LncRNA CDKN2B-AS1 sponges miR-28-5p to regulate proliferation and inhibit apoptosis in colorectal cancer

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Abstract. Long noncoding RNA (lncRNA) CDKN2B-antisense RNA 1 (AS1) functions as a tumor oncogene in numerous cancers. However, the roles and mechanism of CDKN2B-AS1 in colorectal cancer (CRC) have not been explored. The present study aimed to investigate whether and how CDKN2B-AS1 contributes to CRC progression. The data revealed that CDKN2B-AS1 expression was upregulated in CRC tissues. Loss-of-function assays demonstrated that CDKN2B-AS1 in CRC modulated cell proliferation and apoptosis, which was mediated by cyclin D1, cyclin-dependent kinase (CDK) 4, p-Rb, caspase-9 and caspase-3. Bioinformatics analysis and luciferase reporter assays indicated direct binding of microRNA (miR)-28-5p to CDKN2B-AS1. Moreover, the results herein revealed that the expression of miR-28-5p was negatively correlated with that of CDKN2B-AS1 in CRC tissue. Moreover, CDKN2B-AS1 acted as a miR-28-5p competing endogenous RNA (ceRNA) to target and regulate the expression of URGCP. These findings indicated that CDKN2B-AS1 plays roles in CRC progression, providing a potential therapeutic target or novel diagnostic biomarker for CRC.

Introduction

Colorectal cancer (CRC) was the 3rd most common malignancy in Australia in 2017 overall, with estimated all-age incidence rates of 67.3 per 100,000 in males and 49.4 per 100,000 in females. Emergent evidence has suggested that the incidence of colorectal cancer in people under 50 years of age ('early-onset colorectal cancer') is rising in high-income countries (1). Among the various molecular drivers of CRC, genetic and epigenetic alterations are the main cause of the hereditary and sporadic forms (2). Factors involved in CRC include the characteristics and properties of tumors that provide information about the prognosis. Prognostic and predictive biomarkers are selection factors that facilitate treatment decision making and treatment response prediction, respectively, in patients (3).

Previous studies have revealed that noncoding RNAs directly act as tumor suppressors or oncogenes at the transcriptional or post-transcriptional level to regulate the occurrence and development of tumors (4,5). Long noncoding RNAs (lncRNAs) are an interesting class of transcripts longer than 200 nucleotides that have functions other than coding proteins (6,7). Emerging evidence has revealed that lncRNAs play a role in a variety of human cancers and can even be used as predictive biomarkers or therapeutic targets for cancer treatment (8,9). LncRNA CDKN2B-antisense RNA 1 (AS1) has already been implicated in the advancement of various tumors (8,9). The lncRNA CDKN2B-AS1/miR-141/cyclin D network has been revealed to regulate the progression and metastasis of renal cell carcinoma (8). The lncRNA CDKN2B-AS1/miR-324-5p/ROCK1 axis has been revealed to regulate cell cycle progression in laryngeal squamous cell cancer (10). MicroRNAs (miRNAs) are small noncoding RNAs 18-25 nucleotides in length that function by regulating target genes. Studies have demonstrated that the differential expression of miRNAs has an important relationship with the occurrence, development and prognosis of CRC (10-12). LncRNAs act as sponges/competing endogenous RNAs (ceRNAs) to regulate the post-transcriptional regulation of miRNAs and are factors in the etiology of numerous diseases (12). Thus, this study focused on the differential expression of CDKN2B-AS1 and related miRNAs in CRC.

CDKN2B-AS1 may play different roles during carcinogenesis in cancers (8-10). In the present study, the biological role and functions of CDKN2B-AS1 were investigated in CRC. The expression of CDKN2B-AS1 was detected in CRC tissues and cancer cell lines. Moreover, functional experiments were designed to reveal the effects

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Abbreviations: CRC, colorectal cancer; lncRNA, long noncoding RNA; miRNAs or miRs, microRNAs; ceRNAs, competing endogenous RNAs; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; URGCP, upregulator of cell proliferation

Key words: CDKN2B-AS1, miR-28-5p, colorectal cancer, proliferation, apoptosis

of CDKN2B-AS1 on the malignant behavior of CRC cells. Furthermore, the relevant underlying mechanisms of CDKN2B-AS1 in CRC were discussed in depth. Collectively, it was determined that CDKN2B-AS1 may be involved in the progression of CRC through the regulation of proliferation.

Materials and methods

CRC patients and tissue samples. The 69 collected CRC tissues and their corresponding adjacent non-tumor tissue samples (sampled at more than 5 cm from the tumor, 48 males and 21 females; age range, 30-65 years) were obtained from Qingdao Municipal Hospital (Qingdao, China) between May 2018 and May 2019 and stored at -80˚C for further study. The inclusion criteria was as follows: The patients had to be diagnosed with colon cancer by pathological diagnosis and had to be aged between 20 and 75 years. Patients were excluded if they suffered from other malignant tumors or if they had been treated with radiotherapy or chemotherapy within 3 months before enrolment. The study was performed in accordance with clinical study protocols and the principles of the Declaration of Helsinki (modified 2018). All patients provided informed consent for research purposes. The procedures in the present study were approved (approval no. 2018-R-32) by the Qingdao Municipal Hospital Ethics Review Committee.

Cell lines and culture conditions. Human CRC cell lines (LOVO, HCT-116, DLD-1, SW480, and RKO), a human normal cell line (NCM460) and 293T cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. The cells were cultured in Roswell Park Memorial Institute (RPMI-1640; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 atmosphere.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from CRC tissues and CRC cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) was used to reverse transcribe mRNA to cDNA according to the manufacturer's instructions (temperature protocol: 42˚C for 60 min, 70˚C for 5 min and then maintained at 4˚C). The relative expression level of CDKN2B-AS1 was detected by a SYBR-Green PCR Master Mix kit (Takara, Biotechnology Co., Ltd.) according to the manufacturer's instructions. RT-qPCR was performed at 95˚C for 1 min, 40 cycles at 95˚C for 10 sec and 58˚C for 40 sec. The sequences of the specific primers were as follows: CDKN2B-AS1 forward, 5'-GACTTC TGTTTTCTGGCCACC and reverse, TCGGGAAAGGAT TCCAGCAC-3'; upregulator of cell proliferation (URGCP) forward, 5'-CGCAATCATCTCCTTCCATT-3' and reverse, 5'-GATTTGGGAGAAGTAGCCCC-3'; GAPDH forward, 5'-GGTGCTGAGTATGTCGTGGAGTCTA-3' and reverse, 5'-TCTTGAGGGAGTTGTCATATTTCTC-3'. GAPDH expression was used as an endogenous control, and the $2^{-\Delta\Delta Cq}$ method (13) was used to calculate the results.

Bioinformatics analysis. lncRNASNP2 (http://bioinfo.life.hust.edu.cn/lncRNASNP#!/mirna) and TargetScan 7.2 (http://www.targetscan. org/) were used to predict the putative target genes of CDKN2B-AS1 and miR-28-5p.

