

Original Article

Hypoxia-induced m6A demethylase ALKBH5 promotes ovarian cancer tumorigenicity by decreasing methylation of the lncRNA RMRP

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Abstract: Ovarian cancer is one of the most lethal and drug-resistant gynecological diseases. Among the various post-transcriptional RNA modifications, N6-methyladenosine (m6A) has been implicated in several malignancies, including breast cancer. Recently, the biological significance of long noncoding RNA (lncRNA) methylation has garnered significant attention. The N6-methyladenosine (m6A) demethylase ALKBH5 (Alkylation Repair Homolog Protein 5) has been shown to promote ovarian cancer development by reducing the methylation of the lncRNA RMRP. In this study, we found that a hypoxic microenvironment induces an increase in ALKBH5 expression in ovarian cancer. Both in vitro and in vivo investigations demonstrated that ALKBH5, which is overexpressed in human ovarian cancer, promotes carcinogenesis. Furthermore, using bioinformatics analysis, we predicted interactions between ALKBH5 and lncRNAs, confirming RMRP as a potential binding lncRNA for ALKBH5. ALKBH5 was found to upregulate RMRP expression via demethylation. Knockdown of RMRP in ovarian cancer cell lines led to a decrease in cell growth and migration. Additionally, we demonstrated that the inhibition of ovarian cancer by ALKBH5 knockdown is partially mediated by RMRP suppression. In conclusion, our findings reveal a novel mechanism in which ALKBH5 promotes ovarian cancer by demethylating the lncRNA RMRP, suggesting its potential as a therapeutic target for the disease.

Keywords: m6A, ALKBH5, RMRP, OC

Introduction

Ovarian cancer (OC) is the gynecologic malignancy with the highest death rate worldwide [1, 2]. Current treatment strategies, such as surgery and chemotherapy, often prove ineffective, particularly in advanced stages of the disease (FIGO stages III/IV) [3]. In the past studies, at the molecular, cellular, and tissue levels, significant progress has been achieved in understanding the processes of OC formation. Significant progress has been made in understanding OC development at the molecular, cellular, and tissue levels. Both genetic (oncogenes and tumor suppressor genes) and epigenetic processes (DNA methylation, microRNAs, and other non-coding RNAs) have been implicated in OC pathogenesis. Multiple mechanisms, including driver mutations, epigenetic processes, tumor plasticity, and the hypoxic

microenvironment, collectively contribute to the phenotype and treatment resistance of OC, undermining the efficacy of targeted therapies [4]. Hypoxia, a key component of the tumor microenvironment, is a major driver of tumor growth [5]. It can induce a more aggressive phenotype, enhance tumor invasiveness, and promote tumor growth and metastasis [6]. Numerous studies have shown that hypoxia-inducible factor 1 (HIF1) upregulates the transcription of genes involved in tumor motility, formation, metabolic reprogramming, angiogenesis, invasion, and metastasis in hypoxic OC cells [7, 8]. Our current study reveals that hypoxia augments the expression of the m6A demethylase ALKBH5 in OC cells in an HIF1-dependent manner.

Epigenetic regulation of gene expression, including N6-methyladenine (m6A) modifica-

tion, may play a pivotal role in OC development [9]. ALKBH5, a critical N6-methyladenosine (m6A) eraser protein that regulates nuclear RNA export, metabolism, and gene expression, is involved in tumor formation [10]; it has been associated with cancer development and has potential implications for cancer therapy and prognosis. For example, ALKBH5 has been shown to enhance endometrial cancer proliferation and tumorigenicity by modulating IGF1R expression [11-13], and to promote colon cancer growth by reducing NEAT1 lncRNA methylation [12]. Previous studies have also demonstrated that ALKBH5 activates the nuclear factor-kappa B pathway, thereby promoting ovarian carcinogenesis in a model tumor micro-environment [14]. Our findings corroborate these observations, showing that ALKBH5 regulates the proliferation and invasion of OC cells, consistent with its established role in promoting OC.

Long non-coding RNAs (lncRNAs) are RNA transcripts exceeding 200 nucleotides in length that do not encode proteins [15]. Despite their non-coding nature, lncRNAs have been found to participate in and regulate a variety of cellular processes, including protein modification, posttranscriptional regulation, and transcription regulation, thereby contributing to tumorigenesis [16]. For instance, the non-coding RNA RMRP, initially identified in Cartilage-Hair Hypoplasia (CHD), has been linked to stomach cancer [17] and implicated in OC by modulating miR-580-3p/MICU1 signaling, influencing paclitaxel sensitivity [18]. Despite the established role of lncRNAs in OC, studies investigating the regulation of lncRNA methylation by ALKBH5 in OC are scarce.

In this study, we report an increased expression of ALKBH5 in hypoxia-induced OC and identify, for the first time, that RMRP is a downstream target regulated by ALKBH5 in an m6A-dependent manner in OC. These novel findings offer valuable insights into potential targeted therapies, prognosis, and outcomes for OC.

Materials and methods

In silico analysis using the K-M plot dataset and StarBase v2.0 database

We looked at the OS and PFS of over 1000 individuals listed in the K-M plot dataset (<http://kmpplot.com/analysis/>).

In order to anticipate the associations between lncRNAs and ALKBH5 in OC, we used StarBase v2.0 (<http://starbase.sysu.edu.cn/starbase2/>).

Clinical samples collection

Tissues were collected from patients admitted to the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Wannan Medical University between January 2019 and 2021. A human OC tissue microarray found 276 instances of OC. None of the patients had undergone chemotherapy, radiation, or any other anti-cancer treatment prior to surgery. All tissues were collected with informed consent, and the study was approved by the Wannan Medical College Research Ethics Committee.

Cell culture and transfection

Cell lines A2780 and SKOV-3 were obtained from the Chinese Academy of Science's Cell Bank, while OVCAR3, HO8910, and OVCAR8 were sourced from the Shanghai Cancer Institute. The cell lines ES-2 and A2780 were cultured in RPMI 1640 media (Gibco, Beijing, China) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin (all from Gibco), and incubated at 37°C in a 5% CO₂ atmosphere. To simulate hypoxic conditions, cells were placed in a Billups-Rothenberg modular incubator chamber and flushed with a gas mixture containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen for 2 minutes at 2 psi. For mechanistic studies, short hairpin RNAs (shRNA) for ALKBH5 (shALKBH5), si-HIF-1, si-RMRP, and corresponding negative controls were transfected into cultured SKOV-3 and OVCAR-3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. All transfection plasmids were provided by GenePharma Corporation (Suzhou, Jiangsu, China).

