detected by a colorimetric method. b,c Expression of ALKBH5 mRNA and protein in MDSCs of mice at different times (weeks) after tumor cell implantation. d,e Expression of FTO mRNA and protein in MDSCs of mice at different times (weeks) after tumor cell implantation. f,g Expression of ALKBH5 mRNA and protein in WT-MDSCs after treated with TCCM derived from CT26 cells. *Ns: nonsignificant; *p < 0.05; **p < 0.01; ***p < 0.001.*

4.2. ALKBH5 weakened the inhibitory effect of MDSCs on T-cell proliferation

The specific siRNA (si-ALKBH5) or ALKBH5 –lentiviral over-expression vector (LV-ALKBH5) was introduced into TU-MDSCs (SupplementaryFig.1a, b). The function of MDSCs inhibition on CD4$^+$T-cell proliferation after ALKBH5 was knocked down was significantly increased (Fig. 2a). On the other hand, the function of MDSCs on CD4$^+$T-cell proliferation after overexpression of ALKBH5 was significantly lower than the control group (Fig. 2b). These results indicated that

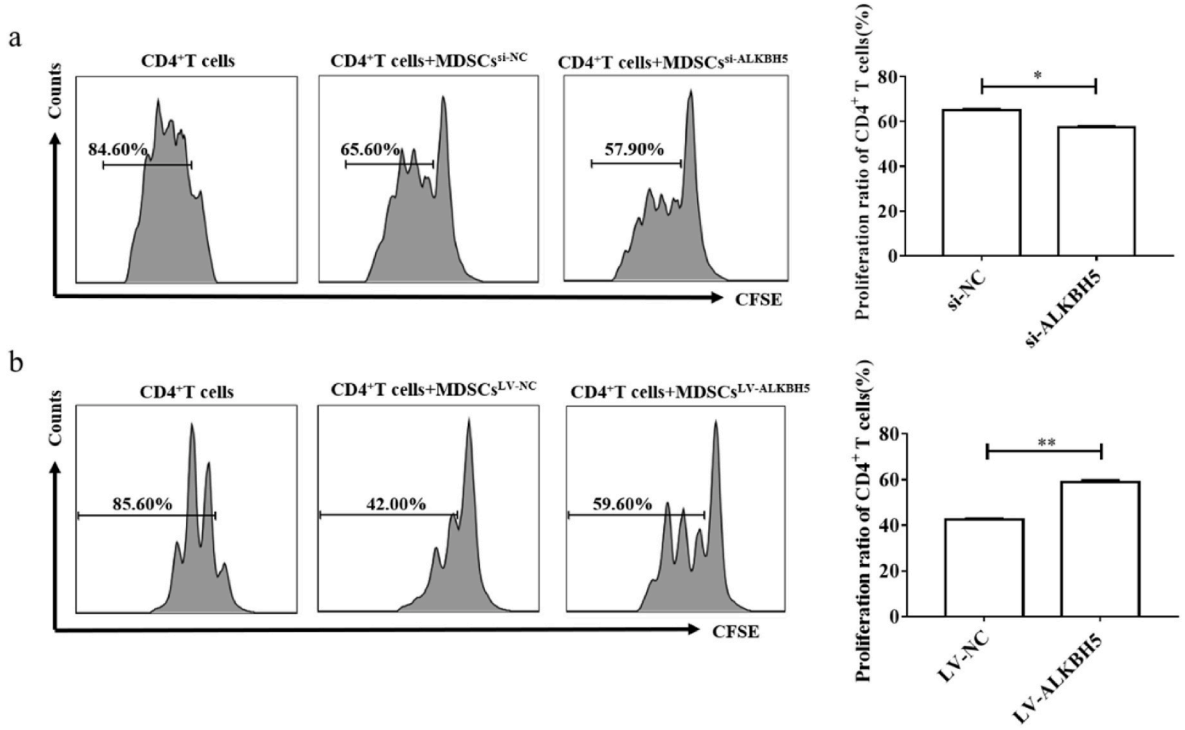

Fig. 2. ALKBH5 weakened the function of MDSCs on CD4$^+$T-cell proliferation. a The ability of MDSCs inhibitation on proliferation of CD4$^+$T-cells after knockdown of ALKBH5 was evaluated by flow cytometry. b The ability of MDSCs inhibitation on proliferation of CD4$^+$T-cells after after overexpression of ALKBH5 was evaluated by flow cytometry. **p < 0.05; **p < 0.01.*

ALKBH5 could weaken the immunosuppressive function of MDSCs in vitro.

