

Original Article

m(6)A demethylase ALKBH5 inhibits cell proliferation and the metastasis of colorectal cancer by regulating the FOXO3/miR-21/SPRY2 axis

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Received May 10, 2021; Accepted August 2, 2021; Epub October 15, 2021; Published October 30, 2021

Abstract: Objective: Colorectal cancer is a common malignancy worldwide. This research aimed to investigate the role of α -ketoglutarate-dependent dioxygenase alkB homologue 5 (ALKBH5), a N⁶-methyladenosine (m(6)A) demethylase, on the cell proliferation and metastasis of colorectal cancer. Methods: The interaction relationship between FOXO3, miR-21, and SPRY2 were predicted by starBase 2.0 and determined using RIP, CHIP, and dual-luciferase reporter assays. Quantitative reverse transcription PCR (RT-qPCR) and western blot were used to measure the gene and miRNA expressions of ALKBH5, FOXO3, miR-21, and SPRY2. The cell proliferation was determined using CCK8 and colony formation assays. The metastatic abilities were measured using wound healing and transwell assays. Results: In colorectal cancer, downregulated ALKBH5 is related to poor prognosis. Rescued ALKBH5 suppresses the proliferation and metastasis of colorectal cancer cells. The role of ALKBH5 is achieved by reducing the m(6)A modification of forkhead box O3 (FOXO3), which enhances its stability. FOXO3 targets miR-21 and increases the SPRY2 expressions. The antitumor effects of ALKBH5 can be blocked by FOXO3 knockdown, which is reversed by the miR-21 inhibitor. Conclusion: ALKBH5 plays an antitumor role in colorectal cancer by regulating the FOXO3/miR-21/SPRY2 axis, providing a new direction for colorectal cancer therapy.

Keywords: α -ketoglutarate-dependent dioxygenase alkB homologue 5, forkhead box O3, miR-21, N⁶-methyladenosine modification, colorectal cancer

Introduction

Epigenetic alterations, such as histone acetylation, DNA methylation and RNA methylation are involved in carcinogenesis and thus serve as clinical biomarkers for the diagnosis, prognosis, and treatment of cancer [1]. N⁶-methyladenosine (m(6)A) is a common modification existing in mRNA molecules [2]. The stability, location, structure, and alternative polyadenylation and splicing of mRNA can be regulated by mRNA m(6)A modification [3]. α -ketoglutarate-dependent dioxygenase alkB homologue 5 (ALKBH5), a m(6)A methyltransferases which belongs to the AlkB family, is involved in the installation of m(6)A. By regulating the mRNAs of certain oncogenes, ALKBH5 plays important roles in cancer [4]. For example, ALKBH5 promotes the growth of renal carcinoma cells by regulating the expression of AURKB and plays

an antitumor role in pancreatic cancer by activating PER1 post-transcriptionally [5]. On the other hand, the non-coding mRNA expression is also controlled by ALKBH5 [6]. Therefore, ALKBH5 is regarded as an independent prognostic indicator in cancer patients [7].

Colorectal cancer is a common malignancy with a high mortality worldwide. Distant metastasis is the major cause of poor prognoses in colorectal cancer [8]. To date, an effective treatment strategy for colorectal cancer, especially metastatic colorectal cancer, is still lacking [9]. Forkhead box O3 (FOXO3) is a member of the FOXO family which play crucial roles in various types of cancer by regulating cell proliferation, metabolism, apoptosis, and metastasis. Among them, FOXO3 is generally considered a tumor suppressor and is associated with better outcomes and prognoses [9]. In colorectal cancer,

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Table 1. The sequences of the reagents used in the cell transfection

	Sequence (5'-3')
miR-21 mimics	UAGCUUAUCAGACUGAUGUUGA
mimics NC	UCGCUUGGUGCAGGUCGGGAA
miR-21 inhibitor	UCAACAUCAGUCUGAUAGCUA
inhibitor NC	CAGUACUUUUGUGUAGUACAA
FOXO3 shRNA	ATGGACAATAGCAACAAGTATAC
SPRY2 shRNA	CACACTTATTTTATTTTGGAAAG
FOXO3-WT	CGCCCACTGGGGACTCATGCAGCGG
FOXO3-MUT	CGCCCACTGGGGCCTCATGCAGCGG
MiR-21-WT	UAGCUUAUCAGACUGAUGUUGA
MiR-21-MUT	UUCGAAUACAGACUGAUGUUGA
SPRY2-WT	GACCCAUGUAUUGCAUAAGCUA
SPRY2-MUT	GACCCAUGUAUUGCUAUUCGAA

FOXO3 can reverse the drug resistance to 5-fluorouracil, and increased expressions of FOXO3 attenuate cell proliferation [10]. Also, the enhancement of FOXO3 represses the migratory and invasive capacities of colorectal cancer cells [11].

The transcriptional expression of FOXO3 is regulated by many factors, such as phosphorylation, acetylation, methylation, and microRNAs (miRNAs) [12]. More than 60% of RNA modification belongs to m(6)A which is a conservative post-translational modification. Proteins with the removal and recognition abilities of m6A can be divided into three categories: writers, erasers, and readers, respectively [13]. “Writers” is the m(6)A methyltransferase complex, including methyltransferase-like 3, methyltransferase-like 14 and Wilms tumor 1-binding protein. “Erasers”, the demethylases, are composed of fat mass, the obesity-associated protein, and ALKBH5. The “readers” category is the m(6)A binding proteins, including the YTH domain protein family, the nuclear heterogeneous protein HNRNP family, and the IGF2BP protein family [14]. However, the biological relationship between ALKBH5 and FOXO3 in colorectal cancer is not clear.

Previous studies indicated that m(6)A modification stabilizes FOXO3 in liver cancer, luteinized granulosa cells, and the inflammatory response [15-17]. Although the understanding of m(6)A in colorectal cancer is very limited, increasing evidence shows the potential role of m6A in the regulation of colorectal cancer [18]. In addition,

m(6)A-modified non-coding RNAs have been reported in the progression of colorectal cancer [19, 20]. Here, we investigated the modulatory role of ALKBH5 in colorectal cancer and its miRNA/FOXO3-related mechanisms, in order to determine whether ALKBH5 can serve as a candidate target for colorectal cancer therapy.

Materials and methods

Cell lines and clinical tissues

Colorectal cancer cell lines (HCT-116, Caco-2, SW480, and SW620) and normal human fetal colonic mucosa cells (FHC; American Type Culture Collection, ATCC) were incubated with DMEM medium (Gibco, Grand Island, USA) containing 11% fetal bovine serum (FBS; Gibco) at 37°C with 5% CO₂. The clinical specimens of colorectal cancer tissues and the adjacent normal tissues, which were patient-matched, were derived from untreated, newly-diagnosed patients (n=36). This study was approved by the ethics committee of our hospital. Written informed consent forms were obtained from each subject prior to participation.