Immunohistochemical staining

Tissue samples were embedded, sectioned into 4 µm-thick slices, and stained with ALKBH5 primary antibodies (1:200, Abcam, USA). All immunohistochemical staining procedures were performed according to standard protocols. The stained sections were examined and imaged using a microscope (Axio Imager: Carl Zeiss). The degree of staining was scored under

Table 1. The primers for RT-qPCR

	Forward Primer	Reverse Primer
si-HIF-1 α -1	5'-GAACGTGGAAAAGAAAAGTCTCG-3'	5'-CCTTATCAAGATGCGAACTCACA-3'
si-HIF-1 α -2	5'-CACCACAGGACAGTACAGGAT-3'	5'-CGTGCTGAATAATACCACTCACA-3'
si-HIF-1 α -3	5'-ATCCATGTGACCATGAGGAAATG-3'	5'-TCGGCTAGTTAGGGTACACTTC-3'
sh-ALKBH5-1	5'-CGGCGAAGGCTACACTTACG-3'	5'-CCACCAGCTTTTGGATCACCA-3'
sh-ALKBH5-2	5'-AGTTCCAGTTCAAGCCTATTCG-3'	5'-TGAGCACAGTCACGCTTCC-3'
sh-ALKBH5-3	5'-GCTTCAGGGTATGGGAGTTG-3'	5'-TTCCAGGATCTGAGTGGATAGA-3'
si-RMRP	5'-ACTCCAAAGTCCGCCAAGA-3'	5'-GATACGCTT CTTGGCGGACT-3'

a light microscope, considering both the proportion of positively stained cells and staining intensity. Scoring was as follows: ratio of positively stained cells (0 = negative, 1 = 1-25% of cells, 2 = 26-50% of cells, 3 = 51-75% of cells, and 4 = 76-100% of cells stained) and staining intensity (no staining 0, weak 1, moderate 2, and strong staining 3). The final score of ALKBH5 was determined as positive cell score \times staining intensity. Low expression was defined as a total score < 6 and high expression as a total score ≥ 6 . These scores were determined in a blinded manner by two senior pathologists and presented as the mean of the two scores.

RT-qPCR

Total RNA from cells and tissues was extracted using the TRIzol method (Invitrogen, Grand Island, NY). Complementary DNA (cDNA) was synthesized using an Aidlab cDNA synthesis kit (Beijing, China). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the SYBR Green qPCR Mix (Aidlab) on an ABI 7900HT (Thermo Fisher Scientific, Waltham, MA). All experiments were repeated in triplicate. The results were calculated using the 2Ct method. The primers for RT-qPCR are listed as **Table 1**.

Western blotting

Total cellular proteins were extracted using a complete protein extraction kit (Beyotime, China). Cell lysates were separated and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies and then species-specific secondary antibodies. Primary antibodies were ALKBH5 (1:1000, Abcam, USA) and GAPDH (1:1000, Abcam, USA). Species-specific secondary antibodies were stained at 1:1000 (CST, USA).

Cell viability assay (CCK8 assay)

SKOV-3 and OVCAR-3 cells were cultured in medium containing 10% serum and seeded in 96-well plates at a density of 2000-3000 cells per well. Five wells were allocated for each of the two groups studied. At 0, 24, 48, and 72 hours, each well received a 10 μ L addition of Cell Counting Kit-8 (Dojindo, Japan). Cell viability was determined using an absorbance microplate reader at a wavelength of 450 nm (BIO-TEK).

Transwell migration and invasion assays

Transwell migration assays and invasion assays were conducted using BD Biosciences chambers (with a 24-well insert and an 8- μ m pore size) as per the manufacturer's instructions. Cells were seeded in the top chambers of the Transwell device in 200 μ L serum-free Dulbecco's modified Eagle's medium at a density of 5×10^4 cells. The bottom chambers were filled with 0.5 mL Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 24 hours, cells on the underside of the membrane were labeled and counted under a microscope in five high-power random fields.

Wound healing assays

Wound healing migration assays were conducted as follows: cells were seeded in a six-well chamber slide in Dulbecco's modified Eagle's medium at a density of 5×10^3 cells per well. Wounds were created in the cell monolayers using a sterile 10 μ L pipette tip. After 48 hours of incubation, images were taken to assess wound closure.

In vivo tumor xenograft model

Stable cell clones, at a density of 5000 cells, were subcutaneously injected into the right

flank of six-week-old male nude (nu/nu) mice (SLAC, Shanghai, China). These clones were infected with SKOV3-Nc and SKOV3-shALKBH5 in 100 μ L of sterilized phosphate-buffered saline. Mice were allowed to recover for six weeks before reinjection. In each group, the tumor weights of six mice were measured and recorded. All mice were euthanized within six weeks post-surgery, following the removal of their tumors. Tumor sizes were measured using Vernier calipers, with the volume calculated as $1/2 \text{ length} \times \text{width}^2$. Tumors were dissected, fixed with phosphate-buffered neutral formalin, embedded in paraffin, and prepared for routine histological evaluation. All experimental procedures involving mice were conducted in accordance with the guidelines of the East China Normal University Animal Care Commission.

Methylated RNA immunoprecipitation experiment (MeRIP)

The total m6A content of total RNA was determined using a complete m6A methylation test kit (EpiGentek, USA). Following total RNA extraction and purification, the attached RNA was cultured with the capture antibody, followed by the addition of the detection antibody and the enhancer solution. The m6A concentration was determined by fluorescence.

RNA immunoprecipitation experiment (RIP)

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore Corporation) was used for the RIP experiments. Anti-ALKBH5 antibodies, as well as IgG control antibodies were used for RIP experiments. The coprecipitated RNAs were detected using RT-PCR.