4.3. ALKBH5 downregulates the expression of Arg-1 in MDSCs

Considering the above results, we then used transcriptome sequencing to screen for effects on the levels of downstream genes after ALKBH5 was knocked down in MDSCs. The expression of 159 genes was significantly changed after ALKBH5 was knocked down, including 122 upregulated genes and 37 downregulated genes (Fig. 3a). We removed genes with extremely low expression and no potential m^6A sites. Then, we focused on screening genes related to the immunosuppressive ability of MDSCs and found that the mRNA levels of Arg-1 and NOS2 (iNOS), the direct effector molecules of the tumor-immunosuppressive function of MDSCs [28,29], were significantly increased after ALKBH5 was knocked down (Fig. 3b).

Si-ALKBH5 or LV-ALKBH5 was introduced into TU-MDSCs. The Arg-1 and iNOS in MDSCs were significantly increased after knockdown of ALKBH5 in transcript levels (Fig. 3c, Supplementary Fig. 2a), consistent with the above sequencing results. In contrast, the expression of Arg-1 and iNOS was significantly decreased after overexpression of ALKBH5 in MDSCs (Fig. 3e, Supplementary Fig. 2c). Moreover, the activity of both Arg-1 and iNOS was increased after knockdown of ALKBH5(Fig. 3d, Supplementary Fig. 2b) but decreased after overexpression of ALKBH5 (Fig. 3f, Supplementary Fig. 2d). These results indicated that the expression of both Arg-1 and iNOS is affected by ALKBH5 in MDSCs.

4.4. ALKBH5 decreasedArg-1 mRNA stability and protein expression

The SRAMP database was used to predict whether Arg-1 mRNA and NOS2 mRNA contain m^6A sites. As shown in Supplementary Fig. 2e, both Arg-1 mRNA and NOS2 mRNA were predicted to contain four high-confidence m^6A sites, suggesting that Arg-1 and NOS2 mRNA expression may be regulated by m^6A modification.

As shown in Fig. 4a and Supplementary Fig. 1f, ALKBH5 can bind Arg-1 mRNA and NOS2 mRNA, which provides the foundation for ALKBH5 to regulate m^6A modification of Arg-1 mRNA and NOS2 mRNA. Then, after knockdown or overexpression of ALKBH5 in MDSCs, MeRIP-PCR was used to measure the m^6A levels in Arg-1 mRNA and NOS2 mRNA. The m^6A level in only Arg-1 mRNA increased with knockdown of ALKBH5(Fig. 4b). When ALKBH5 expression was increased, the m^6A level was obviously decreased inArg-1 mRNA (Fig. 4c), but unchanged in NOS2 mRNA (Supplementary Figs. 1g and h). These results suggested that ALKBH5 decreased the m^6A level in Arg-1 mRNA after binding ALKBH5 but had no effect on the m^6A level in NOS2 mRNA.

Furthermore, we first evaluated whether ALKBH5-mediated m^6A modification affects the stability of Arg-1 mRNA. mRNA synthesis in MDSCs was blocked by treatment with Act-D, and Arg-1 mRNA expression was measured at different time points. Compared with the control group, the degradation rate of Arg-1 mRNA was decreased when ALKBH5 was knocked down (Fig. 4d), but it was significantly increased when ALKBH5 was overexpressed (Fig. 4e). Subsequently, we measured the Arg-1 protein level in MDSCs and found that it was markedly higher in the ALKBH5 knockdown group than in the control group (Fig. 4f); in contrast, it was significantly decreased after overexpression of ALKBH5 (Fig. 4g). These results suggest that ALKBH5 might regulate Arg-1 protein expression by downregulating m^6A modification ofArg-1 mRNA and reducing Arg-1 mRNA stability.

