miR-25-3p serves as an oncogenic in colorectal cancer cells by regulating the ubiquitin ligase FBXW7 function

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Abstract. Accumulating evidence indicates that the dysregulation of microRNAs (miRNAs or miRs), is associated with human malignancies and suggests a casual role of miRNAs in tumor initiation and progression. Even though it has been discovered that a number of miRNAs play significant parts in the development of colorectal cancer (CRC), it is crucial to comprehend the regulatory functions that other miRNAs play in CRC. Based on GSE183437 and GSE156719 microarray data that were obtained from Gene Expression Omnibus database, candidate miRNAs were researched. The oncogenic effects of miR-25-3p in different malignancies have led to its selection for additional investigation in the present study. The expression of miR-25-3p was verified by reverse transcription-quantitative PCR, and its correlation with clinicopathological characteristics in patients with CRC was then investigated. *In vitro* assays were conducted to investigate the influence of miR-25-3p on the proliferative and apoptotic behaviors of HCT116 and Caco-2 cells. The present data revealed that miR-25-3p exhibited one of the most significant upregulations in CRC tissues and cell lines. The expression levels of miR-25-3p were found to be intimately correlated with tumor size, distant metastasis, tumor-node-metastasis stage, and shorter overall survival rate. In terms of functionality, the downregulation of miR-25-3p led to the inhibition of cellular proliferation and the enhancement of apoptosis in both HCT116 and Caco-2 cell lines. The critical tumor suppressor F-box and WD repeat containing domain 7 (FBXW7) was identified as a direct molecular target for miR-25-3p, with an inverse relationship observed between the two in neoplastic tissues. Subsequent studies demonstrated that the tumor suppressive effects of miR-25-3p inhibitor were effectively negated by the silencing of FBXW7. Moreover, the ability of FBXW7 to inhibit the expression of several oncogenes was deemed essential for countering the anticancer effects mediated by miR-25-3p downregulation. These findings posit miR-25-3p as a promising therapeutic target and prognostic indicator for CRC.

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Introduction

Colorectal cancer (CRC) is the third most common malignant tumor globally in terms of incidence and mortality, with >1.85 million diagnoses and 850,000 deaths annually (1). Despite significant progress in surveillance and the development of personalized therapeutic strategies, the 5-year survival rate for patients with CRC remains <20%. Identifying and screening more effective therapeutic agents is therefore of paramount importance in the clinical management of CRC (2).

MicroRNAs (miRNAs or miRs), a class of short non-coding RNAs comprising 20-22 nucleotides, play a pivotal role in gene regulation by targeting mRNAs for translational repression or degradation (3). The dysregulation of miRNAs has been implicated in numerous pathological processes associated with human cancers, including the regulation of cell proliferation and apoptosis (4,5). Several miRNAs have been identified as key players in the pathogenesis of CRC (6,7). For instance, Zhou *et al* (8) demonstrated that miR-483 fostered CRC cell proliferation *in vivo*, while Lin *et al* (9) reported that miR-202, which was under-expressed in CRC tissues, suppressed CRC cell proliferation and invasion by targeting the ubiquitin-like with PHD and RING finger domain 1 (UHRF1) (9). Intriguingly, miR-25 had been recognized as an oncogenic miRNA across a spectrum of malignancies, including those of the lung, stomach and liver (10-12). Although miR-25 is known to be overexpressed in CRC and associated with tumor growth (13), the downstream mechanisms by which it influences the pathological processes of CRC remain to be fully elucidated.

F-box and WD repeat containing domain 7 (FBXW7) is one of the best-studied multiprotein ubiquitin E3 ligases. Due to its involvement in the ubiquitination and degradation of numerous oncoproteins, including c-Myc, Cyclin E, Notch and numerous others, FBXW7 has been demonstrated in numerous studies to act as a tumor suppressor (14-16). Low FBXW7 expression has

been linked to subpar clinical outcomes for patients with CRC, and FBXW7 mRNA expression is considerably downregulated in tumor tissues in CRC (17). Additionally, FBXW7 reduced colon cancer cell migration and proliferation as a result of abnormal enolase 1 expression and activity (18). Despite these findings, the regulatory mechanisms governing FBXW7 in CRC remain to be fully elucidated. Prior studies had suggested that a subset of miRNAs, including miR-223, miR-25, miR-92 and miR-27b, may exert their antitumor effects by directly binding to the 3' untranslated region (3'UTR) of FBXW7, thereby inhibiting its activity and downstream targets across various cancer types (19-22). Consequently, further investigation was warranted to improve understanding of the role and underlying processes of Fbxw7 in the pathogenesis of CRC.

Utilizing microarray data obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/), a comprehensive screening of differentially expressed miRNAs was conducted in the present study. Among these, miR-25-3p stood out as the most significantly upregulated miRNA and was thus selected for in-depth analysis. To elucidate the functional contributions of miR-25-3p in CRC cells and to unravel the molecular mechanisms at play, a series of functional assays were meticulously performed. The current investigation has uncovered evidence suggesting that miR-25-3p may be instrumental in driving the oncogenic processes associated with the development of CRC.

Materials and methods

Cell culture and tissue samples. Human normal colorectal mucosa NCM460 cell line (cat no. SNL-519; SUNNCELL Co., Ltd.), four colon cancer cell lines, namely HCT116 (cat no. CCL-247EMT), SW480 (cat no. CCL-228), SW620 (cat no. CCL-227) and Caco-2 (cat no. HTB-37), and 293T (cat no. CRL-3216) cells were purchased from American Type Culture Collection. These cell lines were cultured in high-glucose DMEM (Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA) in a 37˚C incubator with 5% CO_2.

Between January 2020 and July 2021, 50 CRC tissues and associated normal tissues were obtained from the Department of Colorectal Surgery, Zhejiang Provincial People's Hospital (Hangzhou, China). Before surgery, neither radiation nor chemotherapy had been administered to any of the patients. The adjacent normal tissues that were >5 cm from the tumor's margin were received. The present study was approved (approval no. 2020059) by the ethical committee of the Zhejiang Provincial People's Hospital (Hangzhou, China). Every participant signed a statement of informed consent. Clinicopathological characteristics of patients with CRC (including sex and age distribution) are presented in Table I.

