Long non-coding RNA LINC00858 promotes TP53-wild-type colorectal cancer progression by regulating the microRNA-25-3p/SMAD7 axis

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Received September 21, 2019; Accepted January 21, 2020

DOI: 10.3892/or.2020.7506

Abstract. Long non-coding RNAs (lncRNAs) are involved in colorectal cancer (CRC) progression, however the mechanisms remain largely unknown. The present study aimed to reveal the role and possible molecular mechanisms of a new LNCRNA, LINC00858, in CRC. LINC00858 was increased in CRC tumor tissues, and patients with high LINC00858 expression had a shorter survival time. Knockdown of LINC00858 expression suppressed cell proliferation and induced G_0/G_1 cell cycle arrest and apoptosis in TP53-wild-type CRC cells. Subsequently, using Starbase v2.0 database, miR-25-3p was confirmed to interact with LINC00858 and was downregulated by LINC00858. Reduction of miR-25-3p expression with an inhibitor significantly attenuated the biological effects of LINC00858 knockdown in CRC cells. Furthermore, using TargetScan, SMAD7 was validated to interact with miR-25-3p and was downregulated by miR-25-3p. Lastly, the ectopic overexpression of SMAD7 rescued the suppressive effects of LINC00858 knockdown in CRC cells. Collectively, the results from the present study, to the best of our knowledge, firstly demonstrated a novel LINC00858/miR-25-3p/SMAD7 regulatory axis that promoted CRC progression, indicating LINC00858 as a promising therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is the third most prevalent and the fourth most fatal malignancy worldwide (1). The incidence and mortality rates of this type of malignancy have experienced a marked increase over the past few decades in China (2). The 5-year survival rate has remained >50% in developing countries, although progress has been made in endoscopic screening, surgery and chemotherapy (3). In particular, the prognosis is poor for patients with advanced-stage CRC, with a 5-year survival >10% (3). Therefore, there is an urgent requirement to identify novel molecular mechanisms to develop effective therapeutic strategies for the treatment of CRC.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules, that are over 200 nucleotides in length but the majority are not translated into proteins (4). An increasing number of studies have indicated that lncRNAs are aberrantly expressed in various types of malignancies and engaged in modulating multiple cancer-related biological processes, including cell proliferation, invasion and migration during cancer development (5-7). Several lncRNAs have been reported to play essential roles in oncogenesis and progression of CRC. lncRNA Gata6 has been revealed to sustain stemness of intestinal stem cells and facilitate tumorigenesis of CRC (8). lncRNA GLCC1 was revealed to promote CRC cell proliferation by enhancing glycolysis through stabilization of c-Myc (9). lncRNA APC1 inhibited CRC cell growth, metastasis, and tumor angiogenesis by suppressing exosome production (10). lncRNA OCC-1 suppressed CRC cell growth by destabilization and degradation of HuR protein (11). These aforementioned studies propose that lncRNAs play a controversial role in regulating CRC carcinogenesis and development. Thus, more specific regulatory roles of lncRNAs in CRC require further exploration.

Multiple dysregulated lncRNAs in CRC have been previously identified through RNA-sequencing (12). Here, in the present study the role of a newly identified upregulated lncRNA, LINC00858, in the progression of CRC was investigated. LINC00858 was revealed to be increased in CRC tissue and associated with poor prognosis. LINC00858 is reported to promote cell proliferation, migration and invasion through the microRNA(miR)-22-3p/14-3-3 protein ζ/δ(YWHAZ) axis in TP53-mutant (mut) CRC cell lines (13). However, the functions and mechanisms of LINC00858 in TP53-wild-type (WT) CRC cell lines remain largely unknown. The data from the present study revealed that LINC00858 promoted TP53-WT CRC cell proliferation by regulating SMAD7 through modulation of miR-25-3p. The present study is the first to indicate

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Key words: LINC00858, miR-25-3p, SMAD7, colorectal cancer

that LINC00858 could promote progression of TP53-WT CRC through the LINC00858/miR-25-3p/SMAD7 regulatory axis. LINC00858 may be utilized as a promising diagnostic and therapeutic target in patients with CRC.

Materials and methods

Ethics, patients and clinical tissues. The present study was reviewed and approved by the Ethics Committee of Medical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Fresh paired human CRC tissues and the corresponding adjacent non-cancerous tissues were collected from 120 patients who were treated with surgical resection alone between June 2016 and May 2017 at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. These patients included 72 males and 48 females, and 67 of them were older than 60 years old. Written informed consents were provided from these patients.

Tissue microarrays with survival information were obtained from Shanghai Outdo Biotech Co., Ltd.

Cell culture and reagents. The human TP53-WT CRC cell lines, RKO, HCT116, Colo-678, LoVo, SW48, LS174T and human colonic fibroblast, CCD-112CoN were purchased from American Type Culture Collection (14). All cell lines were ensured to be free of mycoplasma contamination before use. All the cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum, 1% penicillin and streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.) and maintained at 37˚C with 5% CO_2 in a humidified incubator (Thermo Fisher Scientific, Inc.).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized from 1,000 ng total RNA with 5X PrimeScript RT master mix (cat. no. RR036A; Takara Bio Inc.) following the manufacturer's instructions. qPCR was performed using the SYBR Premix Ex TaqTM II kit (cat. no. DRR081A; Takara Bio Inc.) and performed using the LightCycler 96 system (Roche Diagnostics GmbH). β-actin was used as the internal control for normalization. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (15) and each experiment was repeated at least three times. The sequences of the primers used in qPCR were as follows: β-actin forward, 5'-GATTACTGCTCTGGCTCCTAGC-3' and reverse, 5'-CAG CTCAGTAACAGTCCGCC-3'; SMAD7 forward, 5'-TTT GAGGTGTGGTG-3' and reverse, 5'-GAGGCAGTAAGA CAGGGATGA-3'; LINC00858 forward, 5'-CCCAGCTCC TTACACACGTT-3' and reverse 5'-TTCAGAGGCCTGCAT CACTG-3'; miR-25-3p forward, 5'-CAUUGCACUUGUCUC GGUCUGA-3' and reverse, 5'-AGACCGAGACAAGUGCAA UGUU-3'. All the PCR primers were designed and synthesized by Genewiz, Inc.