Cell transfection

For the ALKBH5 and FOXO3 overexpressions, ALKBH5 pcDNA3.1 and FOXO3 pcDNA3.1 were used, and pcDNA3.1 NC was used as the control. For the FOXO3 and SPRY2 knockdown, FOXO3 shRNA and SPRY2 shRNA were used. For the overexpressions or knockdowns of miR-21, the miR-21 mimics, the miR-21 inhibitor, and mimics NC, inhibitor NC were obtained from GenePharma Company (Shanghai, China). The sequences of the above plasmids are shown in **Table 1**. The cells (2~5×10⁴/mL) in a 12 well plate were cultured with FBS-free DMEM medium. The transfection solution was obtained by mixing Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, USA) with FBS-free DMEM medium carefully, and then we added the specific transfection vector. After mixing and standing for 20 min, the transfection solution was added into a 12 well plate. After 48 h, the cells were used for the following experiments.

CCK8 assay

A CCK-8 assay was used to measure the cell viability. The cells (50,000/mL) were diluted

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and then seeded in a 96 well plate. 10 μ L CCK-8 solution (Dojindo, Shanghai, China) were added to each well for 1-3 h reaction. The optical density (OD) value was determined using a Microtitre plate reader (BioTek, Winooski, VT, USA) at 450 nm.

Colony formation assay

The cells were digested with 0.25% trypsin (Gibco), and inoculated into a culture plate for 2-3 weeks. After the colony formation could be observed visually, paraformaldehyde (4%, Sigma-Aldrich) was used to fix the cells for 15 min. The crystalline violet solution (0.1%, Abcam) was then applied for 10~30 min. After washing the extra dye solution and drying, the petri dishes were inverted and covered with a transparent grid. The results were visualized under general microscopy (Leica, Wetzlar, Germany) and the number of clones was counted.

Chromatin immunoprecipitation assay (ChIP)

The interaction between FOXO3 and miR-21 was determined using ChIP assays. The ChIP assays were performed using EpiQuik Chromatin Immunoprecipitation kits (Epigentek Group, USA) following the manufacturer's instructions. Cells at a concentration of 2×10^6 cells/mL were used for the experiment. After adding formaldehyde, the cell suspension was rotated gently for 10 min. Then glycine was incubated for a 5 min crosslink. After centrifuging, the pellets were resuspended in a nuclear lysis buffer containing a protease and phosphatase inhibitor cocktail. The sheared chromatin was obtained using a probe sonicator and then we proceeded to the immunoprecipitation. The samples with FOXO3 primary antibodies were rotated overnight and the dynabeads were incubated for 2 h. After the elution and purification, the samples proceeded with the ChIP qPCR.

Bioinformatics analysis

starBase 2.0 (<http://starbase.sysu.edu.cn>) was used to predict the binding sites among FOXO3, miRNA-21, and SPRY2. The RMVar database (<http://rmvar.renlab.org/>) was used to predict the m(6)A modification of FOXO3.

Dual-luciferase reporter assay

To examine whether ALKBH5 binds to FOXO3, FOXO3 binds to miR-21, or miR-21 binds to SPRY2 directly, dual-luciferase reporter assays were used. FOXO3-WT, and FOXO3-MUT, miR-21-WT and miR-21-MUT, SPRY2-WT and SPRY2-MUT reporter vectors were purchased from Promega (Madison, WI, USA). The assays were described previously [21]. The cells were cultured in 96-well plates until the cell confluence reached 50-70%. Using Lipofectamine 2000 (Invitrogen), the cells were co-transfected with ALKBH5 pcDNA3.1, FOXO3 pcDNA3.1, or an miRNA-21 mimic. Following 48 h of post-transfection, the luciferase activity was estimated using the Dual-Luciferase Reporter Assay System (Promega).

m6A immunoprecipitation-qPCR (MeRIP-qPCR) analysis

The cells were harvested and then centrifugated at 1,500 rpm at 4°C for 5 min. After we removed the supernatant, the cells were mixed with RIP lysis buffer and incubated with the lysate. The m6A antibody (5 mg) containing magnetic beads was rotated for 30 min. The beads were eluted using a RIP buffer twice. After rotation overnight, the beads were washed using a high-salt buffer. The RNA enrichment was determined using qRT-PCR.

Wound healing assay

Cells (6×10^5 /mL) were cultured in a 6-well plate for 12 h. Then, a 10 μ L pipette tip was used to make a scratch on the surface. After 48 h incubation, the cells were washed with PBS, and pictures were taken immediately using an inverted microscope (Leica, Wetzlar, Germany) and then analyzed using ImageJ software (NIH, Bethesda, Maryland, USA) to calculate the relative distance according to the equation $(W_t - W_0)/W_0 \times 100\%$.

Transwell assay

Cells (2×10^5 /mL) were suspended in the upper chamber precoated with Matrigel (BD Biosciences, USA). After the cells passed through the membrane, paraformaldehyde (4%, Sigma-Aldrich, St Louis, MS, USA) was used to fix the cells on the lower side of the chamber and crystal violet (0.1%, Sigma-Aldrich, St Louis, MO,

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Table 2. The primer sequences

	Forward	Reverse
GAPDH	5'-TCCGTGGTCCACGAGAACT-3'	5'-GAAGCATTGCGGTGGACGAT-3'
FOXO3	5'-AACCGCTCCTCAACAGTA-3'	5'-GAAGCAAGCAGGTCTTGA-3'
miR-21	5'-ATGGGCTGTCTGACATTTGGTA-3'	5'-CATTGGATATGGATGGTCAGATGA-3'
ALKBH5	5'-GCTTCAGGGTATGGGAGTTG-3'	5'-TTCCAGGATCTGAGTGGATAGA-3'
SPRY2	5'-TTGCTCGGAAGTTGGTCTAA-3'	5'-TTCCAGGATCTGAGTGGATAGA-3'
U6	5'-CTCGCTTCGGCAGCAC-3'	5'-AACGCTCACGAATTTGCGT-3'

USA) was used. The results were counted in five fields randomly selected using a microscope (Leica, Wetzlar, Germany).