MicroRNA expression profile data from GEO. The microRNA data (accession nos. GSE183437 and GSE156719) were downloaded from GEO databases in NCBI (23). Microarray data of GSE183437 was on account of GPL16791 Platform Illumina HiSeq 2500 (*Homo sapiens*), which included 5 CRC tissues and 5 non-cancerous tumor-adjacent tissues, while GSE156719 was on account of GPL20712 Platform Agilent-070156 human miRNA [miRNA version], which included 3 pairs of colorectal tumor tissues and normal tissues. Differentially expressed miRNAs (DEmiRNAs) were identified through GEO2R online platform (https://www.ncbi.nlm.nih.gov/geo/geo2r/), which is a widely used tool that can be used to analyze data from any GEO series and significance analysis of microarray (SAM), to determine the differential expression of miRNAs among groups. miRNAs were considered to be differentially expressed according to the $P<0.05$ threshold from the limma analysis and median false discovery rate <0.05 from SAM. miRs with the top 46 differences were selected for heat mapping using GeneSpring GX statistical software (version 7.3; Agilent Technologies, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA of the cultured cells and the tissues was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and total RNA was reverse-transcribed to cDNA using the iScript advanced cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. RT-qPCR was performed using SYBR®PrimeScript™ RT-PCR kit (Takara Bio, Inc.) on the Applied Biosystems Quantstudio6 flex (Applied Biosystems; Thermo Fisher Scientific, Inc.). For detection of microRNAs, a universal reverse primer that is complementary to a sequence within the RT stem-loop primer is 5'-GCAGGGTCCGAGGTATTC-3'. The specific forward primers for microRNAs were as follows: miR-25 forward, 5'-CATTGCACTTGTCTCGGT-3'; miR-18a forward, 5'-ACTGCCCTAAGTGCTCCT-3'; miR-92a forward, 5'-AATTATTGCACTTGTCCC-3'; miR-203 forward, 5'-GTGAAATGTTTAGGACCA-3'; miR-143 forward, 5'-TGAGATGAAGCACTGTAG-3'; miR-375 forward, 5'-TTTGTTCGTTCGGCTCGC-3'; miR-29a forward, 5'-TAGCACCATCTGAAATCG-3'; miR-137 forward, 5'-TTATTGCTTAAGAATACG-3'. The other qPCR primers used in the present study were as follows: FBWX7 forward, 5'-CACTCAAAGTGTGGAATGCAGAGAC-3' and reverse, 5'-GCATCTCGAGAACCGCTAACAA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; and GAPDH forward, 5'-TCAACGACCCCTTCATTGACC-3' and reverse, 5'-CTTCCCGTTGATGACAAGCTTC-3'. The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (24).

Transfection. When HCT116 and Caco-2 cells ($5x10^5$ cells/well) in six-well plates reached ~80% confluence, miR-25-3p mimics (20 nM), mimics negative control (mimics NC, 20 nM), miR-25-3p inhibitor (20 nM), inhibitor NC (20 nM), small interfering (si) FBXW7 (50 nM) and si-Scramble (50 nM) were transfected into cells at 37˚C for 24 h using Lipofectamine®2000 according to manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). siRNA, miR mimics and miR inhibitors were provided by GenScript (Nanjing) Co., Ltd. The sequences are as follows: miR-25-3p mimics, 5'-CAUUGCACUUGUCUCGGUCUGA-3'; mimics NC, 5'-CUGUAACUGCUCUGCUGUCGUA-3'; miR-25-3p

Table I. Associations of miR-25-3p expression with the clinicopathological characteristics of colorectal cancer.

Clinicopathological characteristics	All cases (n=50)	Expression level of miR-25-3p		P-value
		High (31)	Low (19)	
Sex				0.771
Male	25	16	9	
Female	25	15	10	
Age, years				0.320
≥60	37	21	16	
<60	13	10	3	
Location				0.812
Proximal colon	30	19	11	
Distal colon and rectum	20	12	8	
Tumor size, cm				0.029
≥5	33	24	9	
<5	17	7	10	
cTNM stage				0.006
I + II	15	5	10	
III + IV	35	26	9	
Distant metastasis				0.011
Absent	28	13	15	
Present	22	18	4	
Histological grade				0.285
Well and moderate	19	10	9	
Poorly	31	21	10	
Lymph node metastasis				0.057
Present	27	20	7	
Absent	23	11	12	

miR, microRNA; TNM, tumor-node-metastasis.

inhibitor, 5'-UCAGACCGAGACAAGUGCAAUG-3'; inhibitor NC, 5'-UAGAUAGUGACGAGAAACCCCG-3'; siFBXW7, 5'-CAAUUGUGUAGACGAUAUACU-3'; si-Scramble, 5'-UAUAUUCUCAUGAACAGGAUG-3'.

The FBXW7 gene was amplified by PCR utilizing human cDNA extracted from adjacent normal tissues. The amplified fragments were cloned into the pcDNA3.1(+) plasmid (Shanghai GenePharma Co., Ltd.), named pcDNA3.1-FBXW7. Transfection was conducted using Lipofectamine®2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

RT-qPCR analysis and western blotting were used to detect the successful knockdown or upregulation of miR-25-3p and FBXW7 expression, respectively.

Cellular proliferation assay. The proliferation of HCT116 and Caco-2 cells was measured using a Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Inc.). After 24, 48 and 72 h post-transfection, CCK-8 reagent (10 μl/well) was added to HCT116 and Caco-2 ($1x10^6$ cells/well), and then incubated at 37˚C with 5% CO_2 for another 3 h. Subsequently, cell proliferation was analyzed at 450 nm, using a microplate reader (Multiskan SkyHigh; Thermo Fisher Scientific, Inc.).

Apoptosis assay. HCT116 and Caco-2 cells ($5x10^5$ cells) were seeded in 6-well plates overnight at 37˚C. Apoptotic rates were determined using the Annexin V-FITC Apoptosis Detection Kit (cat. no. ab14082; Abcam) 48 h post-transfection. Cells were digested with 0.25% trypsin (Millipore Sigma; Merck KGaA), centrifuged at 300 x g for 5 min at 4˚C, resuspended in 20 μl binding buffer, and incubated with 5 μl Annexin V-FITC and 1 μl Propidium Iodide (PI) in a dark room at room temperature for 20 min. The stained cells were then analyzed using BD FACSCalibur Flow Cytometer System (BD Biosciences). Data analysis was performed using BD FACSuite™ software (Version 6.0; BD Biosciences). The results demonstrated healthy viable cells in the lower left quadrant on the scatter plot as ($FITC^-/PI^-$). The lower right quadrant (Q3) represented the early-stage apoptotic cells as ($FITC^+/PI^-$). The upper right quadrant (Q2) represented late-stage apoptotic cells as ($FITC^+/PI^+$). The calculation was made as follows: Apoptotic rate=percentage of early-stage apoptotic cells (Q3) + percentage of late-stage apoptotic cells (Q2).

Caspase 3 activity assay. Caspase-3 activity assay was performed in HCT116 and Caco-2 cells using a Caspase-3

colorimetric assay kit (cat no. 556485; BD Biosciences) according to the manufacturer's protocol.

Luciferase reporter gene assay. Using the online bioinformatics tools TargetScan 7.0 (http://www.targetscan.org/vert_70/) and miRanda (v3.3a; http://www.microrna.org), the binding sites between miR-25-3p and FBXW7 were predicted. The FBXW7 3'-UTR partial sequence, which contains the binding site or mutated binding site, was generated and inserted into a dual-luciferase pGL3 plasmid (Promega Corporation) by GenScript (Nanjing) Co., Ltd. Using Lipofectamine®2000 (Invitrogen; Thermo Fisher Scientific, Inc.), a total of 200 ng of either wild-type (wt)-FBXW7-3'UTR-pGL3 or mutant (mut)-FBXW7-3'UTR-pGL3 reporter plasmids were co-transfected with 40 nM miR-25-3p mimics, mimics NC, miR-25-3p inhibitor, and inhibitor NC into 293T cells. Luciferase assays were performed 48 h post transfection using the Dual-luciferase® Reporter Assay System (Promega Corporation). The luciferase activity was normalized to *Renilla* luciferase activity.