4.5. The effect of ALKBH5 on MDSC function delays the progression of CRC in tumor-bearing mice

Tumor progression in ALKBH5-overexpression MDSC group was significantly slowed (Fig. 5a), and the volume and weight of tumor were lower than control group (Fig. 5b and c). The proportions of $CD4^+$Th1 cells and $CD8^+$cytotoxic T lymphocytes (CTLs) of spleen, local lymph

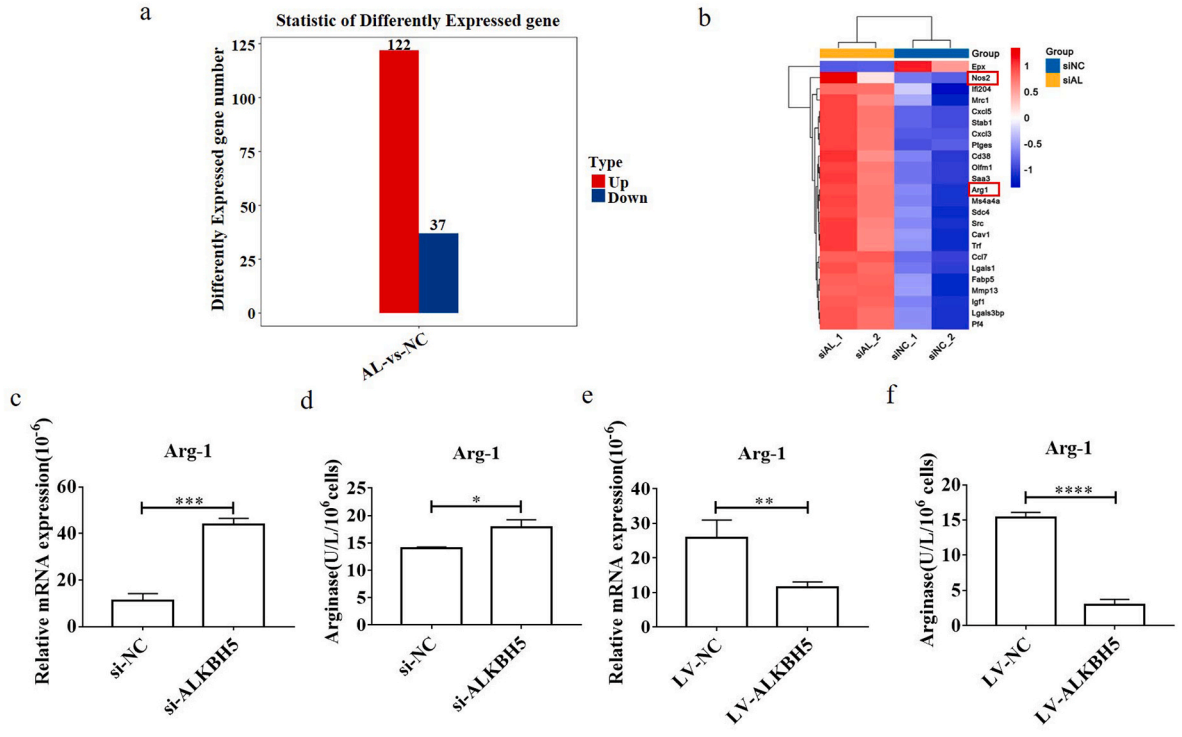

Fig. 3. ALKBH5 downregulates the expression of Arg-1 in MDSCs. a Numbers of differentially expressed genes, the red represents upregulated genes and the blue represents downregulated genes. b Cluster heatmap of the differentially expressed genes. c,d The expression level of mRNA and activity of Arg-1 after ALKBH5 knockdown were detected by qRT−PCR and a colorimetric assay, respectively. e,f The expression level of mRNA and activity of Arg-1 after ALKBH5 overexpression were detected by qRT−PCR and a colorimetric assay, respectively. *Ns: nonsignificant; *p < 0.05; **p < 0.01.*