Cell transfection and luciferase reporter assay. CRC cells were seeded in 6-well plates and cultured to 60-70% confluence for transfection. miRNA mimics, inhibitors or corresponding controls, short hairpin (sh)RNAs and plasmid (Guangzhou RiboBio Co., Ltd.) were transfected using Lipofectamine® 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

For the luciferase reporter assay, cells were cultured to 60-70% confluence for transfection. LINC00858-WT vector or LINC00858-mutant (MUT) vector were created with or without a 3'-untranslated region binding site for miR-25-3p using pmirGLO vector (Promega Corporation) and SMAD7-WT vector or SMAD7-MUT vector were constructed similarly. Cells were co-transfected with miRNA mimics or NC mimics along with luciferase reporter vector using Lipofectamine 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, luciferase activity was detected with a Dual-Luciferase Reporter Assay System (cat. no. E1910; Promega Corp.).

Cell viability and colony formation assays. A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was utilized for measurement of RKO or SW48 cell viability. After cell transfection, the transfected cells were collected and seeded in 96-well plates ($2x10^3$ cells/well). Cells were cultured for 24, 48 and 72 h at 37˚C, the CCK-8 solution (10 μl) was added into the culture medium in 96-well plates. Subsequently, the mixture was incubated at 37˚C for 1.5 h, and the absorbance at 450 nm was measured using a spectrophotometer (BioTek Instruments, Inc.).

For colony formation analysis, transfected cells were seeded into 6-well plates and incubated for 14 days at 37˚C in a humidified incubator with 5% CO_2. Subsequently, the cells were washed in PBS and fixed with 4% paraformaldehyde. The cells were stained with 0.1% crystal violet at room temperature for 30 min (Sigma-Aldrich; Merck KGaA). The stained colonies were viewed using a fluorescence microscope.

Cell cycle and apoptosis analysis. For apoptosis analysis, following transfection for 48 h cells were washed twice with PBS and resuspended in buffer. Annexin-V-fluorescein isothiocyanate apoptosis detection kit (cat. no. 556547; BD Biosciences) was utilized according to the manufacturer's instructions. For cell cycle analysis, the transfected cells were collected and fixed with ice-cold 70% ethanol for 12 h at 4˚C. Subsequently, the cells were stained with Cycle TEST DNA Reagent kit at room temperature for 10 min (cat. no. 340242; BD Biosciences). The cell cycle and apoptosis analysis were performed with flow cytometry using the BD FACSVantage™ SE System.

Protein extraction and western blot analysis. Proteins were extracted using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with EDTA-free protease inhibitor cocktail (cat. no. 04693159001; Roche Diagnostics GmbH) as previously described (16). The suspension was collected after centrifugation at 12,000 x g for 15 min at 4˚C, followed by protein concentration determination using BCA assay. Equal amounts of proteins (20-40 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes using a Bio-Rad semi-dry transfer system. Then the membrane was blocked with 5% skimmed milk. After blocking, the membranes were incubated with primary antibodies (dilution ratio of 1:1,000 with

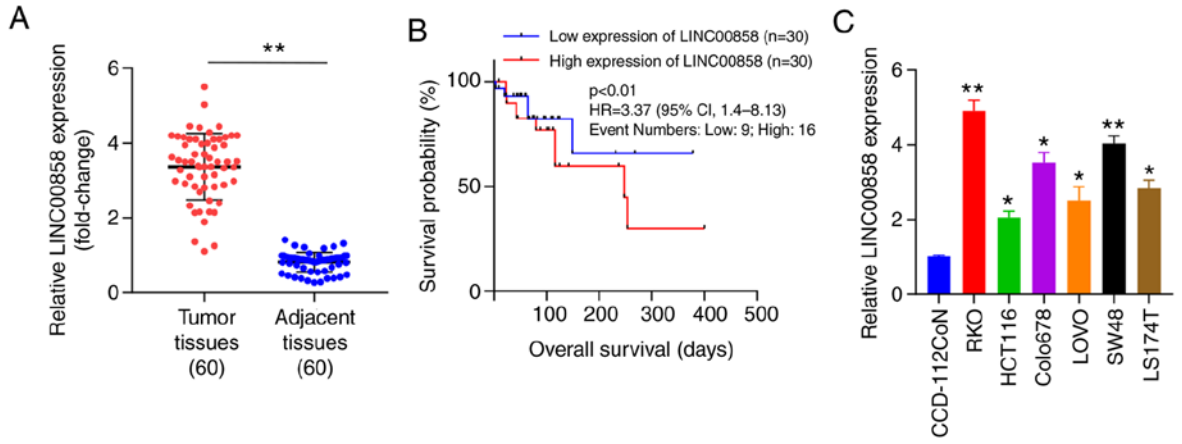

Figure 1. LINC00858 expression in CRC tumor tissues and its clinical significance. (A) Expression level of LINC00858 in CRC tissues (n=60) compared with adjacent normal tissues (n=60) was assessed using qPCR. (B) Kaplan-Meier curves for overall survival in patients with CRC with high (n=30) and low LINC00858 expression level (n=30). (C) LINC00858 expression was analyzed using reverse transcription-qPCR analysis in TP53-WT CRC cell lines (RKO, HCT116, Colo-678, LoVo, SW48 and LS174T) and human normal colonic fibroblast (CCD-112CoN). Data are presented as the mean ± SEM; n≥3. *P<0.05; **P<0.01. CRC, colorectal cancer; q, quantitative; WT, wild-type.

PBST containing 5% BSA) overnight at 4˚C, followed by horseradish peroxidase-labeled secondary antibodies (dilution ratio of 1:5,000 with PBST containing 5% non-fat milk; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands were visualized using the Tanon 4600 imaging system (Tanon Science and Technology Co., Ltd.) with enhanced chemiluminescence (cat. no. 407207; EMD Millipore; Merck KGaA). The primary antibodies used included Smad7 (cat. no. sc-11392; Santa Cruz Biotechnology, Inc.) and GAPDH (product code ab128915; Abcam).