Western blot analysis. Total protein was extracted from cells using the RIPA lysis buffer 48 h after transfection (Beyotime Institute of Biotechnology). A BCA protein assay reagent kit was used to measure the protein concentration. Proteins (25 μg/lane) were separated by SDS-PAGE using 8% gels and then electroblotted on PVDF membranes. The membranes were blocked by soaking in 5% skimmed milk for 1 h at 4˚C overnight. Specific primary antibodies were used to stain the membranes at 4˚C overnight, followed by the corresponding secondary antibody at room temperature for 1 h. The enhanced chemiluminescence (ECL) detection system (Pierce; Thermo Fisher Scientific, Inc.) was used to identify protein bands, and ImageJ software (version 1.46; National Institutes of Health) was used to quantify them. The specific antibodies used in the present study were as follows: FBXW7 (1:200; cat. no. ab105752; Abcam), c-Myc (Y69; 1:1,000; cat no. ab32072; Abcam), Notch 2 (1:1,000; cat. no. ab118824; Abcam), YAP (1:1,000; cat. no. ab52771; Abcam), cyclin E (1:1,000; cat. no. ab33911; Abcam), β-actin (1:1,000; cat no. ab8277; Abcam) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat no. ab6721; Abcam).

Statistical analysis. SPSS 21.0 software (IBM Corp.) was used to conduct the statistical analysis. The data are presented as the mean ± standard deviation. One-way ANOVA was used to analyze comparisons between various groups, and Tukey's post hoc test was used to confirm the results. Using the unpaired Student's t-test, two groups were compared. The correlation between miR-25-3p and clinicopathological features of patients with CRC was analyzed using the chi-square test and Fisher's exact test. The correlation coefficient between miR-25-3p and FBXW7 was calculated using Spearman's rank correlation coefficient. The Kaplan-Meier method was used to create the survival curves, and the log-rank test was used to compare them statistically. The threshold for statistical significance was set at $P<0.05$.

Results

miR-25-3p is upregulated in CRC tissues and associated with worse survival of patients with CRC. To delve into the function of miRNAs in the context of CRC, an analysis of DEmiRNAs from two GEO datasets was conducted (GSE183437 and GSE156719) using the GEO2R online platform (https://www.ncbi.nlm.nih.gov/geo/geo2r/). The present analysis revealed a total of 46 dysregulated miRNAs in CRC tissues, with 20 miRNAs exhibiting increased expression and 26 showing decreased expression relative to the adjacent normal tissues (Fig. 1A). Subsequently, further validation of the five most significantly upregulated miRNAs (miR-18a, miR-92a, miR-25, miR-203 and miR-143) and the three most notably downregulated miRNAs (miR-375, miR-29a and miR-137) was performed across the two GEO datasets. The significantly DEmiRNAs, which were consistent with previous results (25-31), were presented in Fig. 1B, indicating the reliability of the screening results obtained by the microarray analysis. Of these miRNAs, the expression of miR-25-3p was found to be the most significantly increased in the CRC tissue. miR-25-3p has previously been linked to tumorigenesis of CRC (32). By contrast, little is known about the roles of miR-25-3p for CRC. It was therefore decided to focus on miR-25-3p for further molecular analyses, to clarify the previously unknown role of miR-25-3p in CRC.

In order to investigate the relevance of miR-25-3p in CRC, 50 pairs of CRC tissues and adjacent normal tissues were utilized for the validation of its aberrant expression. RT-qPCR analysis confirmed a significant upregulation of miR-25-3p in the CRC tissues as opposed to the adjacent normal tissues. This observation was in alignment with the differential expression patterns observed in the GEO datasets, revealing a consistently differential expression with the GEO datasets (Fig. 1C). Furthermore, the relationship between miR-25-3p expression and clinicopathological features was also analyzed. The samples were divided into high and low miRNA expression groups according to the median expression value as the cutoff point. It was found that high expression of miR-25-3p was closely associated with tumor size, distant metastasis and tumor-node-metastasis stage (Table I). Furthermore, the association between miR-25-3p expression and overall survival rate in CRC was investigated, and the overall survival rate of patients with CRC with high miR-25-3p expression was significantly shorter compared with low miR-25-3p expression (Fig. 1D). Therefore, the findings revealed that miR-25-3p might act as an oncogene in the development of CRC.

Knockdown of miR-25-3p inhibits CRC cell proliferation and promotes cell apoptosis. Taken a step further, to validate whether the altered expression of miR-25-3p was also present in CRC cell lines, the expression of miR-25-3p was detected in HCT116, SW480, SW620 and Caco-2 cell lines and human normal colorectal mucosa NCM460 cell line was used as a control. It was found that miR-25-3p expression was significantly upregulated in CRC cells compared with NCM460 cells, which exhibited the same trend as the results in CRC tissues (Fig. 2A).

To determine the effect of miR-25-3p on CRC cell proliferation, HCT116 and Caco-2 cells were transfected with miR-25-3p inhibitor. Post-transfection with the miR-25-3p inhibitor, a significant reduction in miR-25-3p levels was