Quantitative reverse transcription PCR (RT-qPCR)

The RT-qPCR assays were performed according to a previous study [22]. For the cytoplasm/nuclei RNA extractions, a Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada) was used according to the kit's instructions [23]. In brief, after the RNA collection, reverse transcriptions of the RNA into cDNA were conducted using a reverse transcriptase kit (RevertAid cDNA Synthesis Kit, ThermoFisher Scientific). The primers we used are shown in **Table 2**. The PCR process was conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression levels of RNA normalized to GAPDH or U6 were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The protein extraction (RIPA buffer, ThermoFisher Scientific, Waltham, MA, USA), concentration determination (BCA protein assay kit, ThermoFisher Scientific), separation and transferring to a nitrocellulose filter membrane (Millipore, Boston, USA), incubation with BSA and antibodies were described previously [24]. The primary antibodies, including ALKBH5 (1:1000), FOXO3 (1:2000), SPRY2 (1:1200), and β -actin (1:5000), were obtained from Santa Cruz (USA). The secondary antibodies were obtained from proteintech (USA). The protein expressions were measured using the ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

SPSS 18.0 software was used for the statistical analyses, and the data are presented as the

mean \pm standard deviation ($\bar{x} \pm SD$) from at least three independent experiments. The results were analyzed statistically using analyses of variance and post hoc Student-Newman-Keuls tests at a significance level of $P < 0.05$.

Results

The ALKBH5 expression was downregulated in the colorectal cancer tissues and cell lines

Previous studies have shown that m(6)A demethylase ALKBH5 is associated with poor clinical outcomes in colorectal cancer [25]. Here, we aimed to investigate the effects and potential mechanism of ALKBH5 in colorectal cancer. First, the ALKBH5 expression was evaluated in the tumor tissues and the matched noncancerous tissues. As shown in **Figure 1A, 1B**, the ALKBH5 was inhibited at both the mRNA and protein levels in tumor tissues. Moreover, the expression of ALKBH5 was negatively associated with the stages of colorectal cancer (**Figure 1C**) and the survival rate (**Figure 1D**). The survival rate of the patients with lower ALKBH5 expressions was significantly worse than it was in the patients with higher ALKBH5 expressions. Similarly, both the mRNA and protein expressions of ALKBH5 were decreased in the colorectal cancer cell lines such as in the SW480, HCT-116, Caco-2, and SW620 cells, compared with the expressions in the FHC cells, a normal fetal colonic mucosa cell line (**Figure 1E, 1F**). In conclusion, ALKBH5 was reduced in colorectal cancer.

ALKBH5 suppressed cell proliferation and metastasis of colorectal cancer

In order to assess the effects of ALKBH5, SW480 and HCT-116 cells with low expressions of ALKBH5 were used to transfect ALKBH5 pcDNA3.1. After confirming the overexpression of the posttranscriptional and posttranslational ALKBH5 (**Figure 2A**), the cell proliferation and

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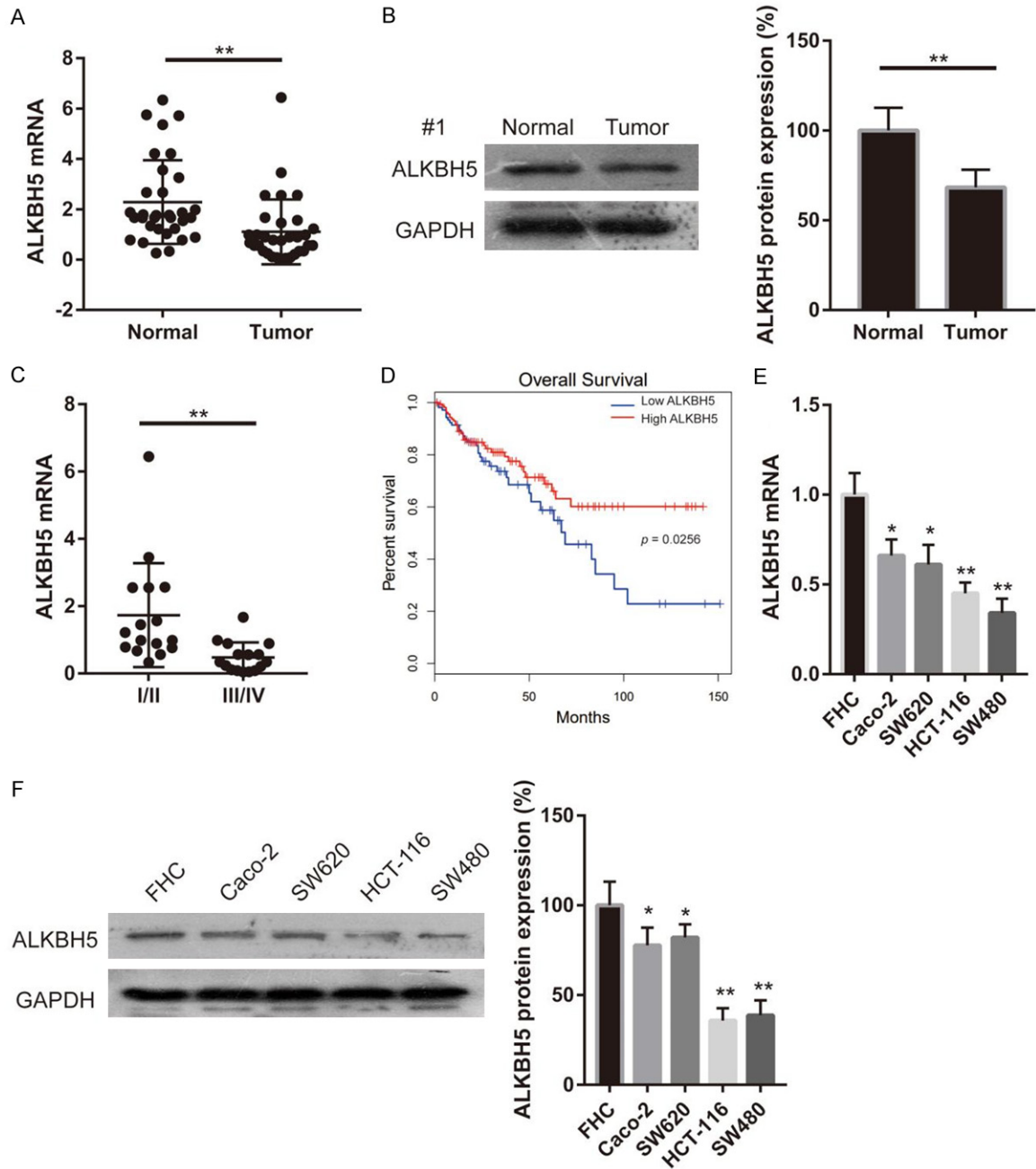