RNA-binding protein immunoprecipitation (RIP) assay. EZ-Magna RIP RNA-Binding Protein Immunoprecipitation kit (cat. no. 17-701; EMD Millipore; Merck KGaA) was used to perform the RNA-binding protein immunoprecipitation assay. Cells were cultured to 80-90% confluence and then harvested. Subsequently, 3 μg of purified antibodies and corresponding IgG were added to the cell lysate, and the mixture was then incubated overnight at 4˚C with rotation. Anti-protein argonaute-2 (AGO2; cat. no. 2897; Cell Signaling Technology, Inc.), and normal rabbit IgG (cat. no. PP64B; EMD Millipore; Merck KGaA) were used for the RIP assay.

Target miRNA prediction. TargetScan V7.0 (http://www.targetscan. org/) provides information concerning humans, rats and other species and predicts target genes by searching the conserved 8 and 7mer sites that match each miRNA seed region (17). StarBase database (http://starbase.sysu.edu.cn) also includes information on the prediction of microRNA targets in the genomes of human and other species, as well as miRNA expression profiles in different tissues and diseases (18). With the two forecasting tools, comparisons of the relative mRNA expression levels among the different groups of samples was performed.

Statistical analysis. Data are presented as the mean ± SEM from three independent experiments. All data were analyzed using the GraphPad Prism v6.0 (GraphPad Software, Inc.). Survival curves were generated using the Kaplan-Meier method, and a log-rank test was used for comparison. Student's t-test, χ^2 test or one-way ANOVA was utilized as appropriate. Both paired and unpaired Student's t-tests were used correspondently. ANOVA was performed to evaluate the differences followed by Tukey's post hoc test. The correlation between the expression levels of two genes was analyzed using Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC00858 is increased in CRC tissues. Dysregulation of LINC00858 has been previously associated with CRC, however, the mechanisms involved remain elusive (12). To validate the dysregulated expression in CRC tissues, the levels of LINC00858 in 60 colorectal tumor tissues and 60 adjacent normal tissues were detected. In accordance with the aforementioned study (13), the expression level of LINC00858 was significantly higher in colorectal tumor tissues compared with that in adjacent normal tissues (Fig. 1A). The relationship between LINC00858 expression level and clinical outcomes of patients with CRC was investigated further (Table I). High expression of LINC00858 was significantly associated with poor overall survival time (Fig. 1B; P<0.001).

LINC00858 knockdown suppresses TP53-WT CRC cell proliferation and induces apoptosis. To further clarify the functional role of LINC00858 in CRC, the following assays were performed. Firstly, LINC00858 expression levels were evaluated in six TP53-WT CRC cell lines (RKO, HCT116, Colo-678, LoVo, SW48 and LS174T) and human normal colonic fibroblast (CCD-112CoN) cells and high expression levels were revealed in all CRC cell lines compared to CCD-112CoN. RKO and SW48 cells were markedly higher compared with the other cell lines (Fig. 1C) (14). Therefore, these two cell lines were selected for further experiments and were subsequently transfected with sh-LINC00858 to knockdown the target lncRNA (Fig. 2A). Subsequently, viability and colony formation assays were performed. Cell proliferation

Table I. Clinical characteristics of patients with CRC.

Characteristics	N	LINC00858 expression		P-value
		Low, n (%)	High, n (%)	
Sex				0.264
Male	72	39 (54)	33 (46)	
Female	48	21 (44)	27 (56)	
Age (years)				0.581
≤60	53	25 (47)	28 (53)	
>60	67	35 (42)	32 (48)	
Size (mm)				<0.001[a]
≤7	74	47 (64)	27 (36)	
>7	46	13 (28)	33 (72)	
Tumor invasion depth				0.007[a]
T1-T2	89	51 (57)	38 (43)	
T3-T4	31	9 (29)	22 (71)	
Lymph node metastasis				0.553
Negative	83	43 (52)	40 (48)	
Positive	37	17 (46)	20 (54)	
Distant metastasis				0.001[a]
Negative	91	53 (58)	38 (42)	
Positive	29	7 (24)	22 (76)	
TNM stage				<0.001[a]
I+II	90	54 (60)	36 (40)	
III+IV	30	6 (20)	24 (80)	

[a]Significant at P<0.05.

was revealed to be significantly suppressed after transfection (Fig. 2B-D). To investigate further, the cell cycle and cell apoptosis analysis was performed. Cell cycle assays revealed that sh-LINC00858 transfection increased the cell population in the G_0/G_1 phase and decreased the cell population in S phase compared with that in the control groups in both CRC cell lines (Fig. 2E and F). Cell apoptosis analysis indicated that downregulation of LINC00858 induced a significant increase in apoptosis of CRC cells compared with that in the control groups (Fig. 2G and H). Collectively, these results revealed that downregulation of LINC00858 suppressed CRC cell proliferation potentially through induction of G_0/G_1 phase arrest and apoptosis.

LINC00858 sponges miR-25-3p in CRC. Previous studies proposed that lncRNAs modulate tumor growth and development by competitively binding to miRNAs (19). Therefore, the potential miRNAs which may have binding sites with LINC00858 were predicted using StarBase database (http://starbase.sysu.edu.cn). As revealed in Fig. 3A, the binding site of miR-25-3p on LINC00858 was predicted by StarBase. This interaction between LINC00858 and miR-25-3p was subsequently validated. Luciferase reporter assays revealed that miR-25-3p mimics significantly reduced the luciferase activity of LINC00858-WT vectors, with no marked change in LINC00858-Mut vectors (Figs. S1B and 3B and C). RIP assays revealed that LINC00858 and miR-25-3p were co-immunoprecipitated by the AGO2 antibody but not the IgG antibody (Fig. 3D). Next, the expression levels of miR-25-3p in human CRC tissues was evaluated. The expression level of miR-25-3p was significantly decreased in CRC tumor tissues compared with that in adjacent normal tissues (Fig. 3E). Pearson's correlation analysis revealed the negative correlation between the expression levels of LINC00858 and miR-25-3p (Fig. 3F). Furthermore, miR-25-3p was revealed to be downregulated in all the cell lines with high LINC00858 expression levels, while in the cell line with a low LINC00858 expression level, miR-25-3p was upregulated (Fig. 3G). In addition, knockdown of LINC00858 significantly increased the expression levels of miR-25-3p in RKO and SW48 cells (Fig. 3H and I). These results indicated that LINC00858 sponged miR-25-3p in CRC.