Subcellular fractionation location

Nuclear and cytoplasmic fractions were separated using the PARIS Kit (Life Technologies) and analyzed according to the manufacturer's instructions.

Statistical analysis

All of the data was gathered from three distinct studies and analyzed using IBM's SPSS 21.0 and GraphPad Prism5 (San Diego, CA) software, respectively. All *P*-values were calculated using two-tailed testing, and in this study, Statistical values less than 0.05 were defined

as significant differences, regardless of how they were calculated.

Results

Hypoxic conditions and HIF-1 α exposure regulate the expression of M6A demethylase ALKBH5

We aimed to investigate the expression of ALKBH5 in hypoxic ovarian cancer (OC) cells. Gene-wide expression profiling was conducted on SKOV-3 cell line exposed to either 20% or 1% O_2 for 24 hours. Notably, ALKBH5 expression was significantly upregulated under 1% O_2 conditions (**Figure 1A**). Further analysis of gene expression profiles in ovarian cancer tissues revealed a substantial upregulation of ALKBH5 (**Figure 1B, 1C**). Examination of ALKBH5 mRNA expression in 10 pairs of OC and adjacent normal tissues showed significantly higher expression in tumor tissues (**Figure 1D**), which was corroborated at the protein level (**Figure 1E**). We treated six OC cell lines (ES-2, OVCAR8, A2780, HO8910, OVCAR3, SKOV-3) with either 20% or 1% O_2 and found that ALKBH5 mRNA levels were significantly increased under hypoxic conditions in all cell lines (**Figure 1F**). Additionally, upon HIF-1 suppression, ALKBH5 expression decreased alongside HIF-1 mRNA expression (**Figure 1G**).

High ALKBH5 expression correlates with adverse clinicopathological features in OC patients

To elucidate the clinical significance of ALKBH5, we performed immunohistochemical analysis on 20 normal ovarian tissues and 276 OC tissues. OC tissues exhibited higher ALKBH5 staining compared to normal tissues (**Figure 2A**), with higher expression observed in advanced FIGO stage OC tissues (**Figure 2B**). Detailed analysis revealed significant variation in ALKBH5 expression in plasmacytic ovarian cancer when comparing various clinicopathologies of OC (endometrial, serous, mucinous, *P* = 0.003). In OC, examination revealed that ALKBH5 expression is strongly related to several unfavorable clinicopathological aspects of the disease, mainly comprising the FIGO stage and lymph node metastasis with ALKBH5 upregulated in patients with more advanced (III-IV) stages (*P* = 0.025) (**Table 2**). Using the KM plotter dataset, which contains survival data from