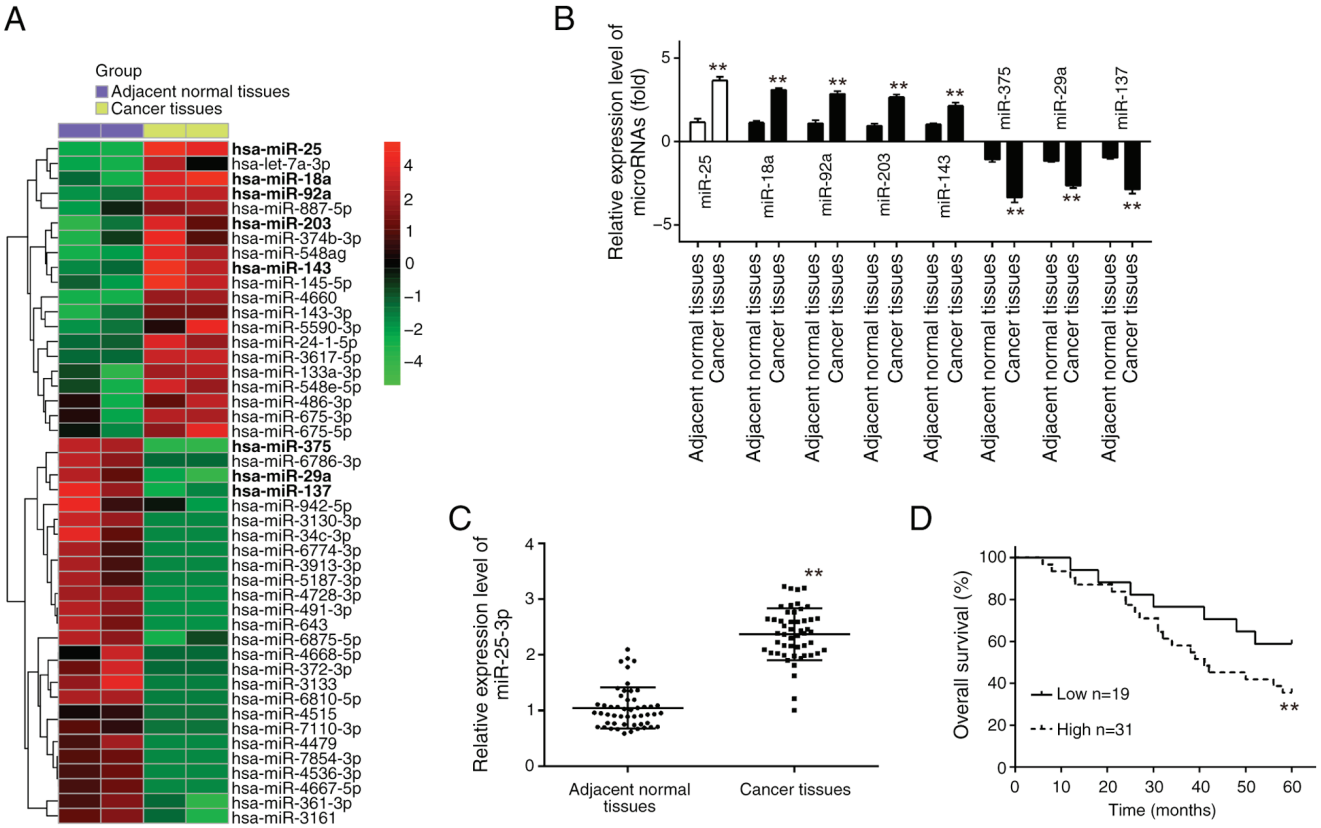

Figure 1. miR-25-3p is upregulated in CRC tissues. (A) Differentially expressed miRNAs were analyzed between CRC cancer tissue and the adjacent normal tissue. Data were retrieved from the Gene Expression Omnibus datasets, with the accession numbers GSE183437 and GSE156719. The color code in the heat map is linear and the expression levels of miRNAs that were upregulated are shown in green to red, whereas the miRNAs that were downregulated are shown from red to green. (B) The expression levels of miR-18a, miR-92a, miR-25-3p, miR-203, miR-143, miR-375, miR-29a and miR-137 were detected in CRC tissues and adjacent normal tissues by RT-qPCR (n=3). Data represent the mean ± SD of three independent experiments. (C) The expression levels of miR-25-3p, were detected in CRC tissues and adjacent normal tissues by RT-qPCR (n=50). Data represent the mean ± SD of three independent experiments. (D) Kaplan-Meier survival curves of patients with CRC according to the expression of miR-25-3p. **P<0.01. miR or miRNA, microRNA; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR.

observed in both HCT116 and Caco-2 cells, as depicted in Fig. 2B. Moreover, the suppression of miR-25-3p led to a significant decrease in cell proliferation and a concurrent increase in the rate of apoptosis in HCT116 and Caco-2 cells when compared with the NC group (Fig. 2C-E). Collectively, these results revealed that knockdown of miR-25-3p suppressed CRC cell proliferation and promoted cell apoptosis.

FBXW7 is a direct of miR-25-3p in CRC. To further explore the possible mechanisms of miR-25-3p in CRC cells, potential target genes of miR-25-3p were searched using TargetScan and miRanda databases. FBXW7 was chosen as a target gene of miR-25-3p in the present study (Fig. 3A). RT-qPCR assay confirmed the successful overexpression or knockdown of miR-25-3p after transfection of miR-25-3p mimics or inhibitor, respectively, as demonstrated in Fig. 3B. As it has been previously reported, FBXW7 is a tumor suppressor with a role in various types of human cancers, including CRC (33-35). Additionally, a luciferase reporter assay was conducted to confirm the interaction between miR-25-3p and the 3'UTR of FBXW7. As illustrated in Fig. 3C, the overexpression of miR-25-3p led to a significant decrease in luciferase activity when the wt-FBXW7 3'UTR was present, whereas the activity of the mut-FBXW7 3'UTR remained unaltered in 293T cells. Conversely, the knockdown of miR-25-3p resulted in an increase in luciferase activity for the wt-FBXW7 3'UTR. Moreover, the overexpression of miR-25-3p in HCT116 and Caco-2 cells led to a significant reduction in both mRNA and protein levels of FBXW7 (Fig. 3D and E), whereas the knockdown of miR-25-3p yielded the opposite effect in mRNA level as shown in Fig. 3D. These results collectively suggested a direct and functional interaction between miR-25-3p and FBXW7. Next, the relationship between miR-25-3p and FBXW7 in CRC tissues was investigated. First, the level of FBXW7 mRNA in four CRC cell lines and 50 patients with CRC was measured by RT-qPCR. The results of RT-qPCR showed that FBXW7 was significantly decreased in the CRC cells and tissues compared with NCM460 cells and the adjacent normal tissues, respectively (Fig. 3F and G). Furthermore, as revealed in Fig. 3H, there was an inverse correlation between miR-25-3p and FBXW7 expression levels in the 50 patients with CRC (r=-0.7989; P<0.01). These data demonstrated that FBXW7 may be a functional target of miR-25-3p in CRC.

Overexpression of FBXW7 inhibits CRC cell proliferation and promotes cell apoptosis. To elucidate the functional role