Knockdown of CDKN2B-AS1 expression. Two si-CDKN2B-AS1 small interfering (si)RNAs (50 nM) targeting CDKN2B-AS1 (si#1 and si#2) were synthesized by Guangzhou RiboBio Co., Ltd. All cells were transfected with siRNAs at room temperature according to the manufacturer's instructions for Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.) for 24-h transfection. miR-28-5p mimics (50 nM) and inhibitors (100 nM) were synthesized by Shanghai GenePharma Co., Ltd. miR-28-5p (50 nM) was transfected into cells at room temperature according to the instructions for Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.) for 24-h transfection. The siRNAs, miR-28-5p mimic, inhibitor and control sequences are provided in Table SI, and si-negative control (NC) (50 nM) used in the present study was scrambled.

Dual-luciferase reporter plasmid transfection. pmiR-RB-REPORT™ plasmids [CDKN2B-AS1-wild-type (WT) and URGCP-WT] and their related mutant plasmids [CDKN2B-AS1-mutant (MUT) and URGCP-MUT] were synthesized by Guangzhou RiboBio Co., Ltd. Cells ($5x10^4$) were seeded into 24-well plates and co-transfected with the reporter plasmids alongside miR-28-5p mimic (50 nM) or NC mimic (50 nM) applying Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequently, 48 h later, the transfected cells were lysed to detect their luciferase activity by employing a dual-luciferase reporter assay system (Promega Corporation). The dual-luciferase reporter assay system was used to assess firefly luciferase activity which was normalized to *Renilla* luciferase activity.

Cell Counting Kit-8 (CCK-8) assay. The proliferation of CRC cells was assessed by CCK-8 assay (Meilun Biotechnology Co., Ltd.). After transfection with siRNA, the cells ($2x10^3$) were plated and cultured at 37˚C in 96-well plates for 24, 48, 72, and 96 h, and then 20 μl CCK-8 solution was added to the wells. To estimate cell proliferation, the absorbance of the cell medium at 490 nm was measured using a multifunctional microplate reader (SpectraMax M5).

Colony formation assay. The cells (1,000/well) were seeded and cultured at 37˚C for 10-14 days after transfection with siRNAs. Subsequently, they were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.5% crystal violet at room temperature for 30 min, and the number of colonies was counted under an inverted light microscope at a magnification of x100 (D850; Nikon Corporation).

In situ hybridization. DIG-labeled LNA-CDKN2B-AS1 probes were synthesized following the manufacturer's instructions by Guangzhou RiboBio Co., Ltd. and the probe sequences are available upon request. In brief, a 5-mm section of paraffin-embedded tissue was fixed with 4% formaldehyde solution, washed, and permeabilized with Triton X-100

solution for 5 min at room temperature. The DIG-labeled LNA-CDKN2B-AS1 probe was incubated with the tissues for hybridization at 37˚C, overnight. Then, the expression was determined using diaminobenzidine solution (1:900; Boster Biological Technology) for 3 min at room temperature, and the staining intensity was observed using a BX51 light microscope (Olympus Corporation). The staining was quantified by counting the number of positive cells at a magnification of x400.

Subcellular fractionation. A nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology) and TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were used to extract cytoplasmic and nuclear RNA according to the manufacturer's instructions. CDKN2B-AS1 expression in the cytoplasmic and nuclear fractions was assessed by qPCR. The thermocycling conditions were as follows: 95˚C for 30 sec, 95˚C for 3 sec and 60˚C for 30 sec, for 40 cycles; 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. GAPDH and U6 were the cytoplasmic and nuclear controls, respectively. The primers of U6 were as follows: forward, 5'-CTCGCTTCG GCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGC GT-3'.

Protein extraction and western blotting. RIPA buffer (Thermo Fisher Scientific, Inc.) containing protease inhibitor cocktail (Roche Diagnostics) was used to extract total protein from CRC tissues and CRC cell lines. The quantification of total protein was conducted with a BCA Protein Assay kit (Sangon Biotech Co., Ltd.). Equal amounts of proteins (20 μg) were separated by 10% SDS-PAGE gels, followed by transferring onto PVDF membranes (Sigma-Aldrich; Merck KGaA). After being blocked with 5% fat-free milk at room temperature for 2 h, the blots were probed with primary antibodies. Primary antibodies anti-cyclin D1 (1:1,000; product no. 55506), cyclin-dependent kinase (CDK)4 (1:1,000; product no. 12790), Rb (1:1,000; product no. 9309), phosphorylated (p)-Rb (1:1,000; product no. 8516), caspase-9 (1:1,000; product no. 9508), cleaved caspase-9 (1:1,000; product no. 9505), caspase-3 (1:1,000; product no. 9662), cleaved caspase-3 (1:1,000; product no. 9664; all from Cell Signaling Technology, Inc.), URGCP (1:3,000; cat. no. PA5-44534; Thermo Fisher Scientific, Inc.), and β-actin (1:1,000; product no. 4970; Cell Signaling Technology, Inc.) were incubated with the PVDF membranes overnight at 4˚C. The PVDF membranes were then incubated with HRP-labeled secondary antibody (1:1,000 dilution; product no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. An enhanced chemiluminescence kit (EMD Millipore) was used to expose the membrane. Images were captured through autoradiography with X-ray film. ImageJ 1.8.0 software (National Institutes of Health) was used to quantify the density of the target bands.

Statistical analysis. The data are presented as the mean ± standard deviation and were analyzed using the statistical software package SAS 8.0 for Windows (SAS Institute, Inc.). The paired Student's t-test was used to analyze the CDKN2B-AS1 levels in 69 paired CRC and control tissues. The unpaired Student's t-test was applied for two-group comparisons of the other assays, and one-way ANOVA with Tukey's post hoc test was used for multiple group comparisons. Two-tailed Pearson's correlation was used to analyze the correlation between CDKN2B-AS1 and miR-28-5p levels. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CDKN2B-AS1 is overexpressed in CRC tissues and cells. qPCR was applied to detect the expression of CDKN2B-AS1 in CRC. The results revealed that CDKN2B-AS1 was highly overexpressed in CRC tissue compared with matched adjacent normal tissue (n=6; $P<0.01$; Fig. 1A). Furthermore, *in situ* hybridization of a DIG-labeled LNA-CDKN2B-AS1 probe revealed that CDKN2B-AS1 was mainly distributed in the cytoplasm of CRC tissue (Fig. 1B). The expression of CDKN2B-AS1 in CRC cell lines (LOVO, HCT-116, DLD-1, SW480 and RKO) was also detected by qPCR. It was revealed that the expression of CDKN2B-AS1 was upregulated in CRC cell lines compared with the normal colonic cell line (n=6; $P<0.01$; Fig. 1C). In LOVO and RKO cells, the expression of CDKN2B-AS1 was markedly higher than that in the other cells (n=6; $P<0.05$; Fig. 1C); hence, the LOVO and RKO cell lines were used for the following study. Two siRNAs (si#1 and si#2) targeting CDKN2B-AS1 were used, and the knockdown efficiency of these siRNAs was investigated in both LOVO and RKO cells. CDKN2B-AS1 expression was significantly downregulated in LOVO cells compared with the control cells (n=6, $P<0.05$; Fig. 1D) and in RKO cells compared with the control cells (n=6, $P<0.05$; Fig. 1D).