ALKBH5 promotes ovarian cancer tumorigenicity

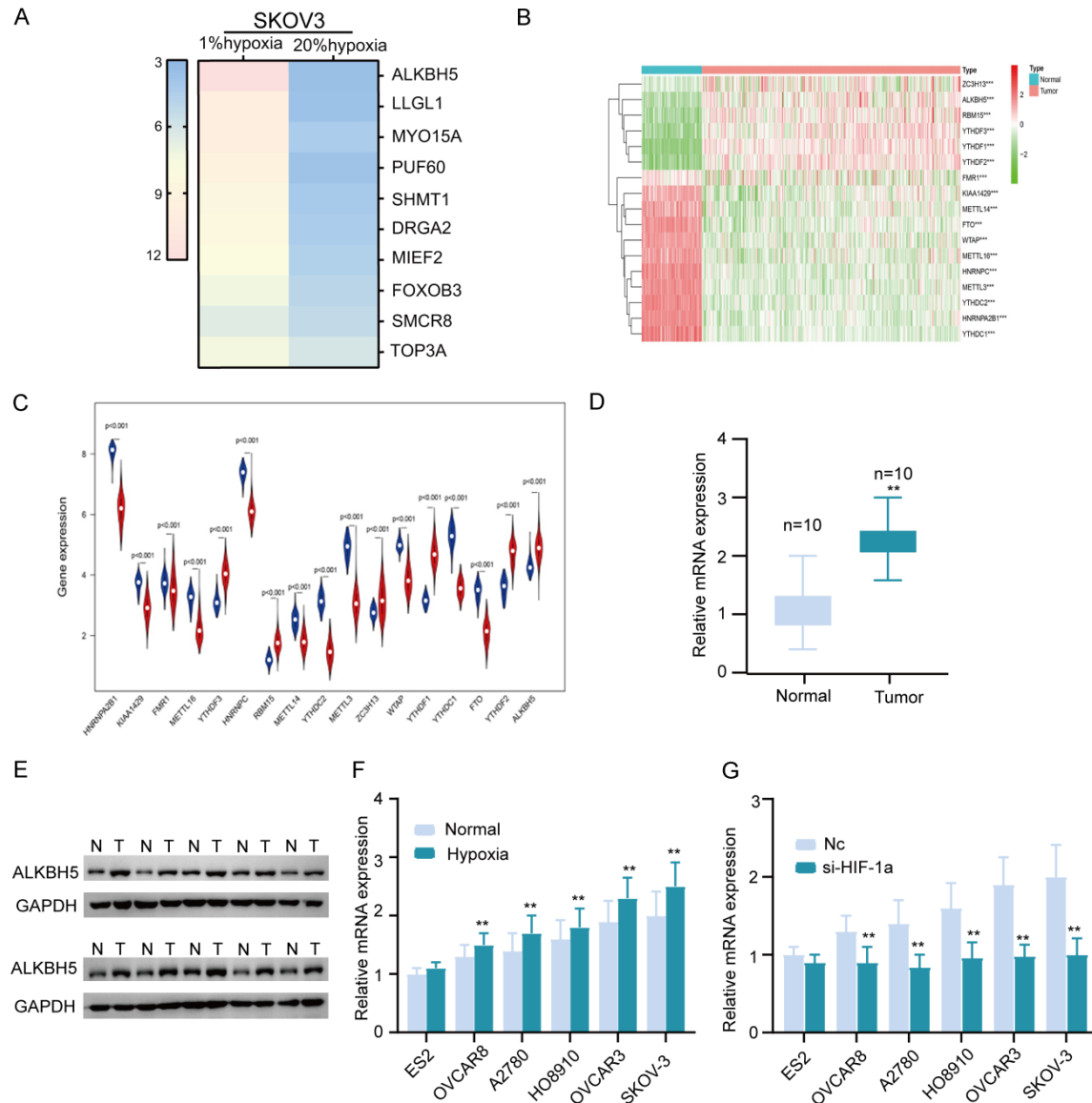


Figure 1. Hypoxia-inducible factor 1 (HIF-1) mediates consistent alteration in m6A demethylase ALKBH5 expression. A: Whole-genome expression profiling of the ovarian cancer cell line (SKOV-3) under 1% and 20% oxygen conditions revealed a significant increase in ALKBH5 expression under hypoxia. B, C: Whole-genome expression profile showing a marked up-regulation of ALKBH5 in OC tissues compared to normal tissues. D: In 10 matched samples, ALKBH5 mRNA was significantly elevated in OC tissues compared to paired normal tissues. E: ALKBH5 protein expression was higher in 10 OC tissues compared to paired normal tissues. F: Six human OC cell lines (ES-2, OVCAR8, A2780, HO8910, OVCAR3, SKOV-3) were exposed to normoxia (20% O₂) or hypoxia (1% O₂) for 24 h, and ALKBH5 mRNA expression was significantly increased under hypoxic conditions, as determined by RT-PCR. G: Knockdown of the hypoxia-inducible factor HIF-1α in OC cell lines led to a marked reduction in ALKBH5 mRNA expression. Data are presented as mean ± SD (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

over 1000 OC patients, high ALKBH5 expression was found to be negatively correlated with overall survival (*n* = 1657, *P* = 0.001) and progression-free survival (*n* = 1436, *P* = 0.001), demonstrating that prognoses was significantly worse for those with higher ALKBH5 levels than for those with lower levels (**Figure 2C**).

Silencing of ALKBH5 suppresses OC cell bio-function in vitro and in vivo

To better understand the biological significance of ALKBH5 in OC cells, we transfected two OC cell lines (SKOV-3 and OVCAR-3) with relatively high ALKBH5 expression with sh-ALKBH5

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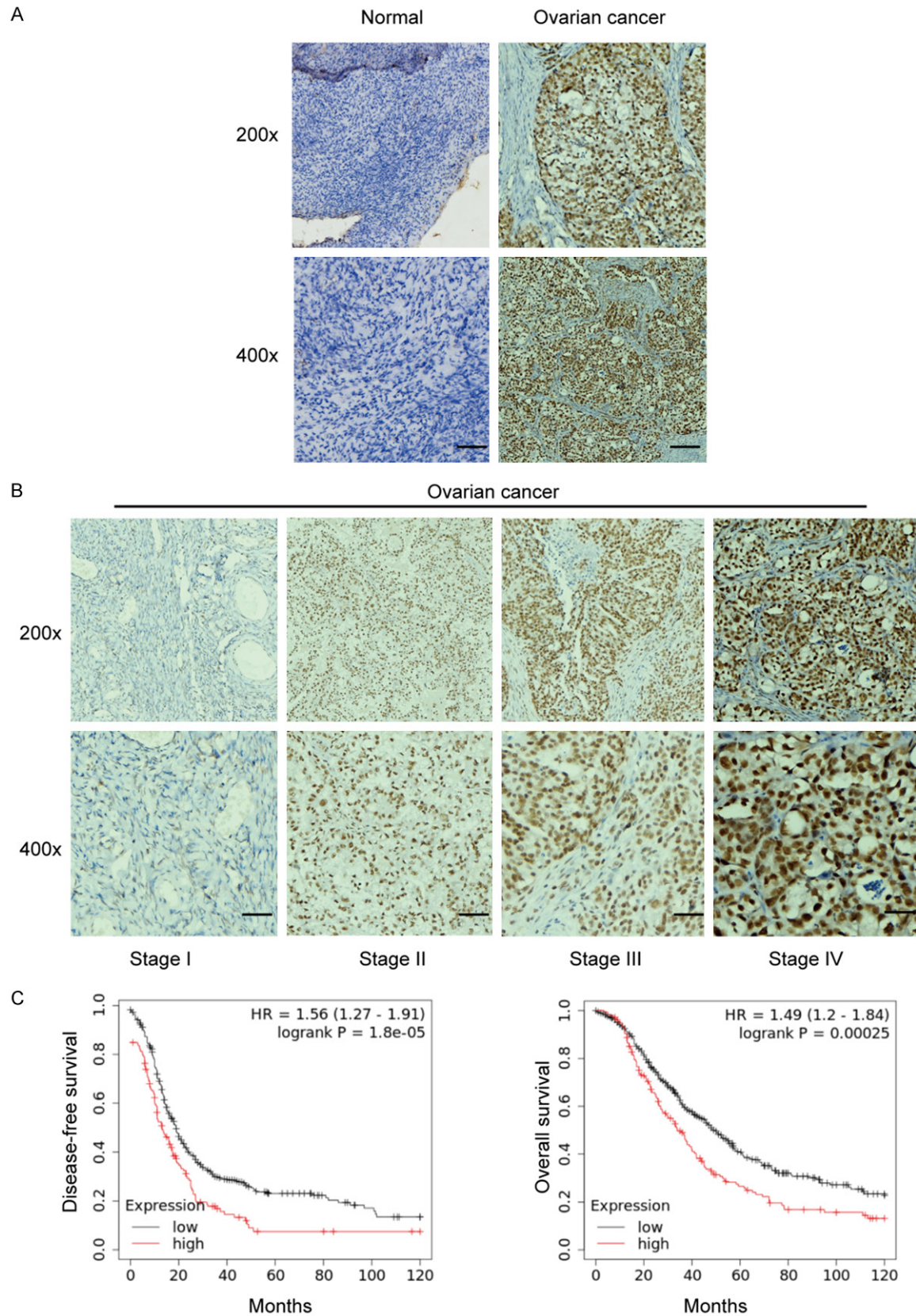


Figure 2. High-level expression of ALKBH5 is strongly associated with advanced clinical stage and lower survival rate in OC patients. A: Immunohistochemical assays of ALKBH5 staining in normal and OC tissues (Original magnifica-

tion, 200 ×, 400 ×). B: Representative images of ALKBH5 staining in different FIGO stages of OC tissues (Original magnification, 200 ×, 400 ×). C: KM plotter dataset analysis demonstrated a negative correlation between ALKBH5 expression and disease-free survival (DFS) and overall survival (OS) in OC patients.