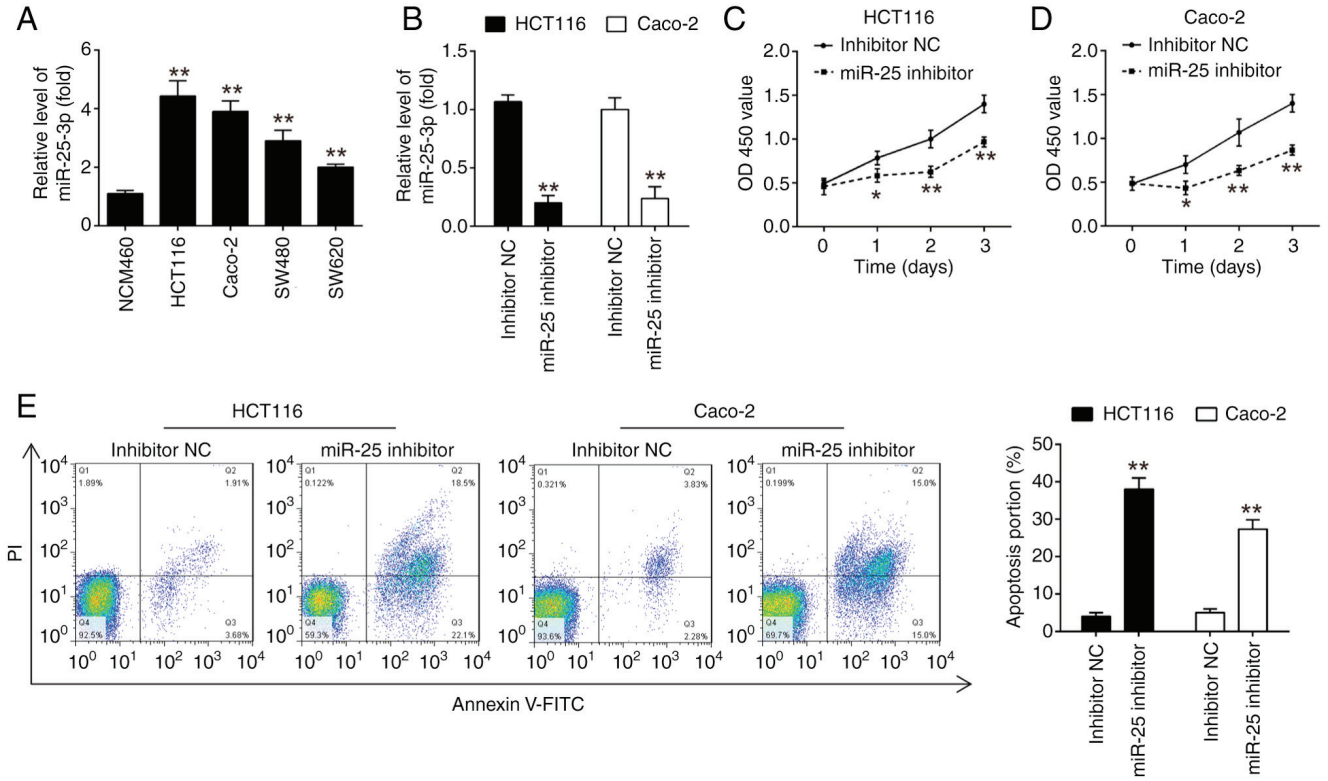

Figure 2. Knockdown of miR-25-3p suppresses the cell proliferation and induces cell apoptosis. (A) The expression levels of miR-25-3p were detected in four colorectal cancer cell lines and a human normal colorectal mucosa cell line (n=3). Data represent the mean ± SD of three independent experiments. **P<0.01 vs. NCM460 (B) HCT116 and Caco-2 cells were transfected with the miR-25-3p inhibitor or inhibitor NC for 24 h, and transfection efficiency was assessed by reverse transcription-quantitative PCR analysis (n=3). (C and D) HCT116 and Caco-2 cells were transfected with the miR-25-3p inhibitor or inhibitor NC for 24, 48 and 72 h, and then cell proliferation was measured using a Cell Counting Kit-8 assay at the indicated time-points (n=3). (E) HCT116 and Caco-2 cells were transfected with the miR-25-3p inhibitor or inhibitor NC for 48 h, and then apoptosis was detected by flow cytometry (n=3). Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. inhibitor NC group. miR, microRNA; NC, negative control.

of FBXW7 in CRC, FBXW7 was overexpressed in CRC cell lines and the impact on cell proliferation and apoptosis was subsequently assessed. For this purpose, plasmids named pcDNA-FBXW7 were constructed and introduced into HCT116 and Caco-2 cells. Western blot analysis confirmed the successful overexpression of FBXW7 following the introduction of the pcDNA-FBXW7 plasmids, as depicted in Fig. 4A. The overexpression of FBXW7 led to a significant reduction of cell proliferation in both HCT116 and Caco-2 cells, as determined by cell proliferation assays, compared with the control group (Fig. 4B and C). Moreover, the apoptotic rate in cells overexpressing FBXW7 was significantly higher than that observed in the control group (Fig. 4D), underscoring the potential of FBXW7 as a tumor suppressor in CRC. Overall, the present findings revealed that FBXW7 overexpression inhibited CRC cell proliferation and enhanced cell apoptosis.

Knockdown of miR-25-3p exerts antitumor effects by regulating FBXW7. It was anticipated that miR-25-3p targets FBXW7 in colon cancer cells to have antitumor effects in light of the aforementioned results showing that overexpression of FBXW7 decreased CRC cell proliferation and enhanced cell apoptosis. HCT116 and Caco-2 cells received simultaneous transfections of si-FBXW7 and miR-25-3p inhibitor. Western blot assay results identified that FBXW7 was successfully knocked down by the si-FBXW7 (Fig. 5A). Then, apoptosis and proliferation of CRC cells were investigated. The findings demonstrated that miR-25-3p inhibitor-transfected HCT116 and Caco-2 cells showed reduced cell proliferation when compared with inhibitor NC-transfected cells; however, these effects were partially diminished by si-FBXW7 (Fig. 5B and C). In the caspase 3 activity assay, HCT116 and Caco-2 cells treated with the miR-25-3p inhibitor exhibited increased caspase 3 activity compared with cells treated with the NC inhibitor. However, the silencing of FBXW7 with si-FBXW7 negated the enhanced caspase 3 activity observed with miR-25-3p inhibition (Fig. 5D and E). A similar pattern of apoptotic effects was observed in HCT116 and Caco-2 cells following transfection with the miR-25-3p inhibitor, which were counteracted by the concurrent knockdown of FBXW7 using si-FBXW7 (Fig. 5F and G). Collectively, the present results indicated that the pro-apoptotic effects of miR-25-3p inhibition on CRC cells are partially mitigated by the suppression of FBXW7.

Knockdown of miR-25-3p influences FBXW7-mediated degradation of the oncogenes. It has been revealed that FBXW7 is responsible for the binding to and the degradation of several oncogenes, including cyclin E, Yap, Notch and c-Myc, and thus inhibits tumor cell proliferation and invasion, to exert its tumor-suppressive effects (36-39). The FBXW7-related oncogenic proteins were detected: cyclin E, Yap, Notch and c-Myc. Compared with the inhibitor NC group, it was discovered that miR-25-3p knockdown significantly reduced the