Knockdown of CDKN2B-AS1 inhibits the proliferation of CRC cells. As the expression of CDKN2B-AS1 was markedly higher in both LOVO and RKO cells than in the other cells, experiments with CDKN2B-AS1 knockdown were performed in these cells. To determine the effect of CDKN2B-AS1 on the proliferation of both LOVO and RKO cells, CCK-8 assays were conducted. As revealed in Fig. 2A, the number of proliferating cells in the si-CDKN2B-AS1 transfection group was decreased compared with that in the NC group (n=6, $P<0.05$; Fig. 2A). Colony formation assays also demonstrated that there were fewer CRC cell colonies in the si-CDKN2B-AS1 group than in the control group (n=6, $P<0.05$; Fig. 2B). To determine the molecular mechanisms of si-CDKN2B-AS1-induced cell proliferation inhibition, cell cycle-related proteins cyclin D1, CDK4, Rb, p-Rb and apoptosis-related proteins (cleaved caspase-9 and cleaved caspase-3) were examined by western blotting. The results demonstrated that the protein levels of cyclin D1, and CDK4 were decreased in CDKN2B-AS1-knockdown CRC cells compared with the NC cells (n=6, $P<0.05$; Fig. 2C and D). Moreover, it is illustrated that p-Rb/Rb expression levels are increased after CDKN2B-AS1 knockdown compared with the negative control cells (n=6, $P<0.05$; Fig. 2C and D). The protein levels of cleaved caspase-9 and cleaved caspase-3 were upregulated in the knockdown cells compared with the NC cells (n=6, $P<0.05$; Fig. 2C and D). Therefore, these results indicated that CDKN2B-AS1 promoted CRC proliferation by regulating cell cycle-related and apoptosis-related proteins in CRC.

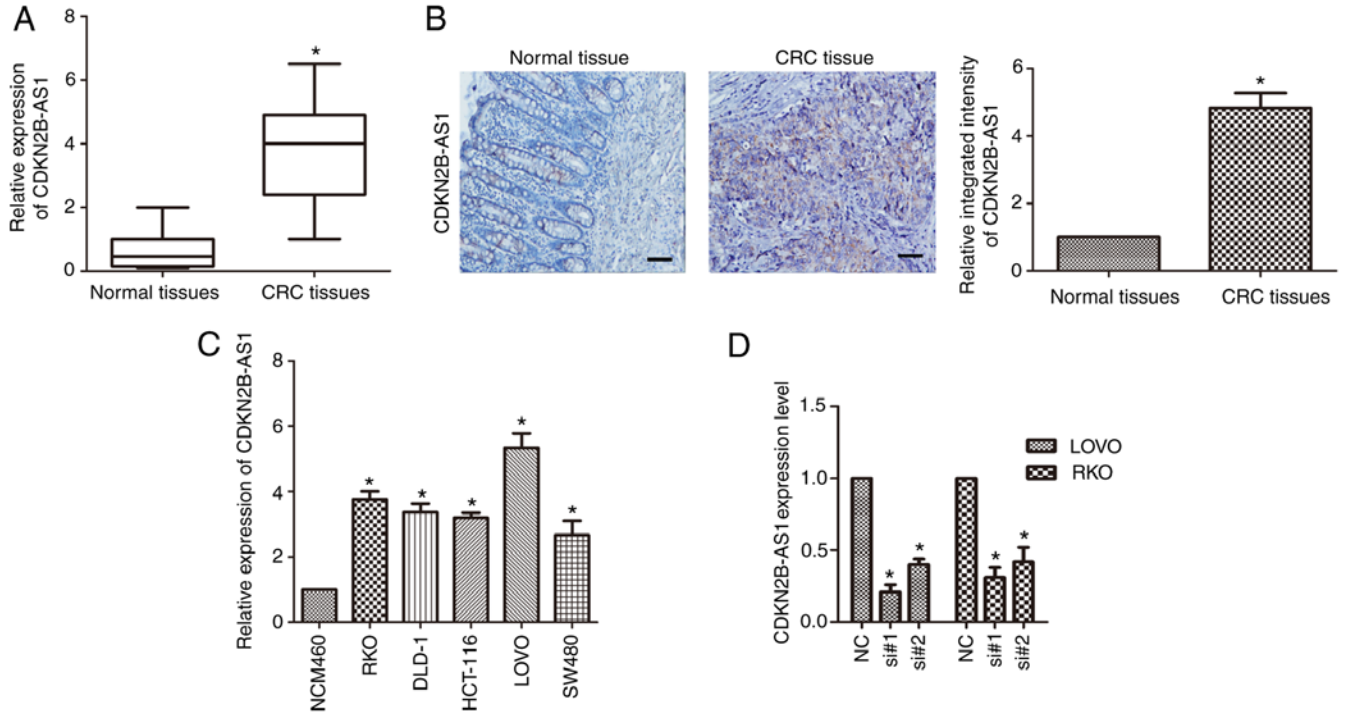

Figure 1. Aberrant CDKN2B-AS1 expression in CRC and cell lines. (A) The expression of CDKN2B-AS1 was significantly elevated in CRC tissues compared with adjacent non-tumor tissues. (B) *In situ* hybridization of a DIG-labeled LNA-CDKN2B-AS1 probe revealed that CDKN2B-AS1 was mainly distributed in the cytoplasm of CRC tissue. (C) The expression of CDKN2B-AS1 in CRC cell lines (LOVO, HCT-116, DLD-1, SW480, and RKO) was detected by quantitative PCR. (D) The knockdown efficiencies in cells transfected with si-CDKN2B-AS1. *P<0.05 vs. NC or NCM460; n=6. CDKN2B-AS1, CDKN2B-antisense RNA 1; CRC, colorectal cancer; si, small interfering; NC, negative control.

CDKN2B-AS1 sponges miR-28-5p in CRC cells. To further explore the molecular mechanism of CDKN2B-AS1 in the malignancy of CRC, a bioinformatics tool (lncRNASNP2; http://bioinfo.life.hust.edu.cn/lncRNASNP#!/mirna) was used to identify potential related miRNAs. The bioinformatics data demonstrated that miR-28-5p is a potential target of CDKN2B-AS1 (Fig. 3A). Subcellular fractionation revealed that CDKN2B-AS1 was principally located in the cytoplasm of CRC cells (Fig. 3B). This binding relationship was further validated by luciferase reporter assay. The results revealed that the luciferase activity of cells co-transfected with miR-28-5p and CDKN2B-AS1-WT reporter plasmids was decreased compared with that of cells transfected with CDKN2B-AS1-MUT alone or co-transfected with CDKN2B-AS1-MUT and miR-28-5p mimic (n=6, P<0.05; Fig. 3C). The expression of miR-28-5p in CRC cell lines was also detected by qPCR. It was revealed that the expression of miR-28-5p was downregulated in CRC cell lines compared with the normal colonic cell line (n=6; P<0.05; Fig. 3D). As the expression of miR-28-5p was markedly lower in both LOVO and RKO cells than in the other cells, the effect of upregulation of miR-28-5p expression was explored in these cells (n=6; P<0.05; Fig. 3D). Conversely, because the expression of miR-28-5p was higher in both SW480 and HCT-116 cells than in the other cells, the effect of downregulation of miR-28-5p expression was explored in SW480 and HCT-116 cells (n=6; P<0.05; Fig. 3D). Moreover, upregulation of miR-28-5p expression induced a reduction in CDKN2B-AS1 levels, whereas downregulation of miR-28-5p caused an increase in CDKN2B-AS1 expression by qPCR results (n=6, P<0.05; Fig. 3E and F). Furthermore, the correlation analysis revealed a negative relationship between miR-28-5p and CDKN2B-AS1 in CRC specimens (P<0.01, r=-0.714; Fig. 3G).