Figure 1. The expression levels of ALKBH5 in the clinical tissues and cell lines of colorectal cancer. A: The mRNA levels of ALKBH5 in the tumor tissues and adjacent normal tissues of colorectal cancer (n=36) were determined using RT-qPCR; B: The protein levels of ALKBH5 in the tumor tissues and adjacent normal tissues of colorectal cancer (n=36) were determined using western blot; C: The association between ALKBH5 and the stages of colorectal cancer (n=36); D: The Kaplan Meier plotter was applied to analyze the survival differences between patients with high and low expressions of ALKBH5 (n=36); E: The mRNA levels of ALKBH5 in the cell lines of colorectal cancer (HCT-116, Caco-2, SW480, and SW620) and the normal fetal colonic mucosa cell line (FHC) were determined using RT-qPCR (n=3); F: The protein levels of ALKBH5 in the cell lines of colorectal cancer (HCT-116, Caco-2, SW480, and SW620) and the normal fetal colonic mucosa cell line (FHC) were examined using western blot (n=3). The asterisk represents a significant difference: *P<0.05, **P<0.01. ALKBH5: α -ketoglutarate-dependent dioxygenase alkB homologue 5; mRNA: messenger RNA; RT-qPCR: quantitative reverse transcription PCR.

metastatic abilities were assessed. CCK-8 and colony formation assays indicated that overex-

pression of ALKBH5 suppressed the proliferation of the SW480 and HCT-116 cells (Figure

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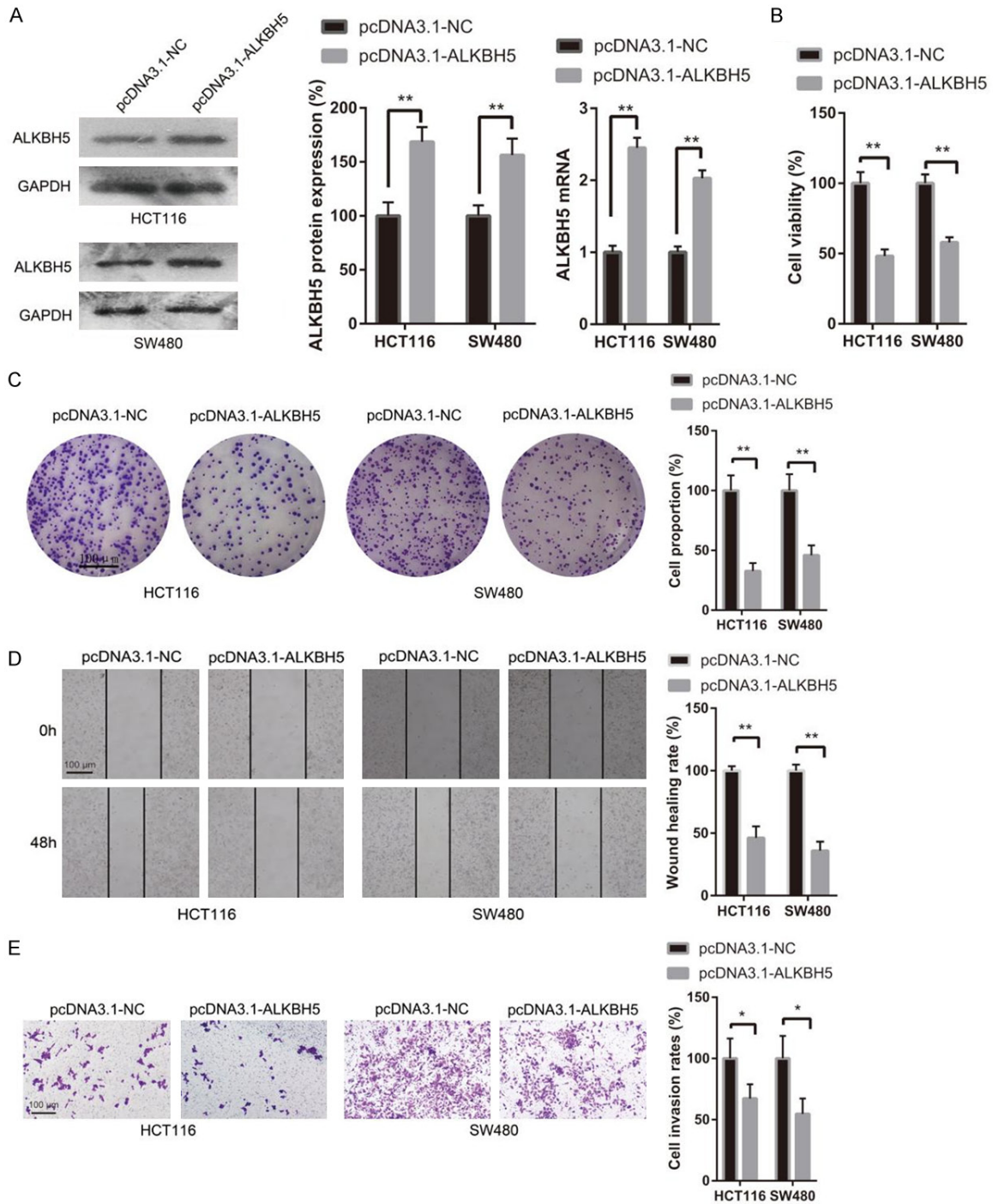


Figure 2. ALKBH5 suppresses the tumor progression of colorectal cancer. A: The expression of ALKBH5 in HCT-116 and SW480 cells with an overexpression of ALKBH5 was determined using western blot and RT-qPCR (n=3); B: Cell viability was determined using CCK8 assays in HCT-116 and SW480 cells with an overexpression of ALKBH5 (n=3); C: Cell proliferation was determined using colony formation assay in the HCT-116 and SW480 cells with an overexpression of ALKBH5 (scale bar: 50 μ m, n=3); D: Cell migration was determined using wound healing assays in the HCT-116 and SW480 cells with overexpressions of ALKBH5 (scale bar: 100 μ m, 200 \times , n=3); E: Cell invasion was determined using transwell assays in the HCT-116 and SW480 cells with an overexpression of ALKBH5 (Scale bar: 100 μ m, 200 \times , n=3). The asterisk represents a significant difference: *P<0.05, **P<0.01. ALKBH5: α -ketoglutarate-dependent dioxygenase alkB homologue 5; mRNA: messenger RNA.

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2B, 2C). In addition, SW480 and HCT-116 cells with enhanced ALKBH5 both showed reduced cell migration and invasion (**Figure 2D, 2E**). These results suggested that ALKBH5 plays an antitumor role in colorectal cancer.