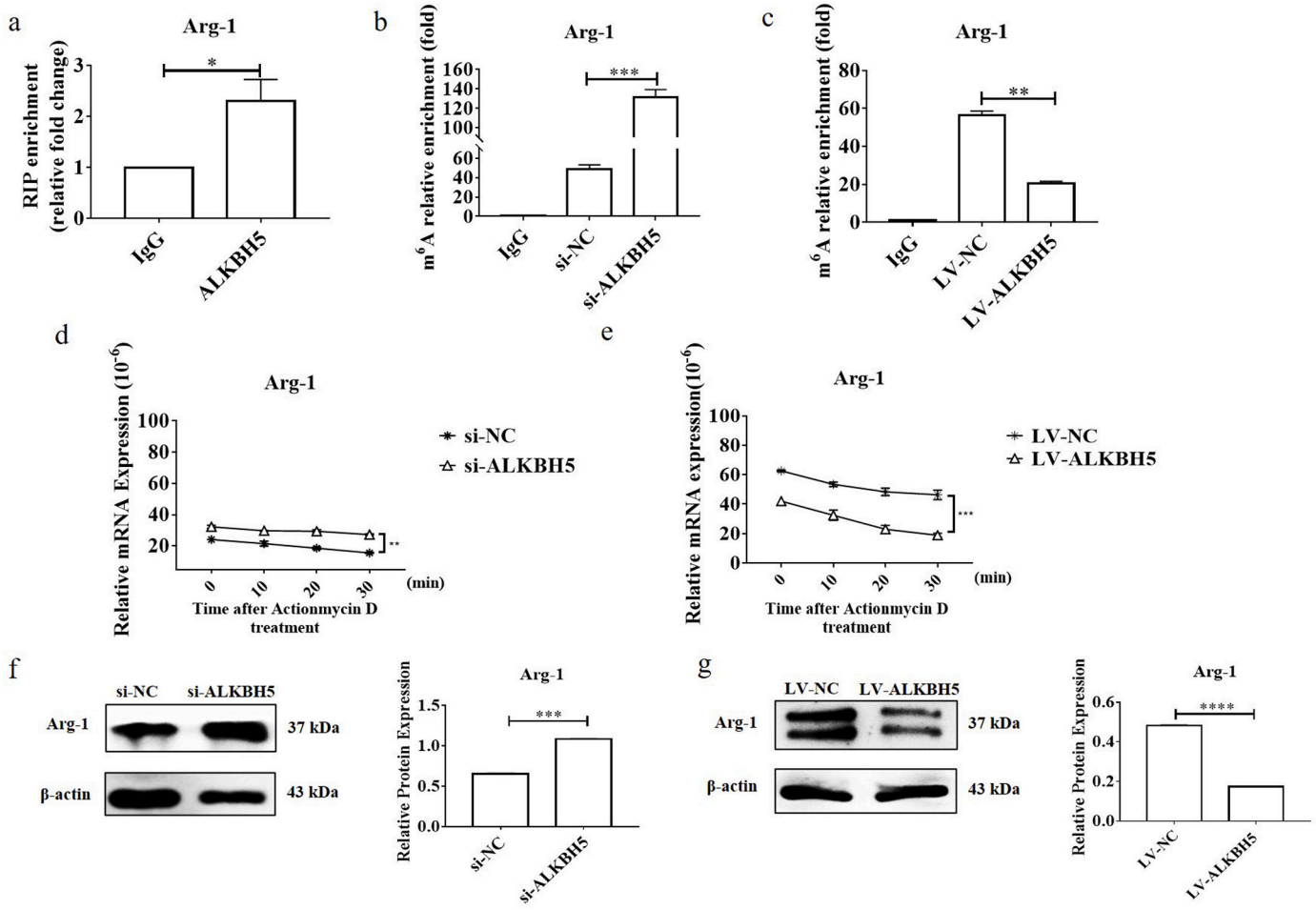

Fig. 4. ALKBH5 decreased Arg-1 mRNA stability and protein expression. a RIP was used to verify the binding of ALKBH5 to Arg-1 mRNA in MDSCs. b,c The m^6A level of Arg-1 mRNA in MDSCs was evaluated by MeRIP after ALKBH5 was knocked down or overexpressed of. d,e Stability of Arg-1 mRNA in MDSCs after ALKBH5 was knocked down or overexpressed. f,g Protein expression of Arg-1 in MDSCs after ALKBH5 was knocked down or overexpressed. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

nodes and tumor tissues were examined in the above groups of mice. The proportions of $CD4^+$ Th1 cells and $CD8^+$ CTLs in mice after the administration of MDSCs overexpression ALKBH5 were markedly higher than those in the control group (Fig. 5 d, e, f) ($P<0.05$). This finding suggests that ALKBH5' effect on the function of MDSCs may indirectly delay tumor progression. In contrast, when ALKBH5 expression in MDSCs was knocked down, the tumor progression rate was significantly accelerated (Supplementary Fig. 3).

5. Discussion

Numerous studies have revealed that ALKBH5 is closely related to tumor progression, both promoting and inhibiting tumor development. On the one hand, ALKBH5 has been reported to play a promotive role in breast cancer, CRC, gastric cancer, liver cancer and other cancers. On the other hand, studies have proven that ALKBH5 plays an inhibitory role in cancers, such as bladder cancer, ovarian cancer, gastric cancer, and CRC. In addition, ALKBH5 also plays a role in regulating tumor progression by regulating the tumor microenvironment, while MDSCs play a vital role in promoting tumor progression in the complex tumor microenvironment. Drug resistance induced by MDSCs is mediated mainly by inhibition of T-cell immunoreactivity and may lead to poor prognosis inpatients. Therefore, exploring more relevant regulatory mechanisms of MDSCs will help to provide a theoretical basis for future immunotherapy targeting MDSCs.