CDKN2B-AS1 function is suppressed by miR-28-5p. The results revealed that the binding relationship between CDKN2B-AS1 and miR-28-5p raised the possibility that miR-28-5p levels may be the intermediate between CDKN2B-AS1 and its oncogenic effects. Among the examined siRNAs, siRNA-1(si#1) exhibited the highest knockdown efficiency of CDKN2B-AS1 in cells, thus, this siRNA was selected in this experiment. To examine this possibility, CCK-8 and colony formation assays were performed. After transfection with the miR-28-5p inhibitor, CCK-8 assays revealed that the decrease in the number of live cells was partly attenuated in CDKN2B-AS1-knockdown CRC cells (n=6, P<0.05; Fig. 4A). The miR-28-5p inhibitor caused a significant increase in colony formation, whereas CDKN2B-AS1 knockdown abrogated this increase in colony formation (n=6, P<0.05; Fig. 4B and C). These data indicated that the CRC cell proliferation effects of CDKN2B-AS1 were mediated by miR-28-5p.

URGCP is a target of miR-28-5p. The potential targets of miR-28-5p were investigated to reveal the mechanism underlying the effects of miR-28-5p. It was revealed by bioinformatics tools that URGCP is a target of miR-28-5p (http://www.targetscan.org/vert_72/; Fig. 5A). After transfection with the miR-28-5p mimic, the expression of

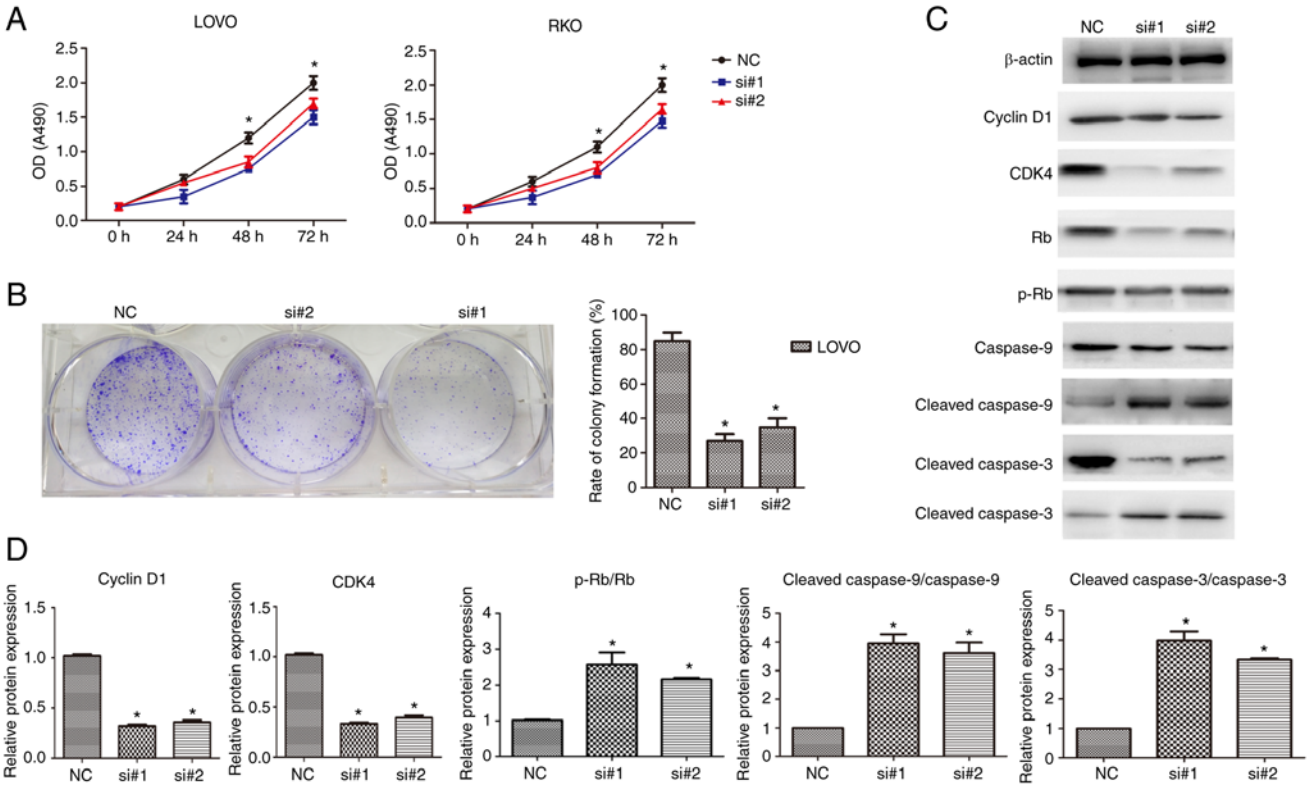

Figure 2. CDKN2B-AS1 knockdown inhibits the proliferation of colorectal cancer cells. (A) Knockdown of CDKN2B-AS1 impaired cell proliferation, as revealed by Cell Counting Kit-8 assay. (B) The rates of colony formation in the control (NC) and CDKN2B-AS1-knockdown groups. (C and D) Cell cycle-related proteins (cyclin D1, cyclin-dependent kinase 4, and phosphorylated Rb and apoptosis-related proteins (cleaved caspase-9 and cleaved caspase-3) were examined by western blotting. *P<0.05 vs. NC; n=6. CDKN2B-AS1, CDKN2B-antisense RNA 1; CDK4, cyclin-dependent kinase 4; p-, phosphorylated; si, small interfering; NC, negative control.

miR-28-5p was upregulated, while it was downregulated after transfection of the miR-28-5p inhibitor in both CRC cells and 293T cells (n=6, P<0.05; Fig. 5B). To verify the direct binding relationship between miR-28-5p and URGCP, a luciferase reporter system was used. After co-transfection of miR-28-5p mimics and URGCP-WT, the luciferase activity was significantly reduced, while the luciferase activity was not altered when cells were transfected with URGCP-MUT (n=6, P<0.05; Fig. 5C). Moreover, the qPCR and western blot results revealed that the miR-28-5p mimic suppressed the expression of URGCP in LOVO and RKO cells, and the miR-28-5p inhibitor upregulated the expression of URGCP in SW480 and HCT-116 cells (n=6, P<0.05; Fig. 5D-G).