miR-25-3p suppresses CRC cell proliferation by inducing apoptosis. To further explore the role of miR-25-3p in the development of CRC, the following experiments were performed. sh-LINC00858 and miR-25-3p inhibitor were co-transfected into RKO and SW48 cells and the cell viability after transfection was determined. The data in the present study revealed that the cell viability was significantly recovered in the co-transfection group, compared with that in the cells transfected with sh-LINC00858

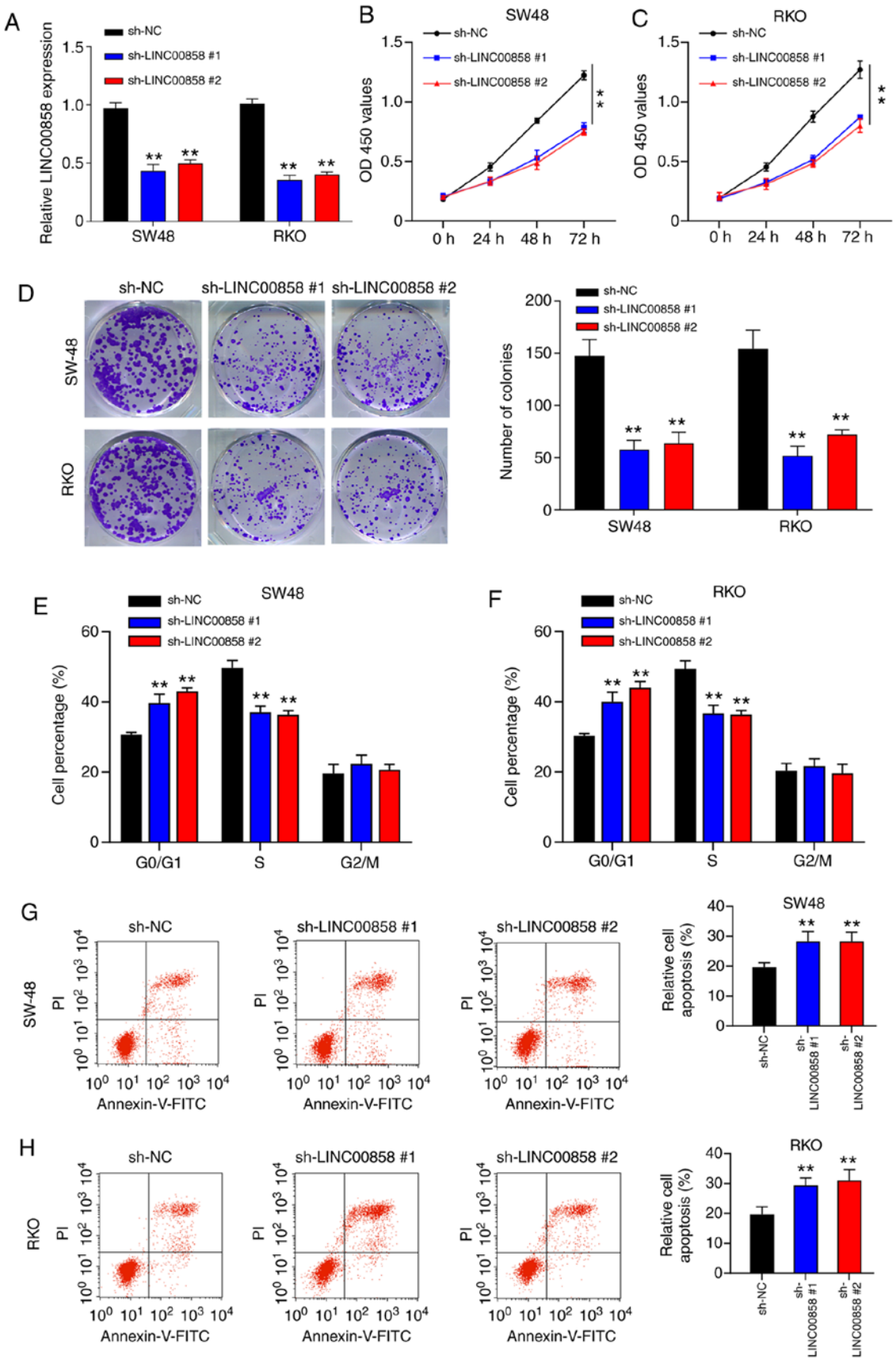

Figure 2. Effect of LINC00858 on proliferation, colony formation, the cell cycle and apoptosis of RKO and SW48 cells. (A) LINC00858 was inhibited by LINC00858 shRNAs, and the expression level of LINC00858 was assessed using reverse transcription-quantitative PCR. (B and C) Cell proliferation was quantified using Cell Counting Kit-8 assays after transfecting LINC00858 shRNAs in (B) SW48 and (C) RKO cells. (D) Colony formation assays were performed after transfecting LINC00858 shRNAs in RKO and SW48 cells for 14 days (left) and the number of cell colonies was quantified (right). (E and F) Quantification of the cell cycle flow cytometric data of (E) SW48 and (F) RKO cells transfected with LINC00858 shRNAs. (G and H) Cell apoptosis was detected using flow cytometry (left) and the percentage of apoptotic cells was quantified (right) in (G) SW48 and (H) RKO cells transfected with LINC00858 shRNAs. Data are presented as the mean ± SEM; n≥3. *P<0.05; **P<0.01. sh, short hairpin; NC, negative control.