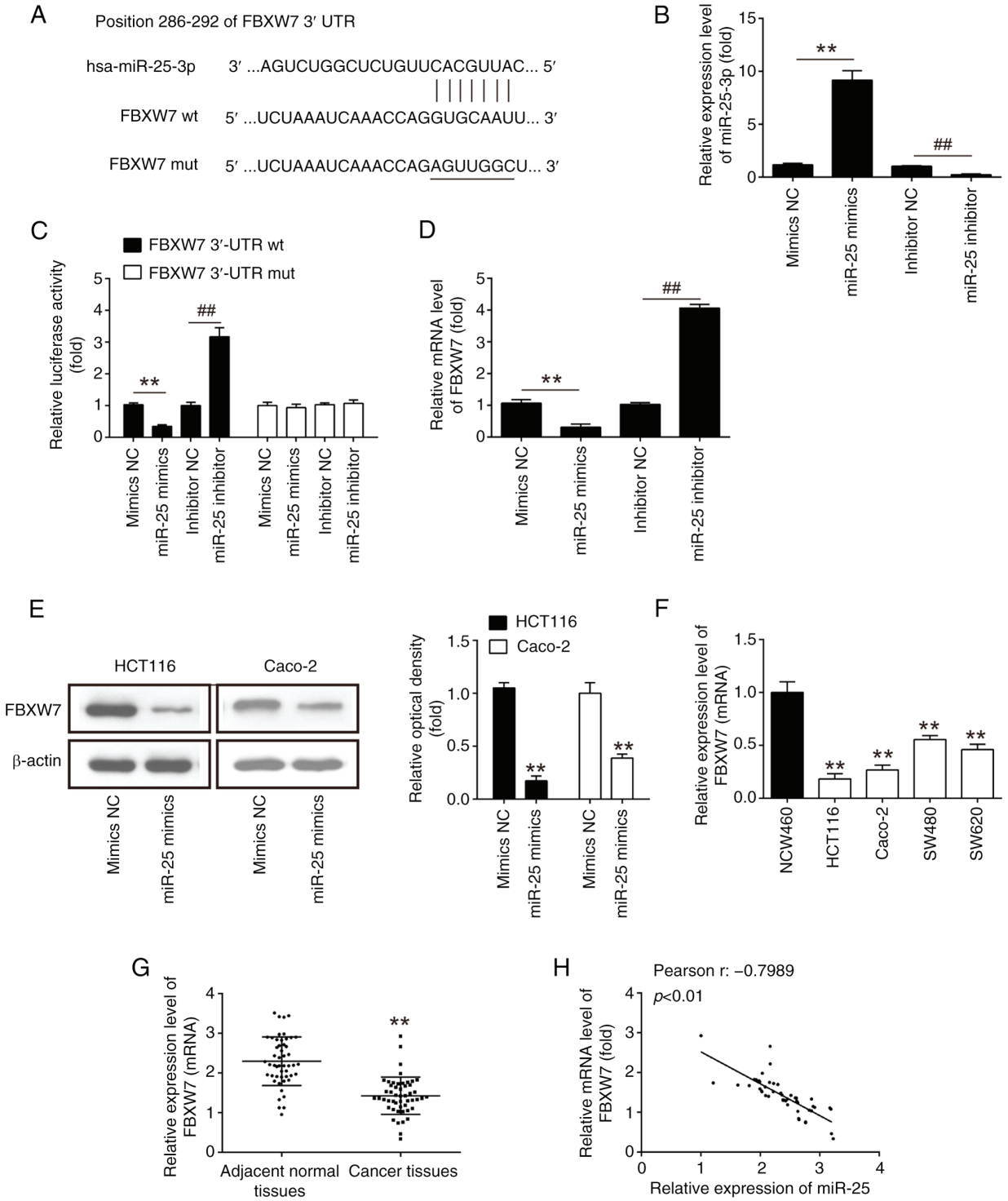

Figure 3. FBXW7 is a direct target of miR-25-3p. (A) Putative binding sites of miR-25-3p and FBXW7. (B) Transfection efficiency of miR-25-3p mimics and inhibitor was assessed by RT-qPCR analysis. (C) The relative luciferase activity of FBXW7 wt or mut 3'UTR in 293T cells following transfection with miR-25-3p mimics, mimics NC, miR-25-3p inhibitor or inhibitor NC, as indicated (n=3). Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. mimics NC; $^{\#\#}$P<0.01 vs. inhibitor NC. (D and E) The mRNA and protein expression of FBXW7 following transfection with miR-25-3p mimics, mimics NC, miR-25-3p inhibitor, or inhibitor NC in HCT116 and Caco-2 cells were measured by RT-qPCR and western blot analysis (n=3). **P<0.01 vs. mimics NC; $^{\#\#}$P<0.01 vs. inhibitor NC. (F) Expression of FBXW7 was examined in four CRC cell lines and NCM460 cells, used as a control, by RT-qPCR analysis (n=3). Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. NCM460. (G) Expression of FBXW7 was measured by RT-qPCR analysis in CRC tissues and adjacent normal tissues (n=50). **P<0.01 vs. adjacent normal tissues. (H) Pearson correlation analysis revealed a negative correlation between the expression of FBXW7 and miR-25-3p (r=-0.7989; P<0.01). FBXW7, F-box and WD repeat containing domain 7; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; wt, wild-type; mut, mutant; UTR, untranslated region; NC, negative control; CRC, colorectal cancer.

expression of cyclin E, Yap, Notch and c-Myc. However, these inhibitory effects were reversed by si-FBXW7 in HCT116 and Caco-2 cells (Fig. 6A-C). All of these findings pointed to the possibility that miR-25-3p functions as an oncogene in CRC cells by suppressing FBXW7 and subsequently indirectly promoting these oncoproteins.