CDKN2B-AS1 regulates URGCP expression via miR-28-5p and regulates the proliferation of CRC cells. After transfection with CDKN2B-AS1-siRNAs with or without a miR-28-5p inhibitor in CRC cells, the qPCR results revealed that knockdown of CDKN2B-AS1 reduced the expression of URGCP, whereas the miR-28-5p inhibitor partly abolished this decreasing effect (n=6, P<0.05; Fig. 6A). Western blot assays revealed that the downregulation of URGCP protein expression in CDKN2B-AS1-knockdown cells was rescued by miR-28-5p inhibitor transfection (n=6, P<0.05; Fig. 6B and C). To further verify whether the CDKN2B-AS1-mediated inhibition of proliferation was dependent on the activation of the miR-28-5p targeting URGCP, western blot analysis was used. The results demonstrated that the expression of cyclin D1 and CDK4, which was decreased in CDKN2B-AS1-knockdown cells, was partly rescued by miR-28-5p inhibitor transfection (n=6, P<0.05; Fig. 6D and E). Moreover, it is illustrated that p-Rb/Rb expression levels are increased after CDKN2B-AS1 knockdown and decreased after miR-28-5p inhibitor transfection. The increases in the levels of the cleaved fragments of caspase-9 and caspase-3 in CDKN2B-AS1 knockdown cells were partly attenuated by transfection with the miR-28-5p inhibitor (n=6, P<0.05; Fig. 6D and E). These data further demonstrated that the proliferation induced by CDKN2B-AS1 depletion in CRC cells was partly mediated by the miR-28-5p targeting URGCP.

Discussion

Accumulating evidence has indicated that lncRNAs can be used as biological markers and therapeutic targets of cancer and play a variety of roles in biological processes related to cancer (14). Recent studies have reported that CDKN2B-AS1 is involved in the occurrence and development of a variety of tumors (15-18). CDKN2B-AS1 has been revealed to regulate the malignancy of renal clear cell carcinoma by mediating NUF2 transcription (16). A recent study also revealed that CDKN2B-AS1-CDKN2B is involved in the molecular genetics of open-angle glaucoma pathogenesis (17). In a study conducted by Akbari *et al* CDKN2B-AS1 was revealed to be related to intrinsic apoptotic genes in CRC (18). However, the detailed functions of CDKN2B-AS1 and the mechanisms through which it exerts its biological functions are not well understood.

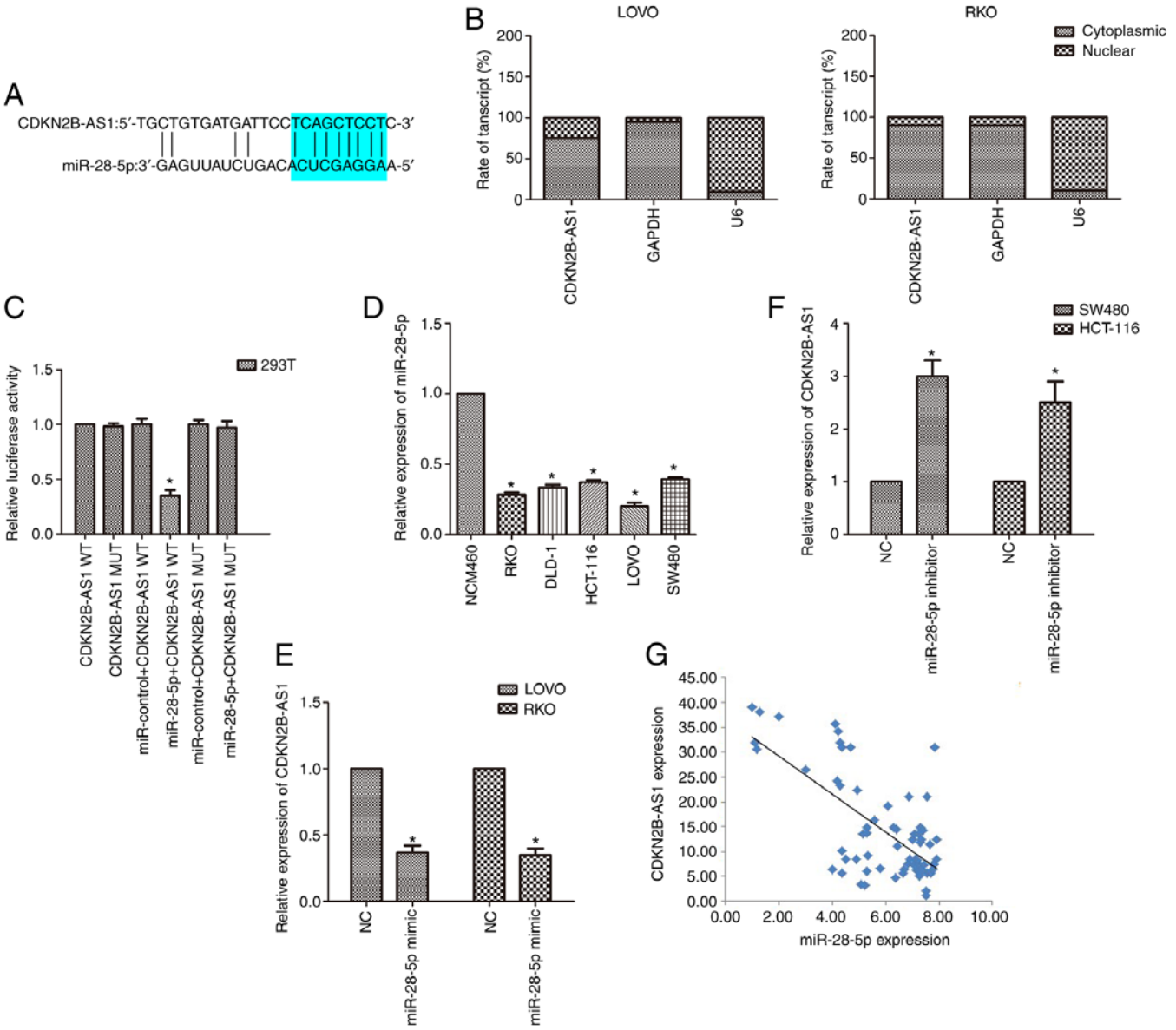

Figure 3. Negative regulation of CDKN2B-AS1 by miR-28-5p. (A) Predicted miR-28-5p binding sites in the CDKN2B-AS1 sequence. (B) Subcellular fractionation revealed that CDKN2B-AS1 was principally located in the cytoplasm of CRC cells. (C) The luciferase reporter assay revealed that the luciferase activity of cells co-transfected with miR-28-5p and CDKN2B-AS1-WT reporter plasmids was decreased compared with that of the cells transfected with the CDKN2B-AS1-MUT reporter plasmid with or without miR-28-5p co-transfection. *P<0.05. (D) The expression of miR-28-5p in CRC cell lines was also detected by qPCR. (E and F) Cells were transfected with a miR-28-5p inhibitor or mimic, and CDKN2B-AS1 expression was analyzed by qPCR. (G) miR-28-5p mRNA levels were plotted against CDKN2B-AS1 expression in CRC specimens (two-tailed Pearson's correlation, r=-0.714; P<0.01). *P<0.05 vs. NC or NCM460; n=6. CDKN2B-AS1, CDKN2B-antisense RNA 1; miR, microRNA; CRC, colorectal cancer; WT, wild-type; MUT, mutant; qPCR, quantitative PCR; NC, negative control.