This study revealed that ALKBH5 expression in MDSCs of CRC-bearing mice was lower than that in wild-type MDSCs, while FTO expression was not significantly different between these MDSCs. After interference with ALKBH5 expression, the function of MDSCs in inhibiting T-cell proliferation was significantly weakened. Moreover, after knockdown of ALKBH5 in MDSCs, there were significant differences in the expression of the effector molecules Arg-1 and iNOS, which are related to the function of MDSCs. Arginine is an amino acid necessary for T cell proliferation. iNOS catalyzes L-arginine to produce nitric oxide (NO), while Arg-1 breaks down arginine into ornithine and urea. Cells like MDSCs expressing iNOS or Arg-1 can consume arginine in TME, causing T cell proliferation and activity to be inhibited. It has been reported that there are other different mechanisms of Arg-1 and iNOS mediated inhibition of T cell proliferation, such as reducing the expression of CD3ζ chain, blocking cells in the G0/G1 phase, promoting T cell apoptosis, blocking the recognition of APC by MHC class II molecules, and reducing the expression of JAK3 and STAT5 in T cells [30]. Our research further delves into how ALKBH5 regulates the function of MDSCs. We found that ALKBH5 could reduce the expression of Arg-1 and iNOS. Further mechanism studies showed that ALKBH5 promoted the degradation of Arg-1 mRNA by reducing the m^6A level of Arg-1 mRNA, thus reducing the level of Arg-1 protein. However, the effect of ALKBH5 on iNOS expression may be mediated via other mechanisms. In summary, our data showed that ALKBH5 was involved in modulating the function of MDSCs and tumor progression, primarily by reducing the m^6A level in Arg-1 mRNA to reducing its stability.