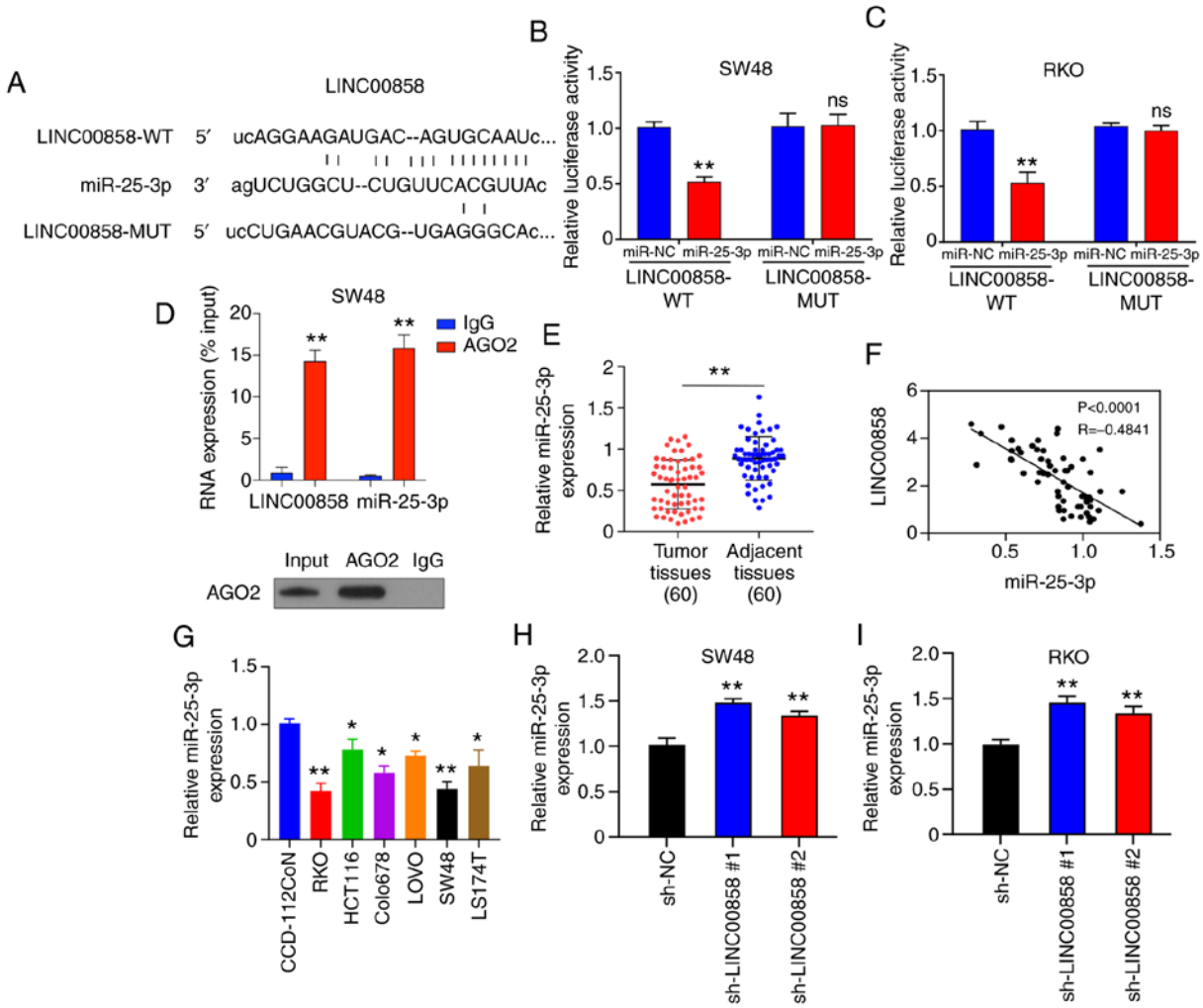

Figure 3. miR-25-3p is a direct target of LINC00858. (A) Potential binding region between miR-25-3p and LINC00858 was predicted using StarBase V2.0 database. (B) miR-25-3p was validated as a direct target of LINC00858 using dual luciferase reporter assays in (B) SW48 and (C) RKO cells. (D) RIP assay confirmed the interaction between miR-25-3p and LINC00858. (E) The expression levels of miR-25-3p in CRC tissues (n=60) compared with that in adjacent normal tissues (n=60) were assessed using qPCR. (F) Correlation between LINC00858 and miR-25-3p expression levels was analyzed using Pearson's correlation analysis. (G) The expression levels of miR-25-3p in TP53-WT CRC cell lines (RKO, HCT116, Colo-678, LoVo, SW48 and LS174T) and human normal colonic fibroblast (CCD-112CoN) were assessed using qPCR. (H and I) The expression levels of miR-25-3p in (H) SW48 and (I) RKO cells transfected with LINC00858 shRNAs. Data are presented as the mean ± SEM; n≥3. *P<0.05; **P<0.01. CRC, colorectal cancer; miR, microRNA; q, quantitative; WT, wild-type; sh, short hairpin; MUT, mutant; AGO2, protein argonaute-2.

alone (Figs. S1C and 4A-C). Next, colony formation assay after the transfection as aforementioned, was performed. Colony formation assay indicated that colony formation was significantly increased after co-transfection of sh-LINC00858 and miR-25-3p inhibitor (Fig. 4D), which was consistent with the cell viability analysis. Cell cycle analysis revealed that concurrent downregulation of LINC00858 and miR-25-3p decreased the cell proportions arrested in the G_0/G_1 stage and increased the cell proportions in the S stage, compared with that in cells downregulated of LINC00858 alone (Fig. 4E and F). Furthermore, the apoptosis assay indicated that cell apoptosis induced by sh-LINC00858 was partially reversed by inhibition of miR-25-3p (Fig. 4G and H). Collectively, LINC00858 promoted the development of CRC, partially by binding to miR-25-3p.