In the present study, it was revealed that CDKN2B-AS1 was upregulated in CRC tissue vs. normal tissue. In addition, the expression of CDKN2B-AS1 in CRC cell lines was detected and it was revealed that CDKN2B-AS1 was overexpressed in CRC cells vs. normal cells. These results indicated that CDKN2B-AS1 could be a useful diagnostic biomarker or therapeutic target in CRC.

The role of CDKN2B-AS1 in CRC was also examined by assessing the biological behavior of CRC cell lines with altered expression of this target. It was revealed by CCK-8 and colony formation assays that the downregulation of CDKN2B-AS1 significantly inhibited the proliferation of CRC cells. Previous studies have confirmed that cyclin D1 promotes the G_1 phase transition of cells by activating CDK4, resulting in increased Rb phosphorylation (p-Rb) (19,20). Assessment of the mechanisms by which CDKN2B-AS1 knockdown affected proliferation revealed that the expression of cell cycle-related proteins (cyclin D1 and CDK4) was reduced in knockdown cell lines. Moreover, it is illustrated that p-Rb/Rb expression levels are increased after CDKN2B-AS1 knockdown. Emerging evidence has indicated that the upregulation of intrinsic apoptotic signals leads to cell death by recruiting and further activating the promoter caspase-9 and effector caspases (caspase-3/6/9) (21). In the present study, it was revealed that CDKN2B-AS1 knockdown induced the apoptosis of CRC cells by activating apoptosis-related proteins (caspase-9 and caspase-3).

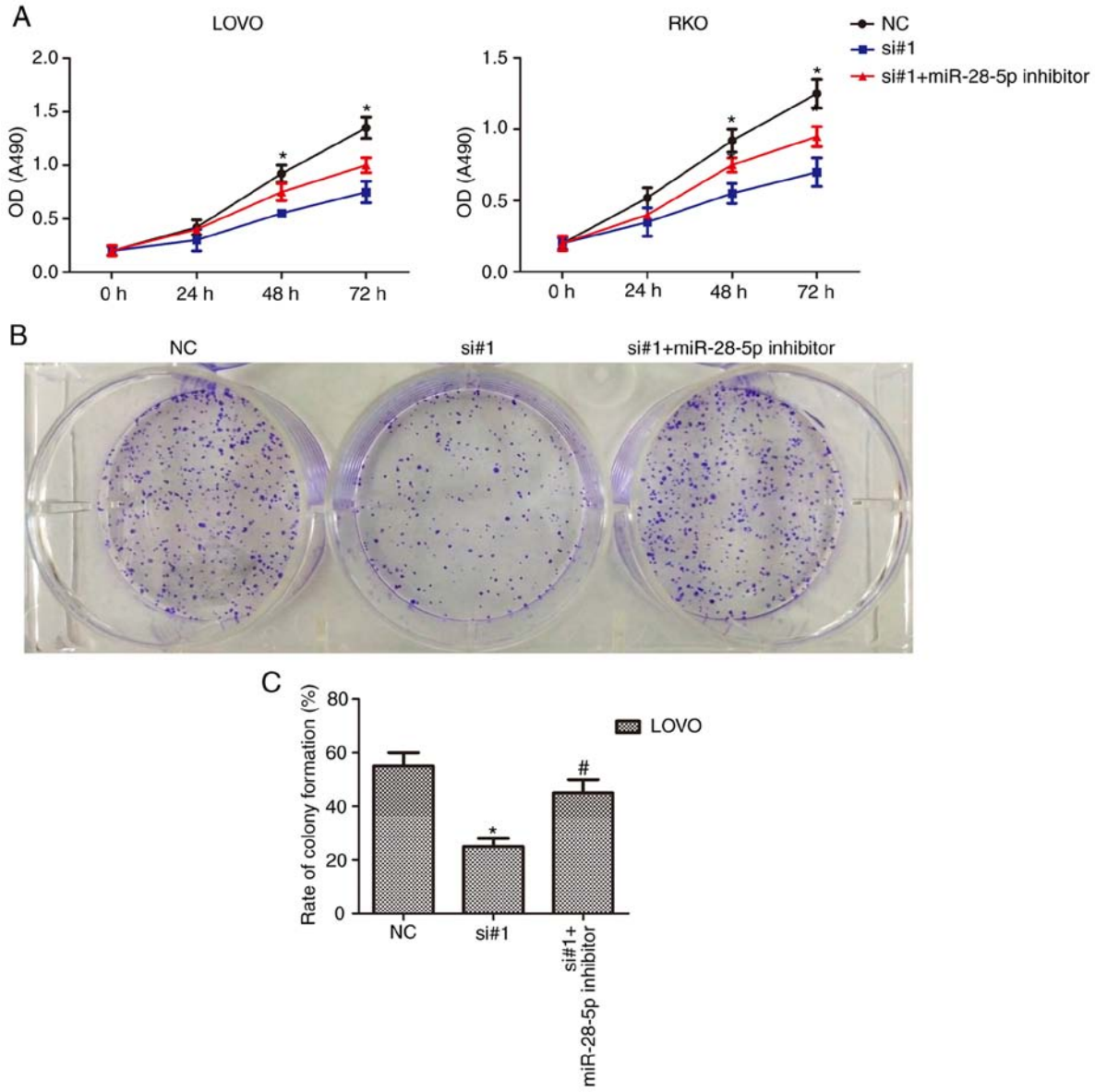

Figure 4. CDKN2B-AS1 function is suppressed by miR-28-5p. (A) The Cell Counting Kit-8 assay revealed that the decreased number of living cells was partly upregulated in CDKN2B-AS1-knockdown colorectal cancer cells after co-transfection with miR-28-5p inhibitor. (B and C) In the colony formation assay, the miR-28-5p inhibitor caused a significant increase in colony formation, whereas CDKN2B-AS1 knockdown abrogated this increase in colony formation. *P<0.05 vs. NC; #P<0.05 vs. si#1; n=6. CDKN2B-AS1, CDKN2B-antisense RNA 1; miR, microRNA; si, small interfering; NC, negative control.