Table 2. The correlation between ALKBH5 expression and the clinical and pathological characteristics of patients

Item		Low expression	High expression	Total	p-value
Age	> 55	50	60	110	0.75
	≤ 55	71	95	166	
	Total	121	155	276	
Histologic subgroups	Serous	45	98	143	0.03
	Clear cell	9	15	24	
	Endometrioid	17	25	42	
	Mucinous	30	37	67	
	Total	101	175	276	
FIGO stage	I	60	110	170	0.025
	II	15	26	41	
	III-IV	21	44	65	
	Total	96	180	276	
Lymph node metastasis	Negative	102	146	248	0.04
	Positive	7	21	28	
	Total	109	167	276	
Grade of serous	Low	19	37	56	0.037
	High	30	57	87	
	Total	49	94	143	

(SKOV-3-sh) or a control vector, Nc. Silencing was confirmed via western blotting (**Figure 3A**). To understand the relationship between ALKBH5 and OC cell growth, proliferation of cells transfected with Nc and sh-ALKBH5 was quantified; knockdown of ALKBH5 significantly diminished the growth rate of OC cells in vitro (**Figure 3B**). Studies using the transwell and the wound healing assay showed that the decrease of ALKBH5 expression significantly reduced the migration and invasion capacity of OC cells compared to the control group (**Figure 3C-E**).

In addition, we performed an in vivo investigation of how ALKBH5 affects proliferation. We inoculated SKOV-3-sh and Nc cells subcutaneously into nude mice and observed them for 6 weeks. Compared to the control group, SKOV-3-sh generated significantly fewer subcutaneous tumors (**Figure 3F**). The average tumor growth rate in SKOV-3-sh mice was much slower than in Nc mice (**Figure 3G**), and tumor weights in SKOV-3-sh animals were significantly smaller than in Nc mice (**Figure 3H**). Our

research showed that silencing ALKBH5 in OC cells decreased both tumor biological activity in vitro and carcinogenesis in vivo.

Demethylation upregulates the production of lncRNA RMRP by ALKBH5

We next examined the molecular mechanisms through which ALKBH5 exerts its biological function in ovarian cancer. We identified the lncRNA RMRP as a potential target of ALKBH5 using StarBase v2.0 screening. We initially observed RMRP expression in six OC cell lines, and interestingly, the level of RMRP expression in OC cell lines was commensurate with ALKBH5 expression (**Figure 4A**). m6A level of RMRP was then identified in numerous OC cells. Our findings show that RMRP m6A enrichment in OVCAR3 and SKOV-3 cells was lower than in ES-2 and OVCAR8 cells (**Figure 4B**). In both OVCAR3 and SKOV-3 cells, we found that ALKBH5 significantly enriched RMRP relative to IgG, METTL3, METTL14, WTAP, and FTO (**Figure 4C, 4D**). Then, using stable RNA interference, we downregulated ALKBH5 expression

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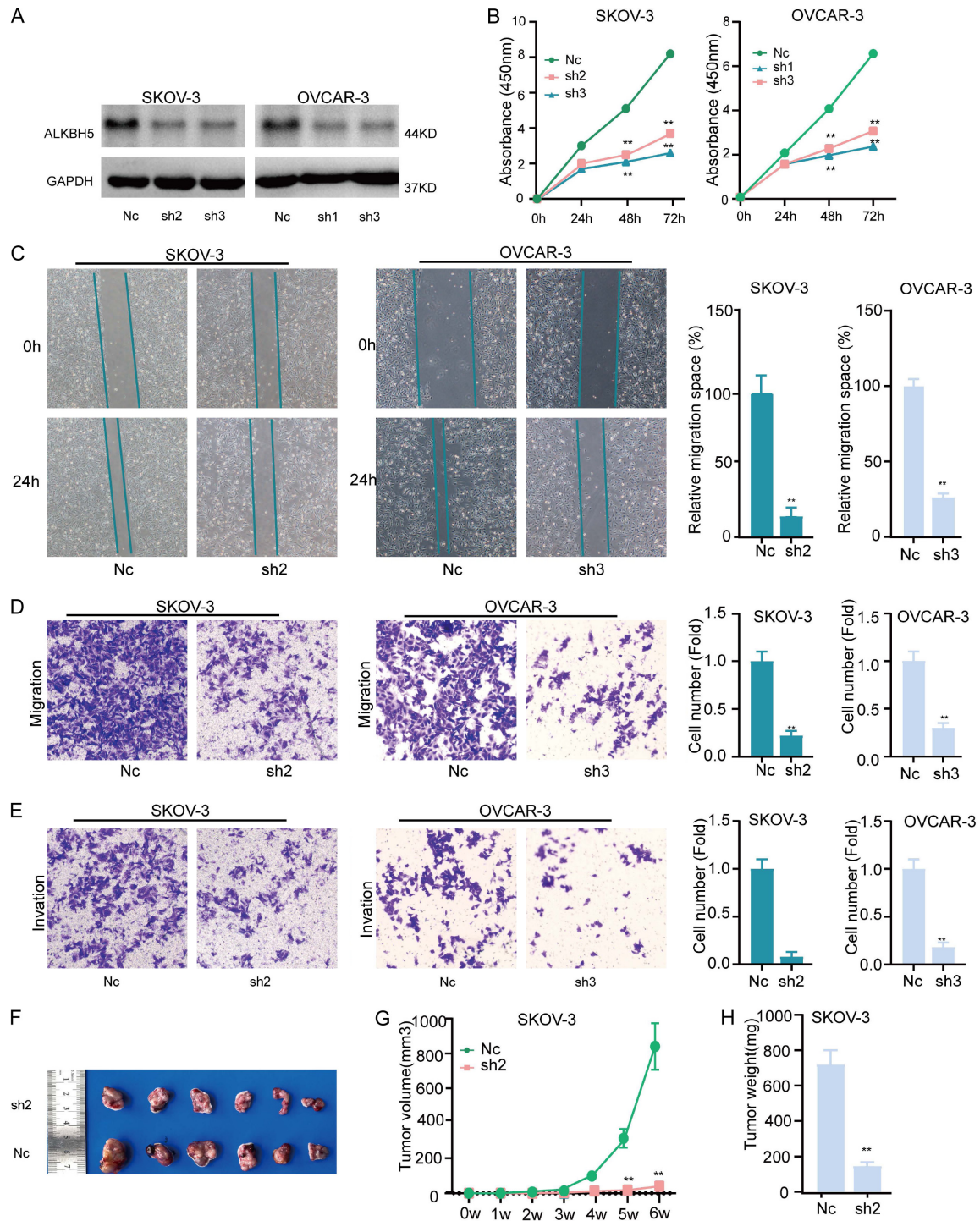