ALKBH5 enhanced the stability of FOXO3 by inhibiting the m(6)A modification

Previous studies have shown that the mRNA stability of FOXO3 is regulated by the m(6)A modification [15, 17]. In agreement, we predicted that there was a m(6)A modification on FOXO3 mRNA using the RMVar database (**Figure 3A**). Furthermore, we found that the m(6)A modification level is more enhanced in colorectal cancer cells than it is in FHC cells (**Figure 3B**). Overexpression of ALKBH5 resulted in an obvious decreased m(6)A modification level in FOXO3 mRNA (**Figure 3C**). We then investigated the potential interaction between FOXO3 and ALKBH5. Overexpressing ALKBH5 distinctly increased the luciferase activity of the FOXO3-WT reporter, with no clear effects observed on the FOXO3-MUT reporter, verifying the binding property between ALKBH5 and FOXO3 (**Figure 3D**). In addition, the overexpression of ALKBH5 promoted the mRNA and protein levels of FOXO3 significantly (**Figure 3E, 3F**). Therefore, FOXO3 is a direct downstream target of ALKBH5.

FOXO3 reduced the expression of miR-21 by direct binding

It was predicted that FOXO3 had a binding site to miR-21 [26]. Using starBase 2.0, FOXO3 was predicted to bind to miR-21 (**Figure 4A**). A dual-luciferase activity reporter assay was conducted. As shown in **Figure 4B**, overexpressing FOXO3 distinctly reduced the luciferase activity of the miR-21-WT reporter, with no clear effects observed on the miR-21-MUT reporter, which verified the binding property between FOXO3 and miR-21. Similarly, a ChIP assay also proved that FOXO3 could interact with miR-21 directly (**Figure 4C**). Moreover, the overexpression of FOXO3 reduced the expression of miR-21 (**Figure 4D**). These results suggested that FOXO3 targets miR-21 directly and regulates its expression negatively.

SPRY2 is a direct target of miR-21

MiR-21 has been reported to be a biomarker in the prognosis of colorectal cancer. Therefore,

the downstream mechanism underlying the ALKBH5/FOXO3/miR-21 axis was then investigated [27, 28]. By using starBase 2.0, it was predicted that miR-21 could bind to Sprouty2 (SPRY2) (**Figure 5A**). A dual-luciferase activity reporter assay also proved that overexpressing miR-21 distinctly reduced the luciferase activity of the SPRY2-WT reporter, with no clear effects observed on the SPRY2-MUT reporter, verifying the binding property between miR-21 and SPRY2 (**Figure 5B**). In agreement, the mRNA and protein levels of SPRY2 were enhanced significantly in the HCT-116 and SW480 cells transfected with the miR-21 inhibitor (**Figure 5C, 5D**). These results proved that SPRY2 is downstream of miR-21 and was regulated by miR-21 negatively.

ALKBH5 inhibited the cell proliferation and metastasis via the FOXO3/miR-21/SPRY2 axis

We found that ALKBH5 suppresses the cell proliferation and migration of colorectal cancer cells (**Figure 1B-E**). Here, we aimed to study whether the antitumor effects of ALKBH5 are achieved by the FOXO3/miR-21/SPRY2 axis. To this end, HCT-116 and SW480 cells with ALKBH5 overexpression were transfected with FOXO3 shRNA, an miR-21 inhibitor, or SPRY2 shRNA. Then, the cell proliferation and metastatic were determined using CCK-8 and Colony formation assays. As shown in **Figure 6A, 6B**, the overexpression of ALKBH5 inhibited the cell proliferation, which could be reversed by the FOXO3 shRNA. Meanwhile, the knockdown of miR-21 blocked the combined effects of ALKBH5 pcDNA3.1 and FOXO3 shRNA, which could be reversed by SPRY2 shRNA. Thus, we confirmed that SPRY2 is the downstream effector of the ALKBH5/FOXO3/miR-21 pathway. Similarly, the cell metastasis, including migration and invasion, measured by the wound healing and Transwell assays displayed the same ALKBH5/FOXO3/miR-21/SPRY2 axis (**Figure 6C, 6D**). Taken together, ALKBH5 suppressed cell proliferation and metastasis in the HCT-116 and SW480 cells by modulating the FOXO3/miR-21/SPRY2 axis. A schematic diagram summarizing our research is shown in **Figure 7**. In our research, ALKBH5 suppressed the cell proliferation and metastasis in HCT-116 and SW480 cells by modulating the FOXO3/miR-21/SPRY2 axis. The detail mechanism was that ALKBH5, a m(6)A Demethylase, could target

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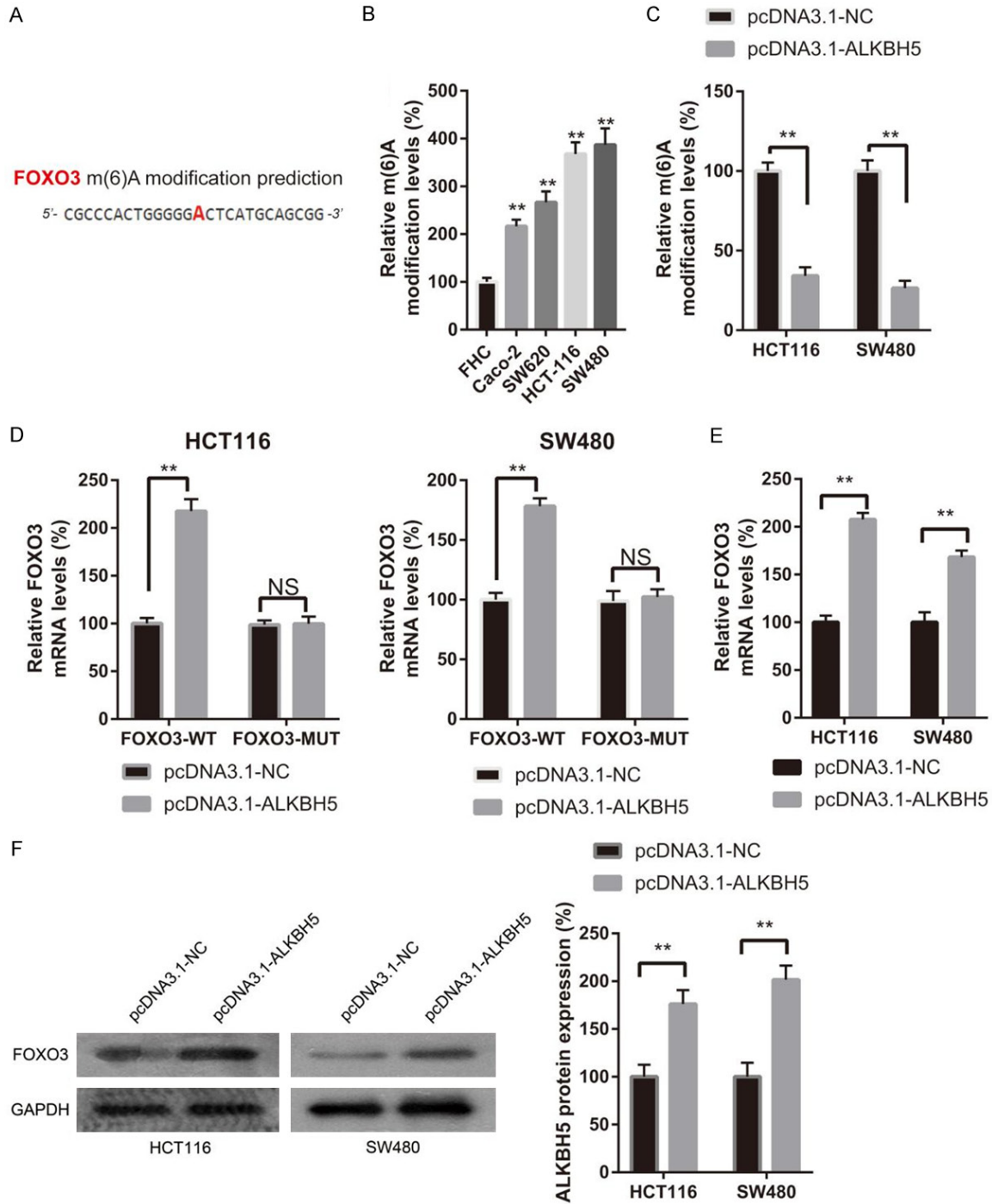