In recent years, increasing evidence has demonstrated the hypothesis that lncRNAs exert their biological effect by acting as ceRNAs to affect the development of cancers (22). The cytoplasmic localization of CDKN2B-AS1 was further demonstrated by quantitative methods using subcellular fractionation experiments, which complemented the hybridization results. The role of CDKN2B-AS1 and miRNA in CRC was determined by bioinformatics analysis and dual-luciferase analysis. It was identified that miR-28-5p directly targeted CDKN2B-AS1. The localization of miR-28-5p in the cytoplasm and its interaction with lncRNAs have been studied in numerous cancers including CRC (23,24). Moreover, the results revealed that the expression of miR-28-5p was negatively correlated with the expression of CDKN2B-AS1 in CRC tissues and cell lines. The results indicated that CDKN2B-AS1 is a sponge of CDKN2B-AS1. Recent studies have revealed that miR-28-5p acts as a tumor suppressor in multiple human cancers, including CRC (25,26). In functional experiments, CCK-8 and colony formation assays revealed that miR-28-5p could partly reverse the effect of CDKN2B-AS1 on CRC cells. Therefore, the effects of CDKN2B-AS1 on CRC cell proliferation can be explained in part by its role as a molecular sponge of miR-28-5p.

In the present study, the mechanism of miR-28-5p was determined by bioinformatics analysis and dual-luciferase analysis. It was identified that miR-28-5p directly targeted URGCP. URGCP, also known as upregulated gene 4 (URG4), exhibits increased expression in osteosarcoma (OS) tissues vs. normal tissues (27). miR-671-5p has been revealed to inhibit gastric cancer cell proliferation and promote cell apoptosis by targeting URGCP (28). URG4/URGCP has also been revealed to enhance the angiogenic capacity of human hepatocellular carcinoma cells *in vitro* via activation of the NF-κB signaling pathway (29). In

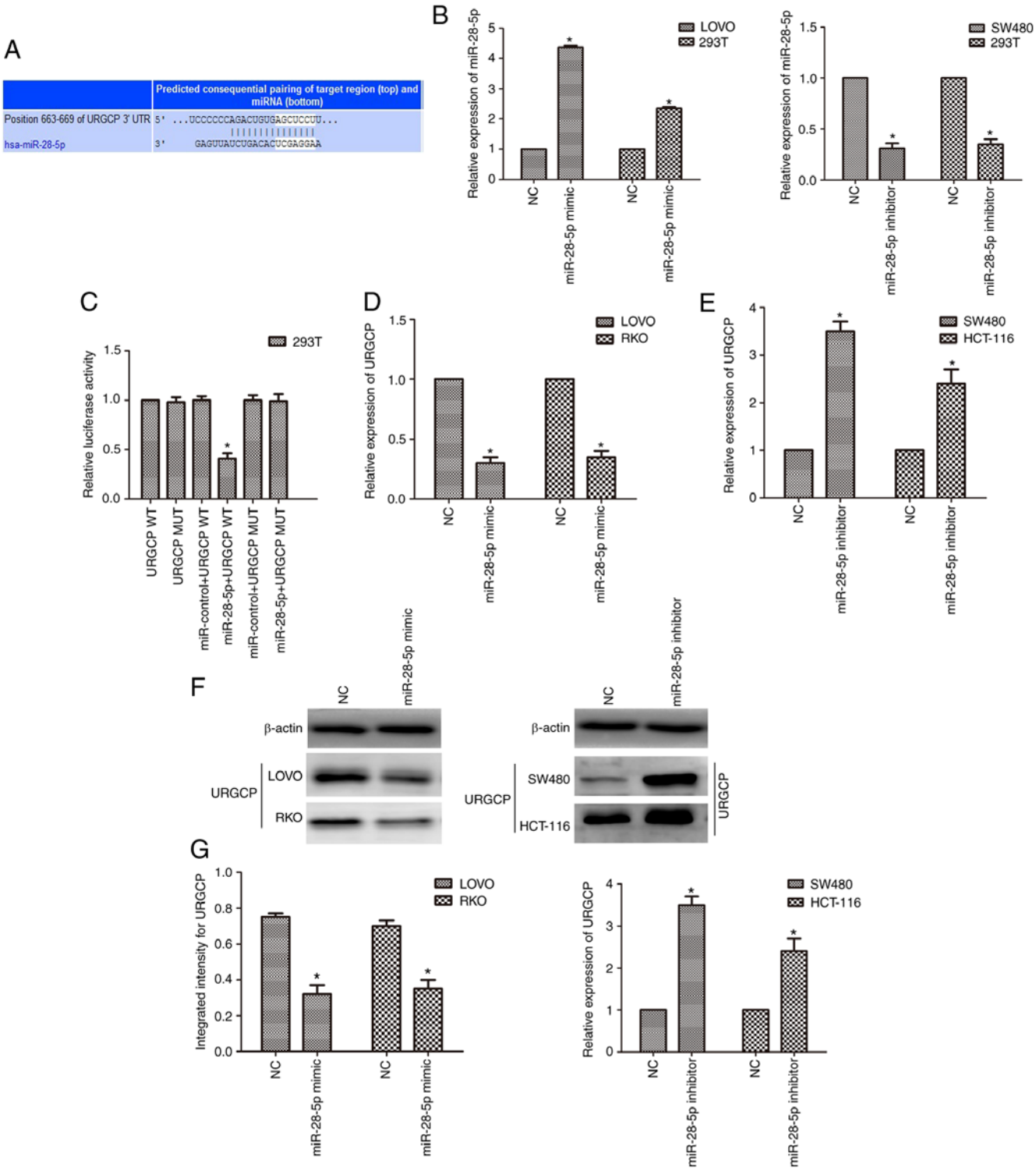

Figure 5. URGCP is target of miR-28-5p. (A) Analysis with bioinformatics tools revealed that URGCP was the target of miR-28-5p. (B) After transfection with miR-28-5p mimic, the level of miR-28-5p was upregulated, and the miR-28-5p level was downregulated after transfection with miR-28-5p inhibitor in both colorectal cells and 293T cells. (C) The luciferase reporter assay revealed that after co-transfection of miR-28-5p mimics and URGCP-WT, the luciferase activity was significantly reduced compared with cells co-transfected with URGCP-MUT. (D-G) The results revealed that the miR-28-5p mimic suppressed the expression of URGCP in LOVO and RKO cells, and the increase of URGCP in SW480 and HCT-116 cell lines when the miR-28-5p inhibitor was used as demonstrated by qPCR and western blot assays. *P<0.05 vs. NC; n=6. URGCP, upregulator of cell proliferation; miR, microRNA; WT, wild-type.

the present study, it was revealed that URGCP was upregulated in CRC. It was next investigated whether URGCP plays a role in mediating CDKN2B-AS1-induced cellular proliferation and apoptosis inhibition in CRC cells. It was revealed that CDKN2B-AS1 sponges miR-28-5p to regulate proliferation and apoptosis via URGCP. To illustrate the mechanism, the expression of proliferation-related proteins and apoptosis-related proteins was also assessed. Western blot analysis demonstrated that the decrease in cycle-related proteins caused by CDKN2B-AS1 knockdown was partly blocked by the miR-28-5p inhibitor.