Figure 3. ALKBH5 silencing suppresses OC cell proliferation, migration, invasion in vitro, and tumor growth in vivo. (A) Western blot assay confirmed the efficiency of ALKBH5 knockdown in SKOV-3 and OVCAR-3 cells. (B) CCK8 assay measured cell growth in the Nc and sh-groups of OC cells at 0, 24, 48, and 72 h. The results showed that ALKBH5 knockdown significantly inhibited the proliferation of SKOV-3 and OVCAR-3 cells in vitro ($P < 0.01$). (C) Representative wound healing images of SKOV-3 and OVCAR-3 cells at 0 and 24 hours, respectively, with statistical analysis of relative migration distance displayed on the right. (D) Representative images of migration assays of ALKBH5 silenced and Nc cells, with the count displayed on the right. (E) Representative images of invasion assays conducted with ALKBH5 silenced and Nc cells, with statistical results displayed on the right (Original magnification, 200 ×). Cell numbers were determined by counting in three arbitrary regions. Data are means \pm SD ($*P < 0.05$, $**P < 0.01$). (F) Representative images of tumor tissues in NC and sh2-ALKBH5 group (n = 6). (G) Tumor growth chart after 6 weeks of inoculation with Nc and ALKBH5 knockdown cells (n = 6). (H) Tumor weights of Nc and SKOV3-sh groups from (G), n = 6. Data are presented as mean \pm SD ($*P < 0.05$, $**P < 0.01$).

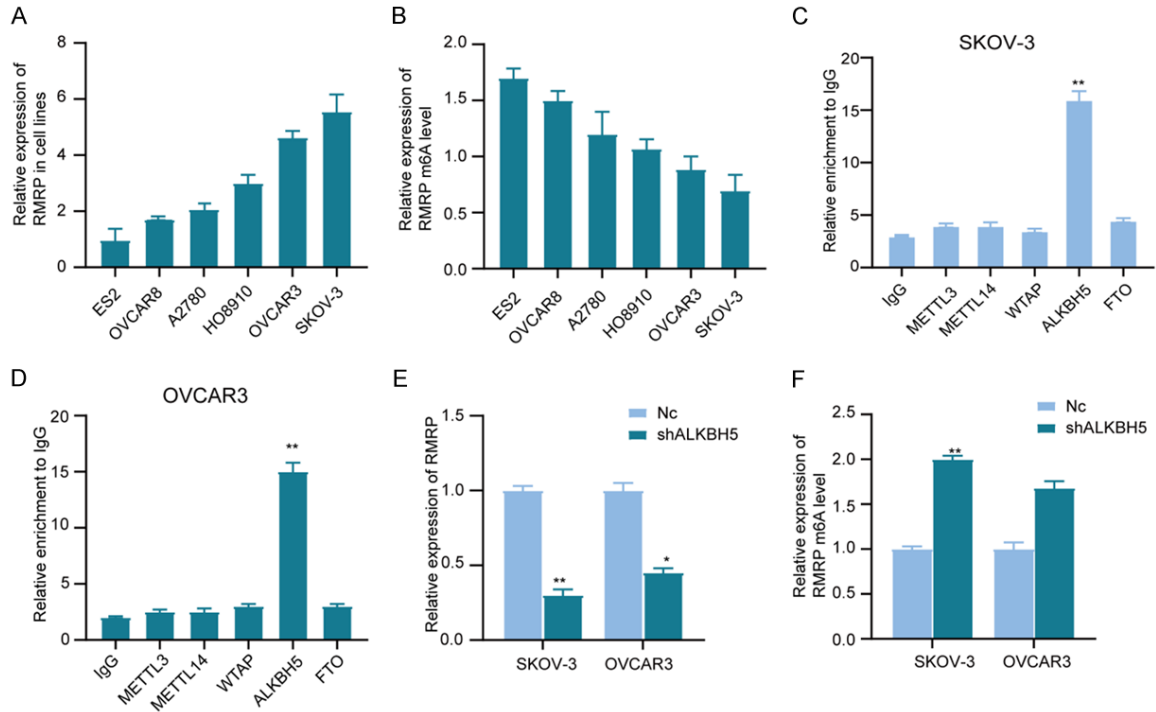


Figure 4. ALKBH5 modulates RMRP expression through a demethylation mechanism. A: RT-PCR was used to detect RMRP expression in 6 human OC cell lines (ES-2, OVCAR8, A2780, HO8910, OVCAR3, SKOV-3) respectively. B: RMRP m6A enrichment was determined in ES-2, OVCAR8, A2780, HO8910, OVCAR3, SKOV-3 respectively; m6A enrichment level was negatively correlated with RMRP expression level. C, D: ALKBH5 significantly upregulated RMRP in OVCAR3 and SKOV-3 cells compared to IgG, METTL3, METTL14, WTAP, and FTO. E: RMRP expression was significantly downregulated in OVCAR3 and SKOV-3 cells following ALKBH5 knockdown. F: ALKBH5 knockdown significantly increased RMRP m6A enrichment in both OVCAR3 and SKOV-3 cells. Data are presented as mean \pm SD (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

in OVCAR3 and SKOV-3 cells, resulting in decreased RMRP expression and higher RMRP m6A enrichment in the corresponding cells (Figure 4E, 4F).