Figure 3. ALKBH5 enhances the stability of FOXO3 by inhibiting the m(6)A modification. A: The predicted m(6)A modification site on FOXO3 using RMVar; B: The m(6)A modification levels were measured using an MeRIP-qPCR analysis (n=3); C: The m(6)A modification levels were measured using an MeRIP-qPCR analysis in the HCT-116 and SW480 cells with an overexpression of ALKBH5 (n=3); D: The interaction between ALKBH5 and FOXO3 was assessed using dual-luciferase activity assays (n=3); E: The mRNA levels of FOXO3 were determined using RT-qPCR in the HCT-116 and SW480 cells with an overexpression of ALKBH5 (n=3); F: The protein levels of FOXO3 were determined using western blot in the HCT-116 and SW480 cells with an overexpression of ALKBH5 (n=3). The asterisk represents a significant difference: **P<0.01. ALKBH5: α -ketoglutarate-dependent dioxygenase alkB homologue 5; mRNA: messenger RNA; RT-qPCR: Quantitative reverse transcription PCR; FOXO3: The forkhead box O3.

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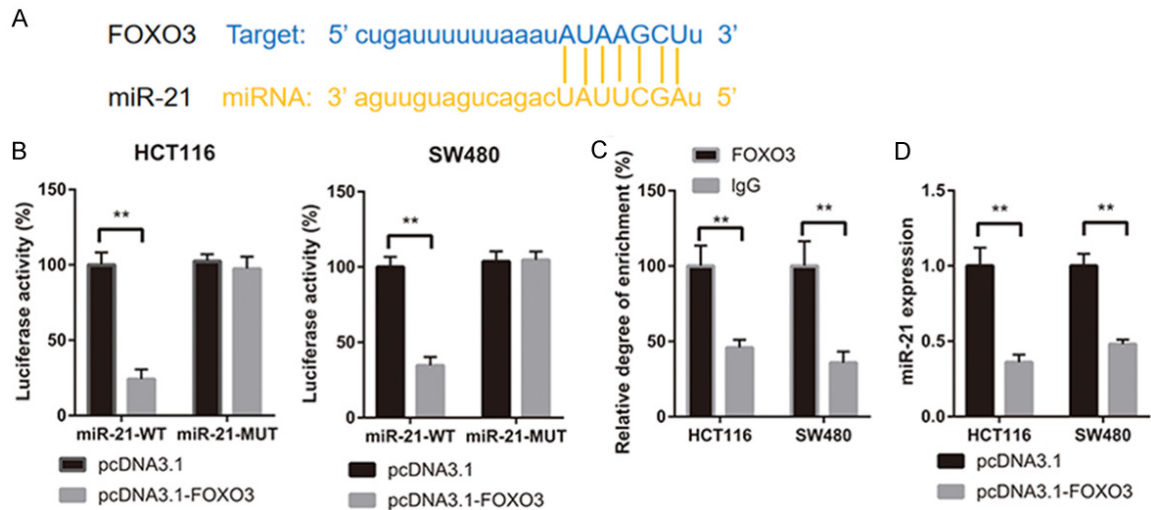


Figure 4. FOXO3 reduced the expression of miR-21 by direct binding. A: The prediction the binding site between FOXO3 and miR-21 using starBase 2.0; B: The interaction between FOXO3 and miR-21 was assessed using dual-luciferase activity assays (n=3); C: The interaction between FOXO3 and miR-21 was measured using ChIP assay (n=3); D: The expression of miR-21 was determined using RT-qPCR in HCT-116 and SW480 cells with an overexpression of FOXO3 (n=3). The asterisk represents a significant difference: **P<0.01. FOXO3: The forkhead box O3; ChIP: Chromatin immunoprecipitation assay.