SMAD7 is the target gene of miR-25-3p. To investigate the potential downstream target of miR-25-3p, TargetScan was used (http://targetscan.org/vert_72) to identify potential targets, which have binding sites with miR-25-3p. As revealed in Fig. 5A, SMAD7 may be the target of miR-25-3p. To investigate the interaction between SMAD7 and miR-25-3p further, luciferase reporter assays were performed. RIP assays revealed that miR-25-3p was co-immunoprecipitated by the SMAD7 antibody but not the IgG antibody (Fig. S1A). miR-25-3p significantly reduced the luciferase activity in SMAD7-WT vector but not in SMAD7-MUT vector (Fig. 5B). The correlation between miR-25-3p and SMAD7 expression levels were explored and a negative correlation between miR-25-3p and SMAD7 with Pearson's correlation analysis was identified (Fig. 5C). These results demonstrated that SMAD7 was a possible downstream target of miR-25-3p.

The LINC00858/miR-25-3p/SMAD7 axis regulates the growth of CRC cells. To verify the relationship between LINC00858, miR-25-3p and SMAD7, RT-qPCR and western

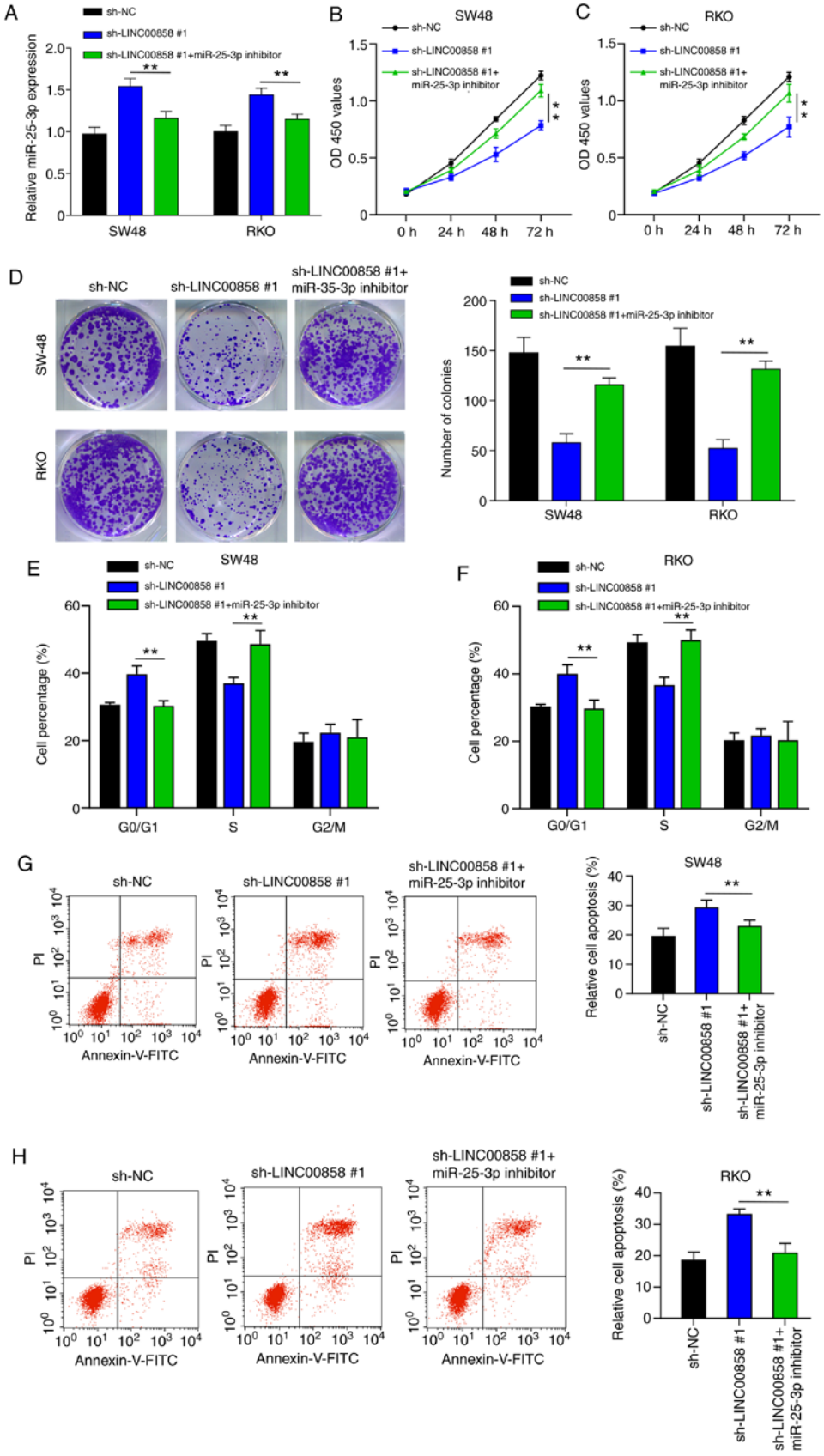

Figure 4. miR-25-3p inhibition reverses the reduced proliferation induced by LINC00858 knockdown. (A) The expression levels of miR-25-3p after transfection with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and miR-25-3p inhibitor in CRC cells. (B and C) Cell Counting Kit-8 assays were conducted to analyze the proliferation of (B) SW48 and (C) RKO cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and miR-25-3p inhibitor. (D) Representative images of colony formation assays (left) and quantification of the number of cell colonies (right). (E and F) Quantification of cell cycle flow cytometry data of (E) SW48 and (F) RKO cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and miR-25-3p inhibitor. (G and H) Quantification of cell apoptosis flow cytometry data of (G) SW48 and (H) RKO cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and miR-25-3p inhibitor. Data are presented as the mean ± SEM; n≥3. *P<0.05; **P<0.01. miR, microRNA; sh, short hairpin; CRC, colorectal cancer; NC, negative control.