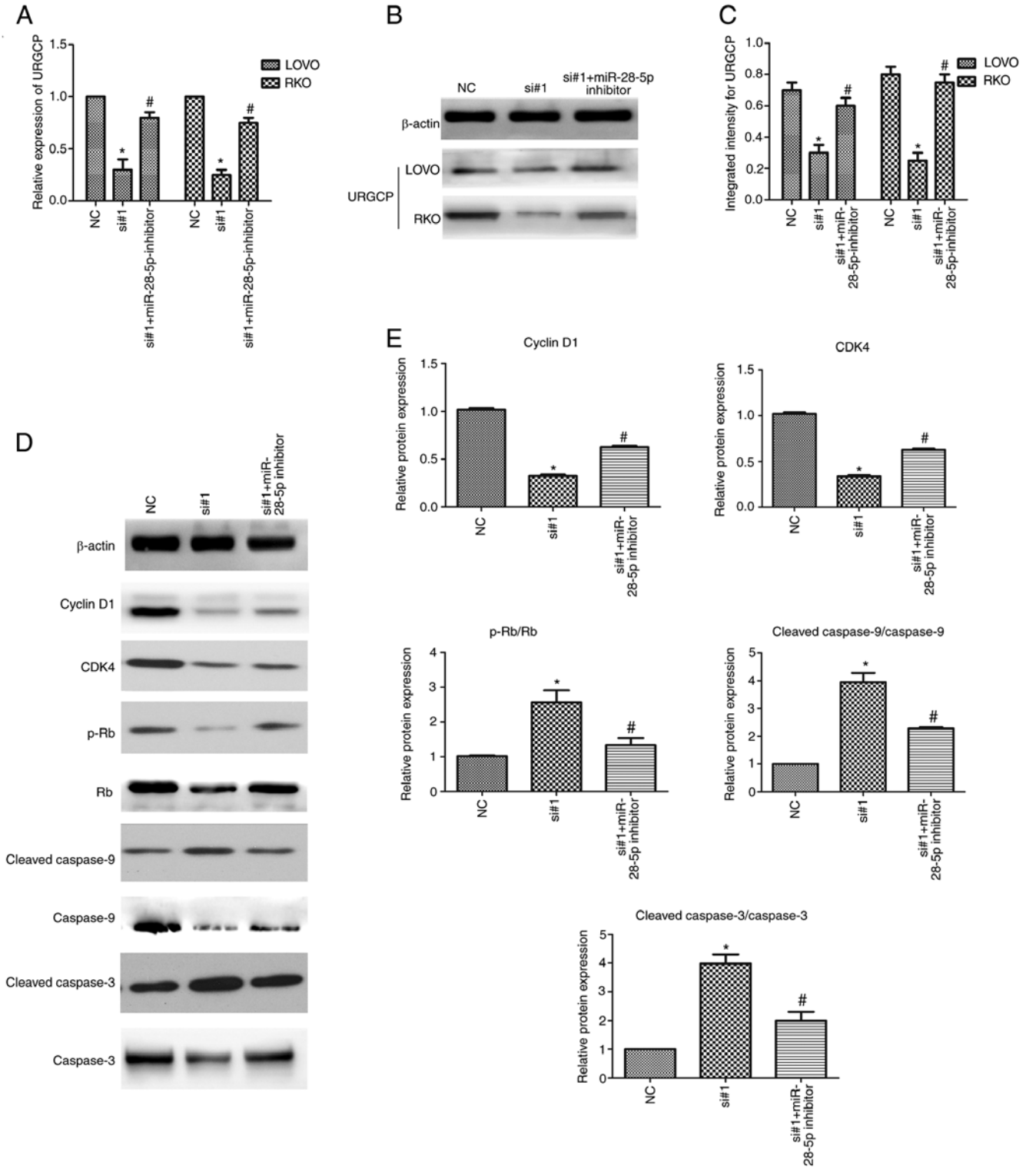

Figure 6. CDKN2B-AS1 regulates URGCP expression through miR-28-5p. (A) The quantitative PCR results revealed that knockdown of CDKN2B-AS1 reduced the expression of URGCP, whereas the miR-28-5p inhibitor could partly abolish this decreasing effect. (B and C) Western blot assays revealed that the downregulation of the URGCP protein in CDKN2B-AS1-knockdown cells was rescued by miR-28-5p inhibitor transfection. (D and E) Western blot analysis demonstrated that the decreases in the levels of cyclin D1 and cyclin-dependent kinase 4 in CDKN2B-AS1 knockdown cells were partly rescued by miR-28-5p inhibitor transfection. Moreover, it is illustrated that p-Rb/Rb expression levels are increased after CDKN2B-AS1 knockdown and decreased after miR-28-5p inhibitor transfection. (D and E) The increases in the levels of the cleaved fragments of caspase-9 and caspase-3 in CDKN2B-AS1-knockdown cells were partly attenuated by transfection with the miR-28-5p inhibitor. *P<0.05 vs. NC; #P<0.05 vs. si#1; n=6. CDKN2B-AS1, CDKN2B-antisense RNA 1; URGCP, upregulator of cell proliferation; miR, microRNA; CDK4, cyclin-dependent kinase 4; p-, phosphorylated; si, small interfering; NC, negative control.

Additionally, our experiments revealed that the upregulation of apoptosis-related proteins caused by CDKN2B-AS1 knockdown was partly suppressed after transfection with the miR-28-5p inhibitor. These data demonstrated that CDKN2B-AS1 depletion-mediated proliferation inhibition and apoptosis induction was partly mediated by URGCP in CRC cells.

While it would be helpful to determine the association of this gene with prognosis in CRC patients, samples collected more than five years after surgery are required and the average period of follow-up is 6 months. The samples collected for the present study were obtained not more than one year after surgery, and these data will be provided in future research. Numerous previous studies on the ceRNA mechanism have used dual luciferase quantitative methods and interaction assays instead of RNA immunoprecipitation (RIP) studies (30,31). Therefore, in the present study, dual luciferase quantitative methods and interaction assays were also used. In our future studies, RIP assays will be performed to further study the ceRNA mechanism. In a number of studies, colocalization experiments of miRNAs and lncRNAs were not performed (32,33), and our study also lacks colocalization experiments of miR-28-5p and lncRNA CDKN2B-AS1. These data will be provided in future research.

In summary, it was revealed that the upregulated lncRNA CDKN2B-AS1 promoted cell proliferation and inhibited cell apoptosis in CRC. Mechanistically, CDKN2B-AS1 functioned as a miR-28-5p sponge to positively regulate URGCP in CRC. The present study revealed the precise role of this regulatory axis and indicated that the CDKN2B-AS1/miR-28-5p/URGCP axis may be a therapeutic target in CRC.

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Availability of data and materials

All the datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

MLM and HYZ conceived the study design. HYZ and SYZ designed the experiments and supervised all research. MLM, SYZ and XLY carried out the experiments and prepared the draft of the manuscript. XLY analyzed the data. All authors read and approved the final manuscript 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

CRC specimens were obtained following the guidelines approved by the Ethics Review Committee of Qingdao Municipal Hospital (Qingdao, China) and written informed consent was obtained from patients in all cases.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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