However, a drawback of this framework is that ALKBH5 could also regulate the expression of iNOS but did not affect m^6A modification of NOS2 mRNA; thus, we speculated that ALKBH5 regulates the expression

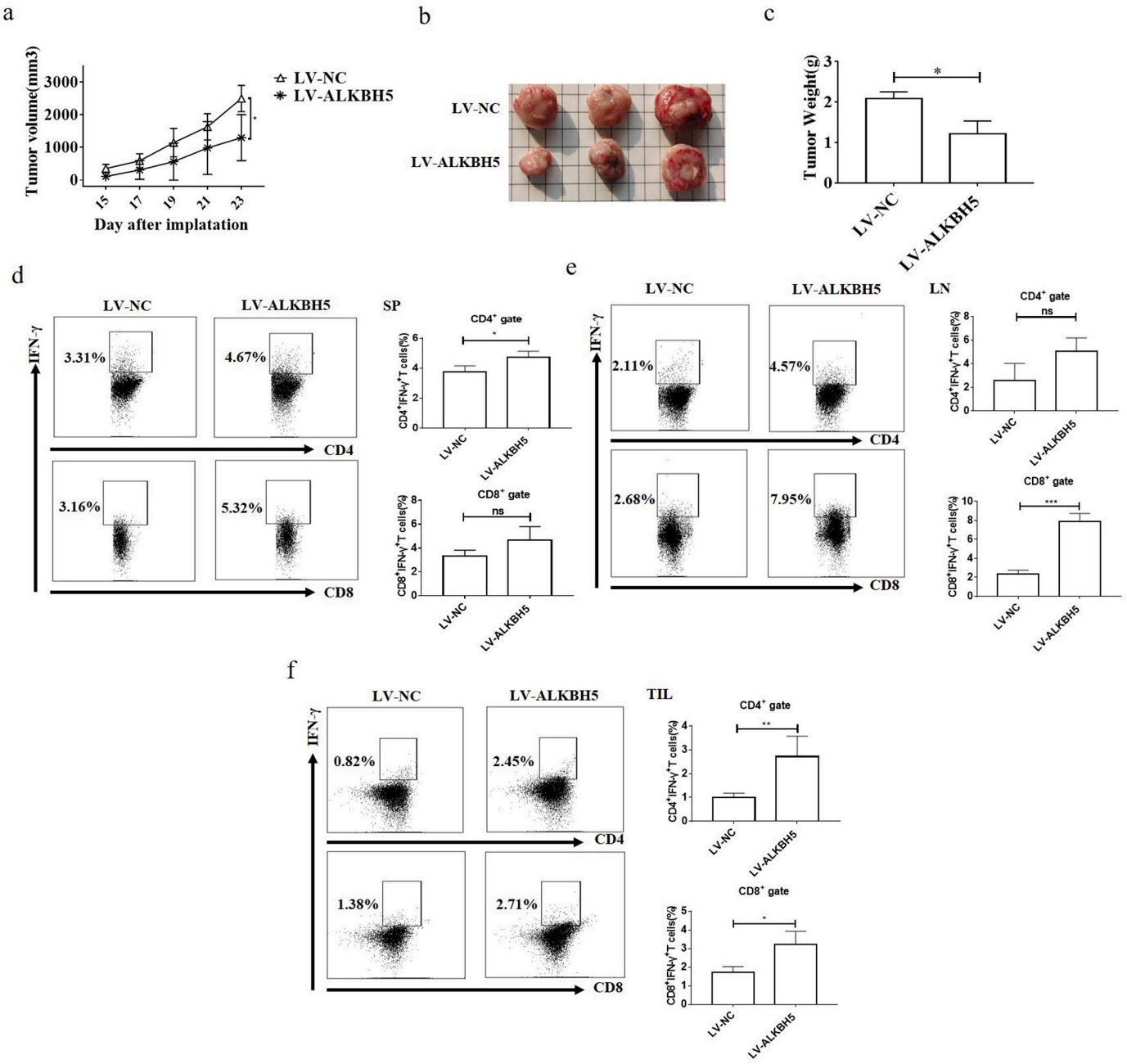

Fig. 5. Overexpression of ALKBH5 in MDSCs delayed the tumor progression rate. a Tumor growth curve after the administration of ALKBH5-overexpression MDSCs. b,c Comparison of tumor size and weight after the administration of ALKBH5-overexpression MDSCs. *d*-f Proportions of Th1 cells and CTLs of spleen, lymph nodes and tumor tissues after the administration of ALKBH5-overexpression MDSCs. *Ns: nonsignificant; *p < 0.05; **p < 0.01; ***p < 0.001.*