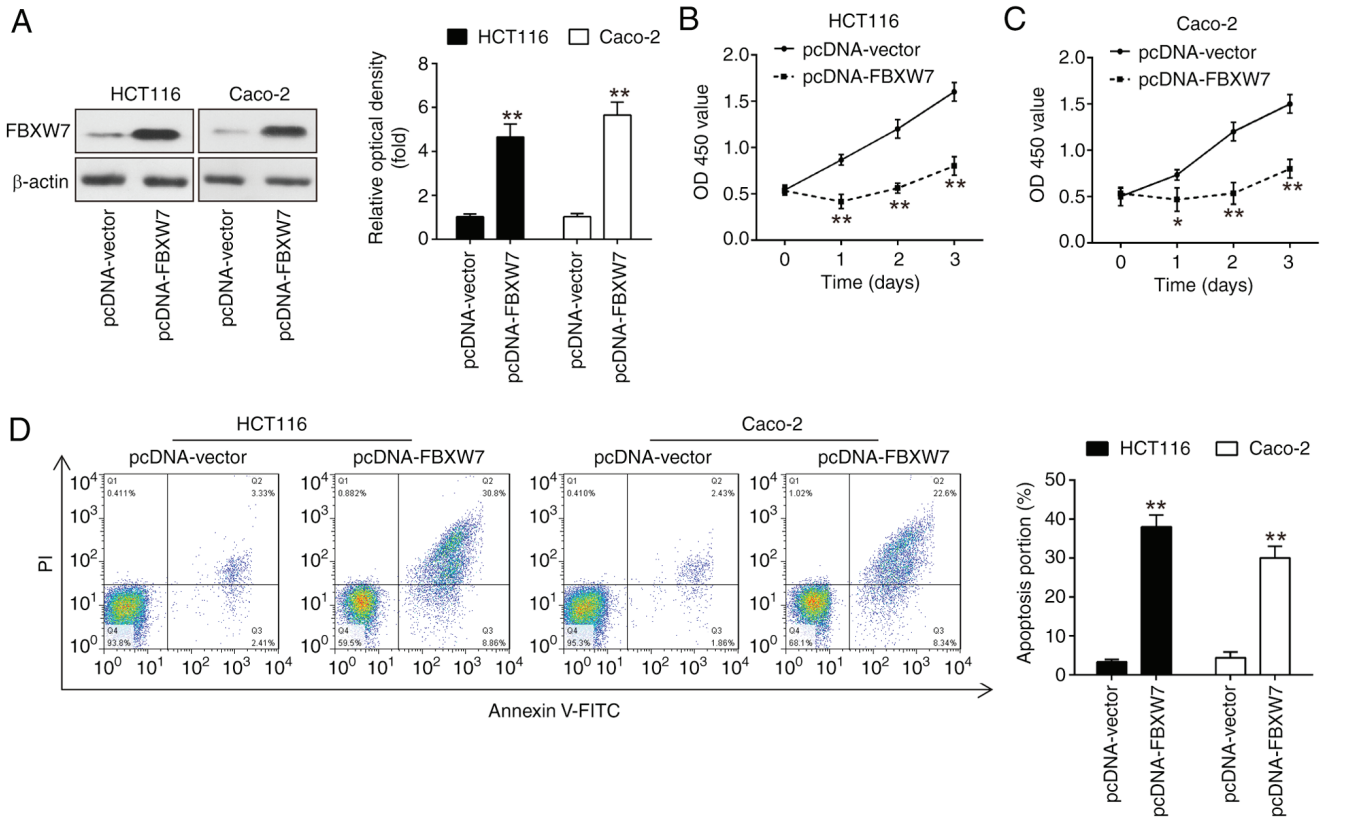

Figure 4. FBXW7 overexpression suppresses cell proliferation and induces cell apoptosis. HCT116 and Caco-2 cells were transfected with the pcDNA-FBXW7 or pcDNA-vector for 48 h, and the cells were used for analysis. (A) Transfection efficiency was assessed by western blot analysis (n=3). (B and C) Cell proliferation was measured using a Cell Counting Kit-8 assay at the indicated time-points (n=3). (D) Apoptosis was detected by flow cytometry (n=3). Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. pcDNA-vector. FBXW7, F-box and WD repeat containing domain 7; NC, negative control; miR, microRNA.

Discussion

In the present study, it was found that miR-25-3p expression was upregulated in CRC tissues and cell lines, and its expression was strongly associated with the overall survival of patients with CRC. *In vitro*, miR-25-3p downregulation significantly reduced cell proliferation and increased cell apoptosis. The oncogenic effects of miR-25-3p may also be partially mediated by the inhibition of FBXW7 on the oncoproteins, as FBXW7 was discovered to be a target of miR-25-3p. These results indicated that miR-25-3p targeting may be a possible treatment strategy for patients with CRC.

Ectopic expression of miRNAs in CRC tissues plays significant roles in the development of CRC, according to mounting evidence (40-42). By targeting FBXL2 and triggering the β-catenin signaling pathway, for instance, Pan *et al* (43) revealed that miR-346-5p may enhance CRC growth both *in vivo* and *in vitro*. By targeting UHRF1, Lin *et al* (44) demonstrated that miR-202 overexpression prevented the growth and invasion of CRC. Due to their high tissue specificity and part in carcinogenesis, miRNAs can be employed for the diagnosis and prognosis monitoring of patients with CRC, making them unique biomarkers for detecting cancer and forecasting patient outcomes. For instance, it has been shown that miR-21-5p, miR-92a-3p and their cluster have great potential for early CRC screening (45). Consequently, identifying more miRNAs that exhibit aberrant expression in CRC and tackling their underlying biological pathways could prove beneficial in devising treatment approaches and identifying CRC. miR-25-3p was found to be one of the most increased miRNAs in CRC tissues after the differentially expressed miRNAs were screened using the GSE183437 and GSE156719 microarray data that were retrieved from GEO and used in the present study. miR-25-3p, which has previously been identified, is important as an oncogene in several human malignancies, including CRC (46). It was identified that miR-25-3p was increased in CRC tissues and that miR-25-3p levels beyond a certain threshold can serve as a molecular predictor for poorer prognosis of patients with CRC.

miR-25-3p was discovered to function as an oncogene in earlier investigations. For instance, Zhang *et al* (47) reported that miR-25-3p targeted PTEN to encourage the migration and invasion of esophageal cancer cells and to suppress apoptosis via the PI3K/AKT pathway. Through targeting EGR2, Yang *et al* (48) demonstrated that miR-25 boosted gastric cancer cell proliferation and prevented their apoptosis. It has been noted that miR-25-3p acts as an oncogenic miRNA in osteosarcoma by targeting Merlin (49). Additionally, it was shown that miR-25-3p, which functions as a tumor suppressor, was downregulated in tongue squamous cell carcinoma (50). These results firmly establish the dual roles of miR-25-3p as an antitumor and carcinogenic molecule in a variety of human malignancies. miR-25-3p expression was previously found to increase the proliferation and migration of human colon cancer cells while inhibiting