Silencing of RMRP inhibits OC proliferation

To better understand the biological role of RMRP in ovarian cancer, we examined its expression in 10 pairs of paired ovarian cancer tissues. The results suggested that RMRP was more highly expressed in tumor tissues than in adjacent normal tissues, coinciding with the extent of ALKBH5 expression in ovarian tissues (Figure 5A). Furthermore, a positive correlation was found between ALKBH5 and RMRP expression in OC tissues using Q-PCR (Figure 5B). In OVCAR3 and SKOV-3 cells, we further investigated the subcellular location of RMRP. As previously described, the amount of RMRP in the nucleus was significantly greater than in the cytoplasm (Figure 5C, 5D). Moreover, the CCK-8 assay revealed that si-RMRP significant-

ly reduced OVCAR3 and SKOV-3 cell proliferation (Figure 5E, 5F).

RMRP can in part rescues the proliferative and invasive effects affected by ALKBH5 silencing

In the above research, we individually investigated the biological roles of ALKBH5 and RMRP in OC. Finally, we examined the involvement of the ALKBH5-RMRP axis in OC biological activity in vitro. We overexpressed RMRP to see whether ALKBH5's biological functions are mediated by RMRP. Through the CCK8 assays, we discovered that RMRP significantly improved the proliferative potential of ALKBH5-silenced OVCAR3 and SKOV-3 cells (Figure 6A, 6B).

Secondly, a transwell experiment revealed that overexpression of RMRP could partially restore the invasion capacity of cancer cells caused by ALKBH5 knockdown (Figure 6C), with statistics shown in Figure 6C. These findings demonstrated that knocking down ALKBH5 partly reduced OC malignant activity through RMRP.

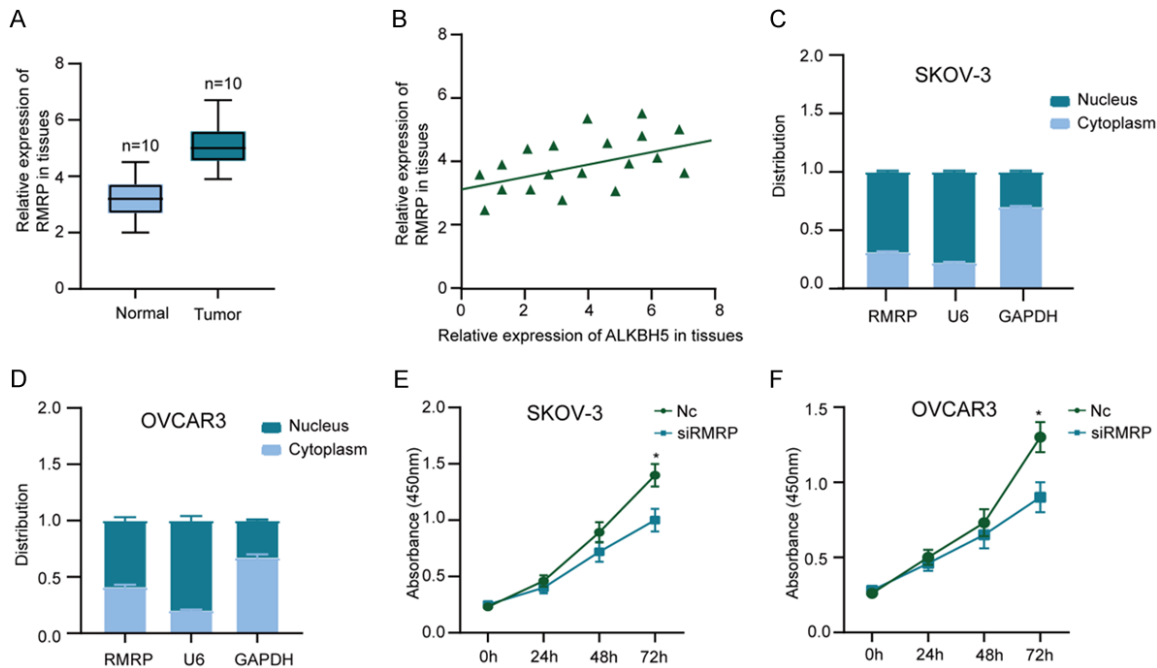


Figure 5. RMRP silencing inhibits OC cell proliferation. A: qRT-PCR assay revealed elevated RMRP expression in 10 paired OC tissues compared to adjacent normal tissues. B: The correlation between ALKBH5 and RMRP expression in OC tissues was measured determined by qPCR. C, D: In both OVCAR3 and SKOV-3 cells, RMRP was predominantly located in the nucleus compared to the cytoplasm. E, F: CCK8 assay assessed cell proliferation of Nc and si-RMRP in OVCAR3 and SKOV-3 cells at 0, 24, 48, and 72 hours. The results demonstrated that RMRP knockdown significantly suppressed *in vitro* proliferation of OVCAR3 and SKOV-3 cells. Data are presented as mean \pm SD (* P < 0.05, ** P < 0.01, and *** P < 0.001).

Discussion

Ovarian cancer (OC) is one of the three primary gynecological cancers in women, characterized by a high fatality rate and significant incidence [19, 20]. Numerous molecular alterations contributing to OC carcinogenesis have been identified to date. Hypoxia, a significant factor in the tumor microenvironment, induces changes in the expression of various tumorigenic genes and tumor suppressors, thereby accelerating tumor progression [21]. In the hypoxic microenvironment of OC, we found that anoxic conditions significantly increased the expression of the m6A demethylase ALKBH5.