of iNOS via other mechanisms. It has been reported that ALKBH5 can regulate the expression of STAT3 in osteosarcoma [31], while STAT3 can regulate the function of MDSCs by promoting the expression of effector molecules such as iNOS [32]. It has also been reported that ALKBH5 inhibits the nuclear export of IL-6 mRNA through its m^6A demethylation [33]. IL-6 can regulate the immunosuppressive function of MDSCs through the STAT3, MAPK and other signaling pathways [34]. STING, a negative regulator of the function of MDSCs, has also attracted much attention [35]. Studies have shown that ALKBH5 can positively regulate STING expression by regulating the STING upstream transcription factor HMGB1 [36]. In conclusion, ALKBH5 may indirectly regulate the expression of iNOS by regulating upstream gene expression and then regulate the immunosuppressive function of MDSCs. However, the specific regulatory mechanism needs more follow-up research.

The regulation of m^6A cannot be achieved without reader proteins. That is, regardless of any change in the level of m^6A on a mRNA, a reader protein is ultimately required to recognize m^6A modification and then shuttle the protein into a "channel" such as degradation or translation. This constitutes a complete collaborative process among m^6A-related molecules. In this paper, the m^6A reader protein required for ALKBH5 to regulate Arg-1 mRNA expression was not definitively identified. It has been reported that YTHDF2 mainly regulates the translation of target mRNAs or promotes their degradation. YTHDF3 is mainly involved in the regulation of target mRNA translation. YTHDC1 is mainly involved in regulating transcript splicing [37]. In contrast to YTHDF2, IGF2BPs have been reported to promote translation by increasing the stability and storage of target mRNAs. Our results showed that the stability of Arg-1 mRNA was reduced after overexpression of ALKBH5, and the m^6A

level inArg-1 mRNA was significantly decreased during this process, suggesting that m^6A modification may help to promote the stability of Arg-1 mRNA. Therefore, we will first consider reader proteins involved in mRNA stability (such as YTHDF2 and IGF2BPs) for further study. In addition, the previous sequencing results of our research group (data not shown) showed that after METTL3 deletion, the m^6A level inArg-1 at the GAACT site was decreased, suggesting that this site may be the main m^6A site in Arg-1 mRNA; however, this hypothesis needs to be confirmed experimentally.

We also found that in addition to affecting Arg-1 and iNOS expression, ALKBH5 knockout resulted in significant differences in the levels of chemotaxis-, differentiation- and development-related molecules in MDSCs. Therefore, does ALKBH5 also play a role in regulating the chemotaxis, differentiation and development of MDSCs? Studies have shown that the chemokine ccl7 secreted by *M*-MDSCs promotes the activation of dormant tumor cells, resulting in the formation of preliminary metastases and eventually leading to tumor metastasis. In addition, chemokine production is also associated with short-term tumor recurrence and distant metastasis. Whether ALKBH5 regulates the secretion of chemokines in MDSCs and whether this regulation is involved in tumor metastasis have not been reported. In the future, this research group will conduct in-depth research on these topics and fully reveal the biological role of ALKBH5 in MDSCs.

CRediT authorship contribution statement

Lili Feng: Writing – original draft, Methodology, Investigation, Formal analysis. **Min Li:** Methodology, Formal analysis. **Jie Ma:** Formal analysis. **Wenxin Wang:** Methodology, Formal analysis. **Shengjun Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Zhenwei Mao:** Formal analysis. **Yue Zhang:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.03.003.

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