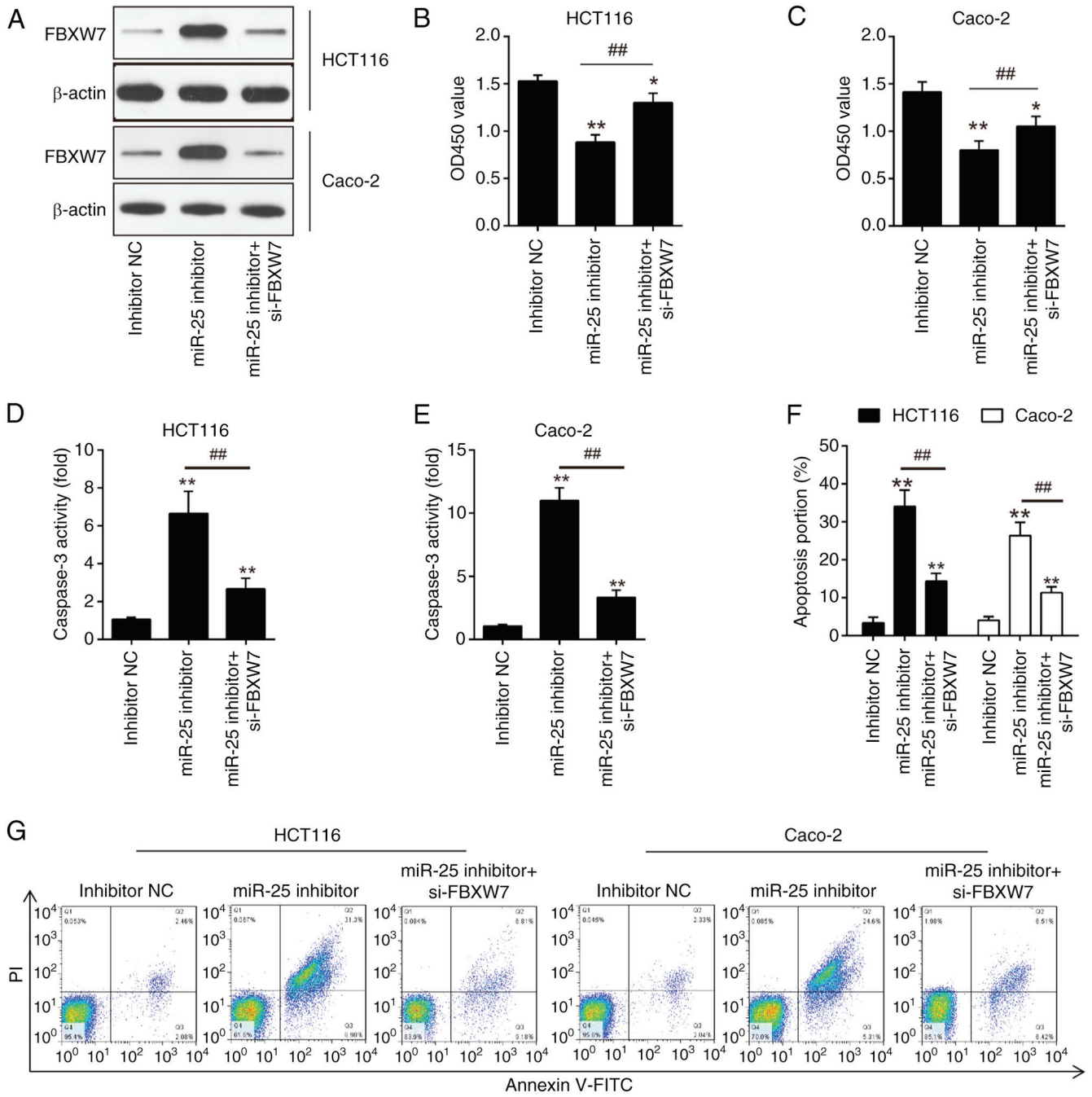

Figure 5. miR-25-3p inhibits cell proliferation and induces cell apoptosis by targeting FBXW7. si-FBXW7 and miR-25-3p inhibitors were co-transfected into HCT116 and Caco-2 cells for 48 h, and the cells were used for analysis. (A) Transfection efficiency was assessed by western blot analysis (n=3). (B and C) Cell proliferation was measured using a Cell Counting Kit-8 assay at the indicated time-points (n=3). (D and E) Caspase-3 activity was detected by a commercial kit (n=3). (F and G) Apoptosis was detected by flow cytometry (n=3). Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. inhibitor NC group; $^{\#\#}$P<0.01 vs. miR-25-3p inhibitor. miR, microRNA; FBXW7, F-box and WD repeat containing domain 7; NC, negative control.

apoptosis in CRC (32). In particular, miR-25-3p is a possible prognostic sign for patients with CRC (51). The present study showed that miR-25-3p knockdown accelerated apoptosis and decreased HCT116 and Caco-2 cell proliferation, indicating that miR-25-3p may function as an oncogene in CRC.

FBXW7, often referred to as hCDC4, serve as the SCF E3 ubiquitin ligase's substrate recognition subunit (52). One of the most frequently dysregulated ubiquitin-proteasome system proteins in human cancer, FBXW7 is a crucial tumor suppressor (53,54). It has been reported that certain oncoproteins including cyclin E, c-Myc, Mcl-1, mTOR, Jun and Notch are degraded by the proteasome under the supervision of FBXW7 (55,56). Abnormal expression of these targets has been detected in several human malignancies, including CRC (22). Previous studies have revealed that the expression of FBXW7 plays a crucial part in the development of CRC (57,58). For instance, Wei *et al* (59) discovered that FBXW7 loss-of-function increased FASN-mediated lipogenesis and encouraged the growth of CRC. The present study revealed that FBXW7 is a target of miR-25-3p, and miR-25-3p knockdown in CRC cells inhibited tumor cell proliferation and enhanced cell death by targeting FBXW7. It was also confirmed

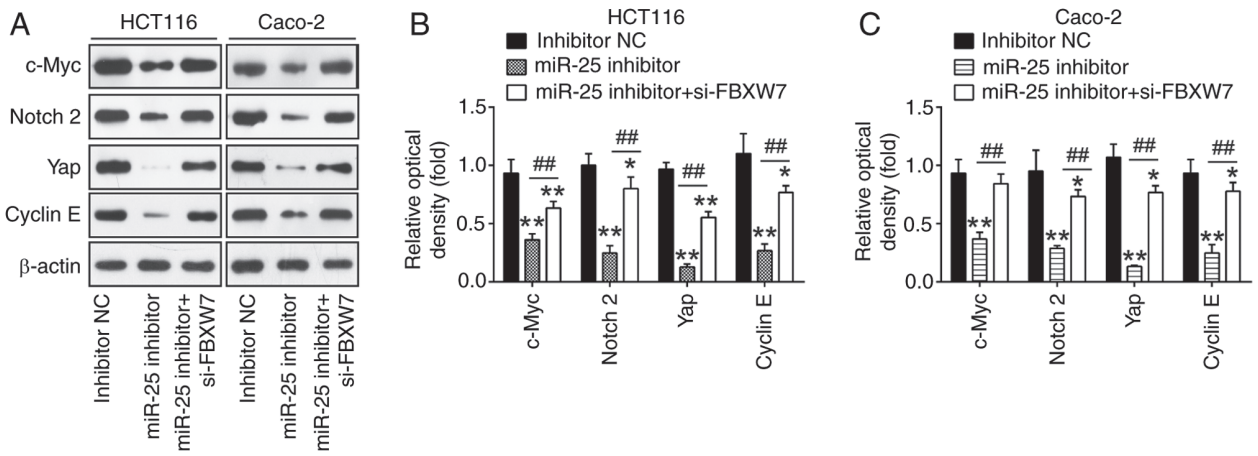

Figure 6. miR-25-3p knockdown suppresses the expression levels of oncoproteins by targeting FBXW7. si-FBXW7 and miR-25-3p inhibitors were co-transfected into HCT116 and Caco-2 cells for 48 h, and the cells were used for analysis. (A) The expression levels of cyclin E, c-Myc, Yap and Notch 2 proteins were determined in HCT116 and Caco-2 cells by western blotting (n=3). (B and C) Protein bands were analyzed semi-quantitatively using ImageJ software and normalized to β-actin density. Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. inhibitor NC group; $^{\#\#}$P<0.01 vs. miR-25-3p inhibitor. miR, microRNA; FBXW7, F-box and WD repeat containing domain 7; si-, small interfering; NC, negative control.

that tumor suppressor actions of FBXW7 are achieved by regulating the proteolytic activity of oncogenic substrates such as cyclin E, Yap, c-Myc and Notch2. In the future, more investigations are needed to fully understand how FBXW7 and its downstream pathways contribute to the development of CRC.

However, there are still some limitations to the present study. Firstly, though not altered as markedly as miR-25-3p, several miRNAs have also been screened; the authors' ongoing experiments will mainly focus on the function and the mechanism of these miRNAs in CRC. Secondly, other genes may be targeted by miR-25-3p; FBXW7 is not unique as a target gene of miR-25-3p. More importantly, the FBXW7 gene will be knocked out in CRC cells to investigate the function of miR-25-3p, and further verify the targeting relationship between miR-25-3p and FBXW7 in animal models. Moreover, it was found that the level of miR-25-3p varies among different CRC cell lines, which may be related to the invasive ability of different tumor cells. In future research, this correlation will be demonstrated to gain a more comprehensive understanding of the important role of miR-25-3p in CRC.

In conclusion, the present study demonstrated that the high level of miR-25-3p was associated with poor prognosis in CRC, suggesting that miR-25-3p might be used as a potential prognostic indicator in clinical CRC. Downregulation of miR-25-3p suppressed the proliferation and promoted apoptosis of CRC cells by targeting FBXW7. These findings demonstrated the carcinogenic function of miR-25-3p and the underlying molecular mechanism behind it, which may open the door to the development of more effective and practical CRC treatments.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YC, BC, ST and HY performed all the experiments and collected the data. HY and ST conceived and designed the study. YC and HY wrote the main manuscript and analyzed the data. HY and ST confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. 2020059) by the ethical committee of the Zhejiang Provincial People's Hospital (Hangzhou, China). Every participant signed a statement of informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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