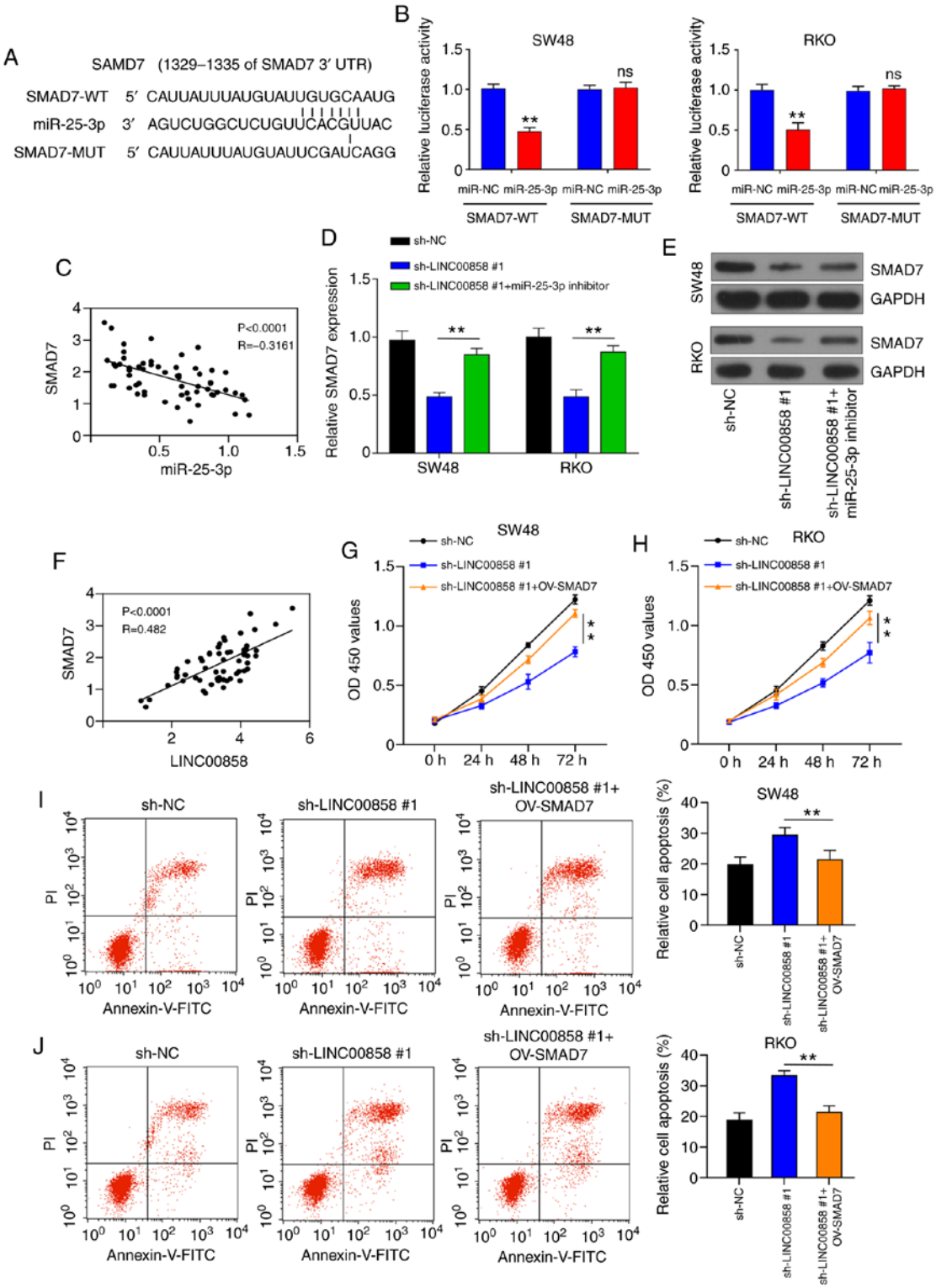

Figure 5. Smad7 is a direct target of miR-25-3p. (A) The potential binding site of miR-25-3p within the 3'-UTR of Smad7 as predicted by TargetScan database. (B) Smad7 was validated as a direct target of miR-25-3p using dual luciferase reporter assays in SW48 and RKO cells. (C) Pearson's correlation analysis was used to analyze the relationship between Smad7 and miR-25-3p expression levels. The expression levels of Smad7 in cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and miR-25-3p inhibitor were evaluated using (D) reverse transcription-quantitative PCR and (E) western blot analysis in RKO and SW48 cells. (F) The correlation between Smad7 and LINC00858 expression levels was analyzed using Pearson's correlation analysis. Cell proliferation of (G) SW48 and (H) RKO cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and Smad7 overexpression plasmid was detected using Cell Counting Kit-8 Kit. (I and J) Quantified cell apoptosis flow cytometric data of (I) SW48 and (J) RKO cells transfected with LINC00858 shRNAs alone or a combination of LINC00858 shRNAs and Smad7 overexpression plasmid. Data are presented as the mean ± SEM; n≥3. **P<0.01. miR, microRNA; sh, short hairpin; UTR, untranslated region; MUT, mutant; WT, wild-type; NC, negative control; OD, optical density.

blot analysis was performed. The results indicated that LINC00858 knockdown downregulated the expression of SMAD7, which was reversed with the addition of miR-25-3p inhibitor (Fig. 5D and E). Correlation analysis also revealed

a positive correlation between LINC00858 and SMAD7 expression levels (Fig. 5F). These data indicated that LINC00858 may regulate proliferation of CRC through the LINC00858/miR-25-3p/SMAD7 axis. To verify our hypothesis, rescue assays were performed. Cell viability assays revealed that LINC00858 knockdown-induced inhibition of cell proliferation was partially reversed by SMAD7 overexpression in RKO and SW48 cells (Fig. 5G and H). In addition, cell apoptosis induced by LINC00858 knockdown was also rescued by SMAD7 overexpression (Fig. 5I and J). Collectively, the data in the present study demonstrated that the LINC00858/miR-25-3p/SMAD7 axis regulated the growth and development of CRC cells.