RNA m6A modification has emerged as a new pattern of epigenetic regulation, alongside DNA histone modifications [22]. The dynamic m6A modification, regulated by m6A methyltransferases and demethylases, participates in numerous biological processes such as protein translation, alternative splicing of RNA, and stem cell pluripotency regulation [23, 24]. Given its critical biological functions, the role of m6A modifi-

cation in human malignancies, including OC, is under extensive investigation. ALKBH5, an m6A eraser protein, selectively removes m6A from target mRNAs and has been implicated in the progression of various cancers. For example, in breast cancer, hypoxia-induced upregulation of ALKBH5 has been shown to demethylate NANOG mRNA, leading to enhanced NANOG expression and the formation of a breast cancer stem cell phenotype [25]. Similarly, ALKBH5 has been found to be highly expressed in glioblastoma stem cells (GSCs), and its knockdown attenuated the proliferative capacity of GSCs [26]. In OC, ALKBH5 has been shown to activate the NF- κ B pathway [14]. Consistent with these findings, our study demonstrated elevated ALKBH5 expression in OC tissues, which was associated with tumor stage and histological grade. Notably, patients with high ALKBH5 expression exhibited significantly shorter overall survival (OS) and progression-free survival (PFS) times, as evidenced by KM-plot dataset analyses. Additionally, increased ALKBH5 expression promoted OC cell proliferation and invasion. These findings revealed

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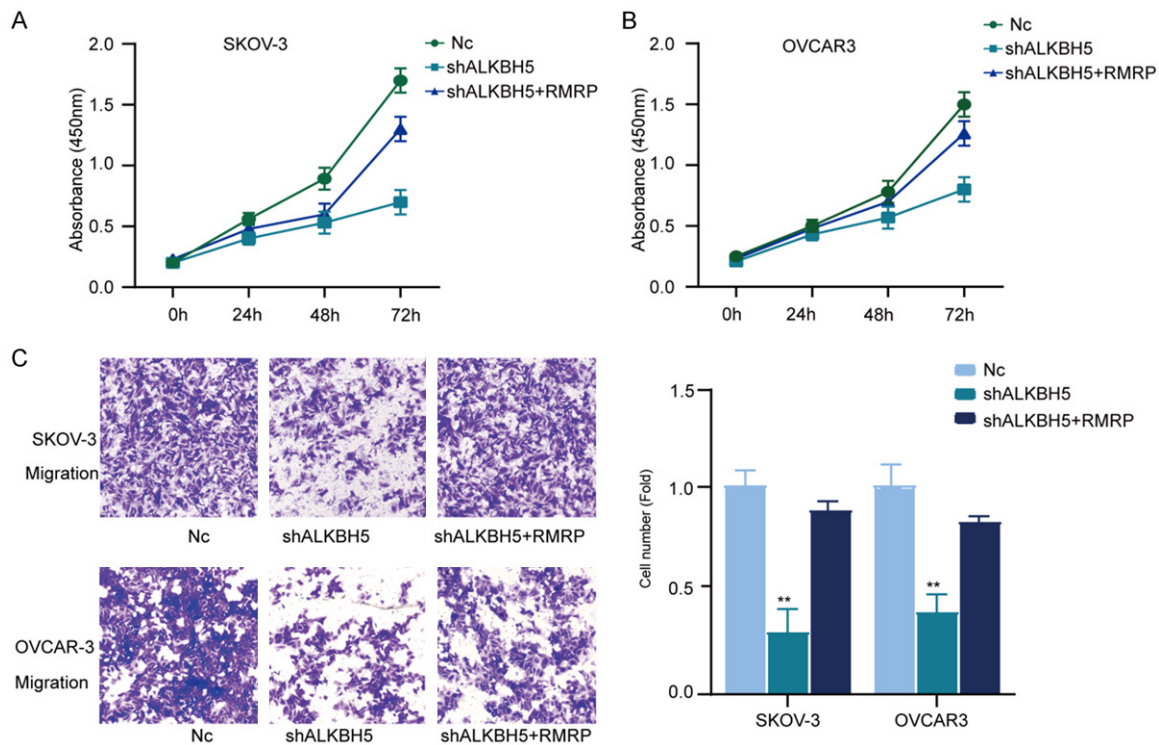


Figure 6. RMRP partially rescues the proliferative and invasive effects of ALKBH5 silencing. A, B: CCK8 assays revealed that RMRP overexpression partially restored the proliferative ability of OVCAR3 and SKOV-3 cells transfected with shALKBH5. C: Transwell assays demonstrate that overexpression of RMRP partially rescued the inhibitory effect on the invasiveness of OVCAR3 and SKOV-3 cells transfected with shALKBH5. Cell numbers were counted in three randomly selected fields and displayed on the right. Data are presented as mean \pm SD (* P < 0.05, ** P < 0.01, and *** P < 0.001).

that high levels of ALKBH could enhance the aggressive nature and proliferative capacity of cancer cells, suggesting that ALKBH5 may serve as a potential therapeutic target in OC.

The most common methylation alteration observed in eukaryotes is the m6A methylation of messenger RNA (mRNA). As the most prominent demethylase, ALKBH5 can demethylate m6A mRNA, affecting mRNA export and stability [27]. Among the diverse post-transcriptional modification targets, long non-coding RNA (lncRNA) is a significant target of m6A modification modulated by ALKBH5. The role of lncRNA methylation in tumor formation has garnered increasing attention in recent years. For example, ALKBH5 has been shown to increase the invasion and metastasis of gastric cancer by reducing the methylation of lncRNA NEAT1 [28]. Similarly, ALKBH5 has been shown to promote colon cancer progression by reducing the methylation of lncRNA NEAT1 [11]. Another study found that ALKBH5 reduces lncRNA-KCNK15-AS1 methylation, inhibiting pancreatic cancer

motility [29]. However, such studies are rarely reported in OC.

By analyzing OC samples with the StarBase v2.0 software, we identified for the first time that the lncRNA RMRP might be a viable target biomarker for the ALKBH5 gene. We found that silencing ALKBH5 increased RMRP m6A enrichment and decreased RMRP expression in OC, similar to previous findings in lung cancer [30]. RMRP has also been implicated in the regulation of cancer progression, particularly OC. A recent study found that RMRP was significantly expressed in OC and associated with OC paclitaxel sensitivity [31]. In our study, RMRP knock-down slowed the growth of OC in vitro, and ALKBH5 silencing was shown to have a proliferation and invasion effect, which was partially reversed by RMRP inhibition.

In conclusion, we found that suppression of ALKBH5 suppressed OC progression by modulating RMRP expression through demethylation. Furthermore, ALKBH5 is increased in OC tis-

sues under hypoxic conditions. These findings have led to the development of effective OC treatment strategies.

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Disclosure of conflict of interest

None.

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