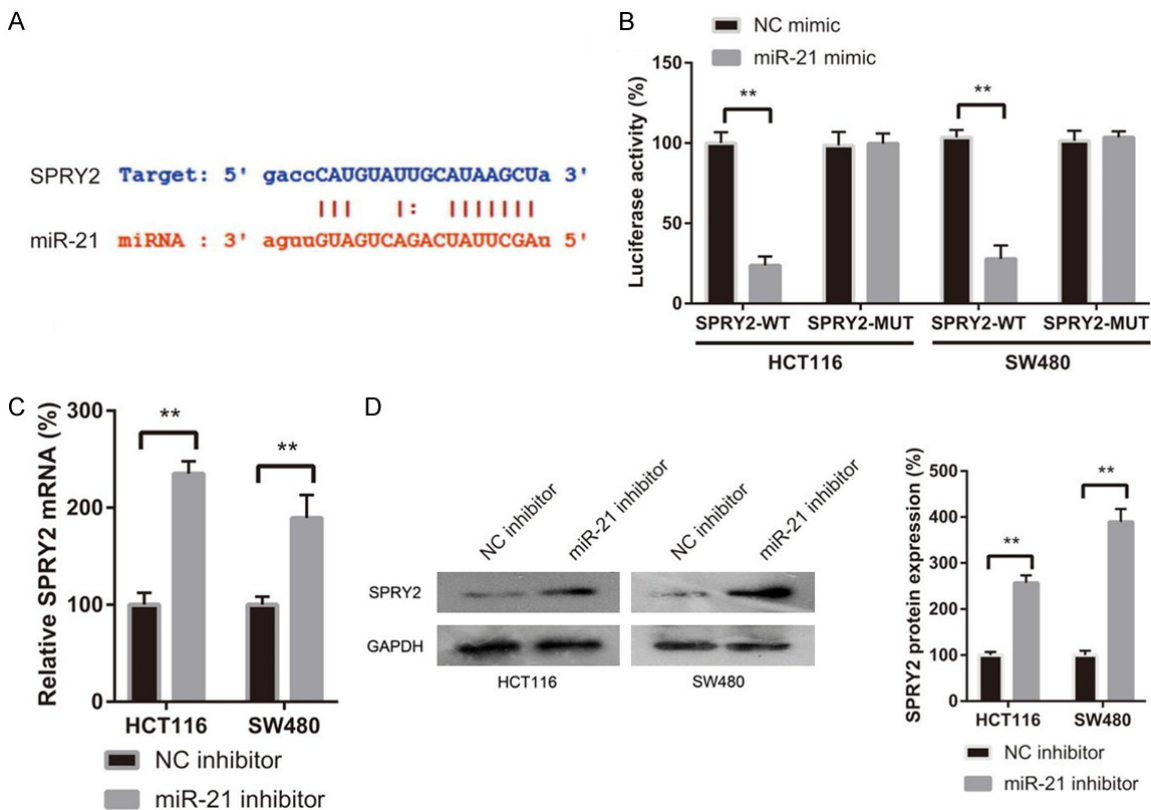
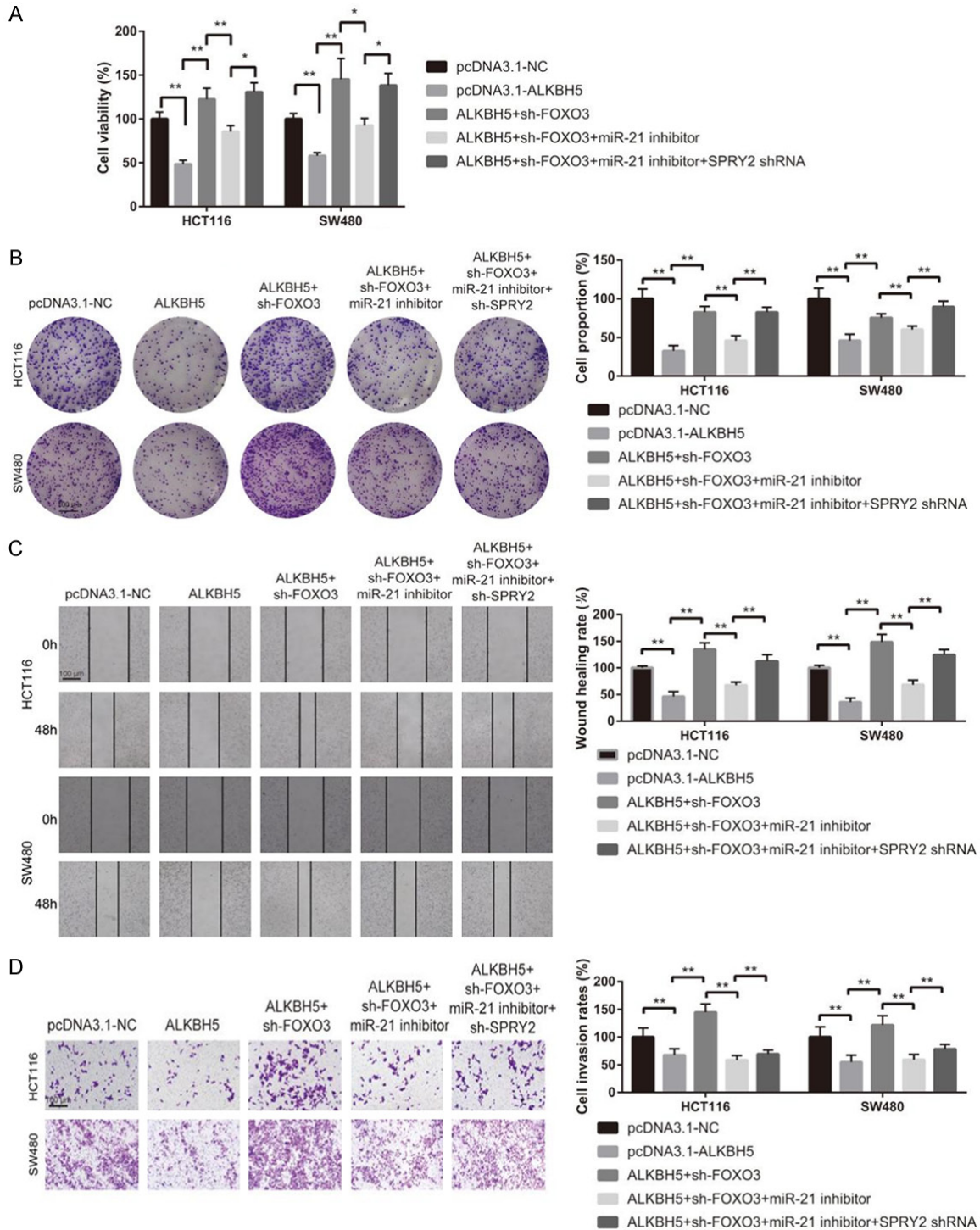


Figure 5. SPRY2 was the direct target of miR-21. A: The predicted binding site between miR-21 and SPRY2 using starBase 2.0; B: The interaction between miR-21 and SPRY2 was assessed using a dual-luciferase activity assay (n=3); C: The mRNA level of SPRY2 was determined using RT-qPCR in HCT-116 and SW480 cells transfected with an miR-21 inhibitor (n=3); D: The protein level of SPRY2 was determined using western blot in the HCT-116 and SW480 cells transfected with omiR-21 inhibitor (n=3). The asterisk represents a significant difference: **P<0.01. mRNA: messenger RNA.