Discussion

lncRNAs are largely reported to be engaged in the initiation and progression of various types of cancers, but the role of lncRNAs in CRC remains to be elucidated (5,11). In the present study, it was revealed that a newly identified lncRNA, LINC00858, was significantly increased in CRC clinical tissues and associated with poor prognosis. The functional data revealed that LINC00858 served as a competitive endogenous RNA to impair miR-25-3p activity, increasing the expression of miR-25-3p target gene, SMAD7 and thus promoting CRC cell growth. Notably, it was also demonstrated that overexpression of SMAD7 reversed the restriction of CRC proliferation induced by downregulation of LINC00858 expression. Collectively, it was established that lncRNA LINC00858 may be used as a potential diagnostic and therapeutic target in CRC.

LINC00858 is a newly identified lncRNA, which is located on 10q23.1 (12). In recent years, several studies have investigated the role of LINC00858 in various types of cancers. In non-small cell lung cancer, LINC00858 promoted cell proliferation and induced cell migration and invasion through the LINC00858/miR-422a/kallikrein-related peptidase 4 axis (20). In lung cancer, LINC00858 was also reported to accelerate the proliferation and invasion through the miR-3182/matrix metalloproteinase 2 pathway (21). In osteosarcoma, LINC00858 was confirmed to sponge miR-139, thereby upregulating CDK14 to promote the tumorigenesis and progression of osteosarcoma (22). Yamada *et al* (12) reported that LINC00858 was upregulated in CRC by RNA sequencing for the first time. Sha *et al* (13) revealed that LINC00858 could promote cell proliferation, migration and invasion in TP53-mutant CRC cell lines (HT-29, HCT-15, SW837 and SW1463) through the miR-22-3p/YWHAZ axis. As is recognized, CRC patients often exhibit diverse phenotypes under the different status of TP53, which in turn indicates different therapeutic approaches for CRC (23-25). However, the expression pattern and role of LINC00858 in TP53-WT CRC cell lines remains largely unknown. In the present study, two cell lines (HCT-116 and SW48) were selected with notably high expression of LINC00858 from seven common TP53-WT CRC cell lines to investigate the possible function of LINC00858 in TP53-WT CRC. It was revealed that LINC00858 knockdown significantly inhibited CRC cell proliferation, consistent with the role of LINC00858 in TP53-mutant CRC. It was further revealed that LINC00858 knockdown induced CRC cell cycle arrest in the G_0/G_1 stage and the cell apoptosis rate was significantly increased. These results indicated that LINC00858 promoted cell proliferation in TP53-WT CRC cell lines.

lncRNAs can act through diverse mechanisms, including genomic targeting, regulation in *cis* or *trans*, and antisense interference (26,27). In recent years, the role of lncRNA as competitive endogenous RNA in multiple types of cancer has gained attention and has been wildly investigated (19). The competitive endogenous RNA can decrease the stability of target miRNA, thereby upregulating the expression of the miRNA target gene (19). In the present study, the potential miRNA that LINC00858 may regulate in CRC progression was explored. It was revealed that LINC00858 contained the binding site of miR-25-3p using bioinformatics analysis and the interaction between LINC00858 and miR-25-3p was further validated using a luciferase assay and RIP. Moreover, downregulation of LINC00858 increased the expression of miR-25-3p. In addition, cell apoptosis induced by LINC00858 knockdown could be reversed by miR-25-3p inhibitor. These results indicated that LINC00858 may exert an oncogenic function by inhibiting the expression of miR-25-3p.

Several studies have reported that SMAD7 plays a pro-tumorigenic role in CRC and downregulation of SMAD7 was correlated with reduced cell proliferation and invasion (28,29). It is also proposed that inhibition of SMAD7 promoted transforming growth factor (TGF)-β-induced EMT in CRC, which indicates that SMAD7 can also play an anti-tumorigenic role in CRC (30). SMAD7 is a negative regulatory protein of the TGF-β signaling pathway (31). SMAD7 can inhibit TGF-β signaling by binding to TGF-β type I receptor (TβRI) and preventing the receptor-Smad phosphorylation and activation, recruiting E3 ubiquitin ligases to TβRI and inducing its degradation and interacting with the regulatory subunit of the protein phosphatase to inactivate TβRI (32-34). Previous studies have indicated that TGF-β can regulate the expression of miRNA (35). Boguslawska *et al* (36) reported that TGF-β can upregulate the expression of miR-25-3p, thus promoting the progression of renal cell carcinoma. However, it remains unknown whether miR-25-3p may in turn modulate the TGF-β signaling pathway in CRC. In the present study, the TargetScan database was used to explore potential targets that could bind with miR-25-3p. It was revealed that Smad7, a TGF-β signaling inhibitory protein, directly interacted with miR-25-3p and this was validated using a luciferase assay and Smad7 expression was downregulated by miR-25-3p. Subsequently, it was revealed that downregulation of LINC00858 could decrease Smad7 expression and this effect could be reversed by the addition of miR-25-3p inhibitor. Finally, it was confirmed that the inhibition of CRC cell proliferation induced by LINC00858 knockdown could be rescued by the overexpression of Smad7. In total, it was demonstrated that the novel lncRNA, LINC00858, could promote the progression of TP53-WT CRC through the the LINC00858/miR-25-3p/Smad7 axis.

In conclusion, it was revealed that LINC00858 was increased in CRC tumor tissues and may be used as a predictive marker of prognosis. Notably, to the best of our knowledge this is the first study to establish that the role of LINC00858 could accelerate the progression of TP53-WT CRC through

the miR-25-3p/Smad7 pathway. Thus, LINC00858 could be adopted as a novel diagnostic and therapeutic target in the management of CRC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant. no. 81974381), the Hubei Province Health and Family Planning Scientific Research project (grant. no. WJ2015MB068) and the Scientific Research Starting Foundation for Returned Overseas Staffs in Tongji Hospital 2016.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QF designed the study. JZ and JT performed the cell experiments and PCR analysis. JZ and QF drafted the manuscript. All authors read and approved the final manuscript for publication and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was reviewed and approved by the Ethics Committee of Medical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Written informed consent for publication was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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