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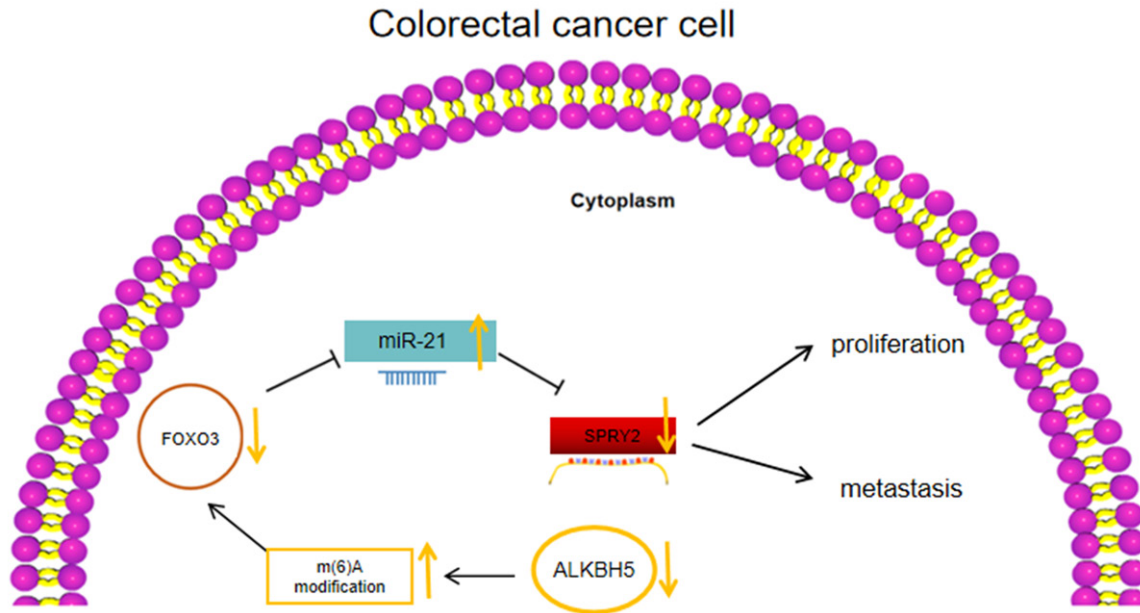


Figure 7. A schematic diagram of the mechanism of ALKBH5 via the FOXO3/miR-21/SPRY2 axis.

FOXO3, and FOXO3 negatively targeted miR-21 which negatively targeted SPRY2.

Discussion

More and more studies have demonstrated that m(6)A modification is involved in the progression of colorectal cancer [25]. Herein, we found that the expression of ALKBH5 was decreased in the tissues and cell lines of colorectal cancer, and is negatively related to the prognosis and the clinical stages. And ALKBH5 exerted an antitumor role by increasing the stability of FOXO3 by decreasing its m(6)A modification. What's more, FOXO3 can negatively regulate the expression of miR-21 by direct binding, and miR-21 can target SPRY2. Therefore, the mechanism of ALKBH5 in colorectal cancer may lie on the FOXO3/miR-21/SPRY2 axis.

More than 60% of RNA modification belongs to m(6)A which is a conservative post-translational modification. Methylases and demethylases are responsible for the dynamic and reversible m(6)A modification. As a demethylase, ALKBH5 is located in the cell nuclei and participates in the assembly/modification of mRNA processing, thus promoting m(6)A mRNA demethylate and regulating the hemostasis and stability of mRNAs [29]. Therefore, the aberrant expression of ALKBH5 may cause a turbulent cellular

process, for example, *Alkbh5*-deletion disturbs the balance of RNA m(6)A methylation and then induces the cerebellum abnormalities in mice [30]. What's more, decreased ALKBH5 levels are related to reduced cell viability in pancreatic cancer [6]. In colon cancer, ALKBH5 is negatively correlated with distant metastasis [31]. This evidence suggests the antitumor effects of ALKBH5 in colon cancer. However, there are also studies showing the oncogenic roles of ALKBH5. The overexpression of ALKBH5 promotes the invasive capability of glioma stem cells [32]. In acute myeloid leukemia, ALKBH5 promotes carcinogenesis and the self-renewal of stem cells by targeting TACC3 [33]. In our research, we proved that ALKBH5 is downregulated and related to poor prognosis in colorectal cancer, indicating an antitumor effect. The overexpression of ALKBH5 can inhibit cell proliferation and metastasis by decreasing the m(6)A modification level of FOXO3 mRNA, which is opposite of what occurs in glioma stem cells. Therefore, the roles of ALKBH5 may be dependent on the specific cell type. The limitation of our study is the absence of *in vivo* evidence from animal experiments. Our results from the *in vitro* cell experiments are still insufficient. In our next study, we will construct animal experimental model of colorectal cancer to prove the antitumor effect of ALKBH5.

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The effects of the tumor suppressor factors, such as FOXO3, have been reported [34]. The overexpression of FOXO3 inhibits the cell proliferation and invasion of cancer cells. However, the opposite effects of FOXO3 cannot be ruled out, as a previous study reported that the high expressions of FOXO3 are negatively related to poor clinical outcomes in patients with hepatocellular carcinoma [35]. Here we showed that the expressions of FOXO3 are upregulated after the overexpression of ALKBH5 and the knock-down of FOXO3 abrogates the inhibitory effects of ALKBH5 on cell proliferation and metastasis, which is consistently with the research on hepatocellular carcinoma. Also, we demonstrated that the effect of ALKBH5 on FOXO3 takes place by inhibiting m(6)A modification. Previous studies have indicated the regulation of m(6)A modification on FOXO3 [15-17], as well as FOXM1, an oncogenic factor, which belongs to the FOXO family [36].

We also investigated the potential downstream mechanisms of ALKBH5/FOXO3. Previous studies found that FOXO3 can induce cell apoptosis by targeting miR-21 in colon and breast cancer [37], for example, miR-21 promotes cell metastasis in gastric cancer [38]. Therefore, miR-21 is regarded as an oncogenic target for cancer therapy [39]. In our research, the effects of miR-21 were proved to be negatively associated with FOXO3. According our prediction and the result from the dual-luciferase activity assay, FOXO3 can target miR-21 which is related to SPRY2. It has been indicated that miR-21 correlates with SPRY2 [40]. The oncogenic role of miR-21 can be achieved by targeting SPRY2 which is reported to inhibit tumor progression by reducing the expressions of VEGF, p-ERK, and HIF-1 α [40]. Finally, we further verified the important role of the FOXO3/miR-21/SPRY2 axis in the antitumor effects of ALKBH5, but we still lack an understanding of its in-depth mechanism.

In conclusion, our research shows that ALKBH5 plays an antitumor role in colorectal cancer by modulating the FOXO3/miR-21/SPRY2 axis, which not only suggests a regulatory effect between hALKBH5 and FOXO3, but also provides a new therapeutic direction for colorectal cancer.

Acknowledgements

This work was supported by the Wenzhou Science and Technology Bureau Project (Y20190404).

Disclosure of conflict of interest

None.

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