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Downregulated LINC00628 aggravates the progression of colorectal cancer *via* inhibiting p57 level

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Abstract. – OBJECTIVE: To uncover the potential function of LINC00628 in influencing the progression of colorectal cancer (CRC) and its underlying mechanism.

PATIENTS AND METHODS: Relative levels of LINC00628 and p57 in CRC tissues and cell lines were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Regulatory effects of LINC00628 and p57 on proliferation, cell cycle, and apoptosis of SW480 and SW620 cells were assessed. Subcellular distribution of LINC00628 in CRC cells was analyzed. Moreover, the interaction between LINC00628 and enhancer of zeste homolog 2 (EZH2) was evaluated by the RNA Binding Protein Immunoprecipitation (RIP) assay.

RESULTS: LINC00628 was downregulated in CRC tissues and cell lines. CRC patients expressing a low level of LINC00628 suffered worse prognosis. The. knockdown of LINC00628 enhanced proliferative ability, prolonged S phase in cell cycle progression, and inhibited apoptosis in SW480 and SW620 cells. LINC00628 was mainly distributed in the nucleus. The RIP assay demonstrated that LINC00628 could bind to EZH2 to upregulate the p57 level. Rescue experiments verified that the overexpression of p57 could reverse regulatory effects of downregulated LINC00628 on proliferative and apoptotic abilities of CRC.

CONCLUSIONS: LINC00628 is downregulated in CRC. It aggravates the progression of CRC by binding to EZH2 to further inhibit the p57 level.

Key Words:
LINC00628, p57, Colorectal cancer, Proliferation, Apoptosis.

Introduction

Colorectal cancer (CRC) seriously threats human health worldwide. As a common digestive tract tumor, the occurrence and progression of CRC are closely related to environmental factors, genetic factors, diet, and lifestyle[1,2]. In developed countries in Europe and America, the morbidity and mortality of CRC rank third in all malignancies[3]. In China, the incidence of CRC is second only to esophageal cancer and gastric cancer of all the digestive tract tumors, presenting an increasing trend each year[4]. Early-stage diagnosis of CRC is insufficient, and a great number of CRC patients lose the optimal chance of surgery. The pathogenesis of CRC is very complex and remains unclear[5,6]. It is necessary to uncover the molecular mechanism of CRC to develop new biological hallmarks and therapeutic targets.

Long non-coding RNAs (LncRNAs) are a class of non-coding RNAs with 200 nt long. They are involved in cellular and biological processes by interacting with DNAs, chromatins, and proteins[7,8]. Some studies[9,10] have uncovered a great number of differentially expressed lncRNAs in many types of tumors, participating in epigenetic modification, transcriptional regulation, protein translation, etc. In tumor progression, lncRNAs could influence many aspects of tumor cell behaviors. Several lncRNAs are differentially expressed in CRC, which is closely linked with malignant growth, distant metastasis, and overall survival[11-14]. CRC-related lncRNAs may be utilized as hallmarks for predicting the prognosis of affected patients.

LINC00628 is located on chromosome 1q32.1 with approximately 1.2 kb in length. Recent reports have shown that abnormally expressed LINC00628 is involved in the development of gastric cancer and breast cancer by inhibiting the proliferative, migratory, and invasive capacities of cancer cells. In gastric cancer, LINC00628 interacts with enhancer of zeste

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homolog 2 (EZH2) in the nucleus and alters histone methylation level, thus regulating cancer cell behaviors[15]. In osteosarcoma, LINC00628 overexpression inhibits the growth and invasion by regulating the PI3K/Akt signaling pathway[16]. In this paper, we mainly discussed the potential biological function of LINC00628 in CRC and the underlying mechanism.

Patients and Methods

Clinical Samples

Paired CRC and adjacent non-tumor tissues were surgically resected from 80 CRC patients admitted in the Tianjin Union Medical Center from 2016 to 2019. Samples were pathologically detected and preserved in liquid nitrogen. This research was approved by the Ethics Committee of Tianjin Union Medical Center. The informed consent was achieved from all the patients.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cells were lysed to harvest RNAs using the TRIzol method (Invitrogen, Carlsbad, CA, USA), and the extracted RNAs were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: LINC00628: F: CAGTGGGGAACTCTGACTCG; R: GTGCCTGGTGCTCTCTTACC; p57: F: GGTGTCTAGGTGCTCCAGGT; R: GCACTCTCCAGGAGGACACA; EZH2: F: TGCACATCCTGACTTCTGTG; R: AAGGGCATTCACCAACTCC; U1: F: CAGGGCGAGGCTTATCCA; R: GCAGGGGTCAGCACATCC.

Cell Culture

Colon epithelial cells NCM460 and CRC cells (HCT116, SW480, SW620, and RKO) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). They were regularly observed and passaged.

Cell Transfection

Cells were inoculated in 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 60% confluence. LINC00628 siRNA, pcDNA3.1-p57, or negative control was dissolved in Lipofectamine 2000, and applied in each well. Transfected cells for 48-72 h were collected.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates with 2.0×10^3 cells per well. At the established time points, 10 μL of CCK-8 solution (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Cycle Determination

Cells were collected and adjusted to 1×10^6 cells/mL. After centrifugation, cells were suspended in 500 μL of pre-cold phosphate-buffered saline (PBS) and incubated in 3.5 mL of pre-cold 70% ethanol at 4°C overnight. On the other day, cells were centrifuged, resuspended in 50 μL of RNaseA, and subjected to 37°C water bath for 30 min. Aftewards, they were incubated with 450 μL of propidium iodide (PI) at 4°C for 30 min. The cell cycle distribution was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis Determination

Cells were collected and adjusted to 1×10^6 cells/mL. They were incubated with AV solution in the dark for 10 min, followed by incubation with 10 μL of PI for another 10 min. At last, the apoptotic percentage was determined by flow cytometry.

5-Ethynyl-2'-Deoxyuridine (EdU)

Cells were fixed in PBS containing 3.7% formaldehyde. Rupture of cell membranes was conducted using PBS containing 0.5% Triton X-100. Afterwards, cells were labeled with EdU solution (R&D Systems, Minneapolis, MN, USA) in the dark for 30 min, and stained with Hoechst 33342 for another 30 min. Images of EdU-labeled and Hoechst-labeled cells were taken under fluorescence microscopy (magnification 40×).

Subcellular Distribution Determination

Cytoplasmic and nuclear fractions were harvested using the PARIS Kit (Life Technologies, Gaithersburg, MD, USA). Cytoplasmic and nu-

clear RNAs were extracted using the TRIzol reagent. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U1 were internal references for the cytoplasm and nucleus, respectively.

RNA Binding Protein Immunoprecipitation (RIP) Assay

2×10^7 cells were collected and processed according to the procedures of Millipore Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cells were incubated with anti-EZH2 or anti-IgG at 4°C overnight. A protein-RNA complex was obtained when intracellular specific proteins were captured by the antibody. Next, proteins were digested by proteinase K, and the RNA molecules were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNA was finally subjected to qRT-PCR for determining the relative level.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software Inc., CA, USA) were used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm SD$). Intergroup data were compared using the *t*-test. The Kaplan-Meier method was introduced for survival analysis. $p<0.05$ considered the difference was statistically significant.

Results

Downregulation of LINC00628 in CRC

We first determined the LINC00628 level in CRC tissues and adjacent non-tumor tissues. LINC00628 was downregulated in CRC tissues (Figure 1A). CRC patients were classified according to tumor staging. QRT-PCR data showed a higher abundance of LINC00628 in CRC patients

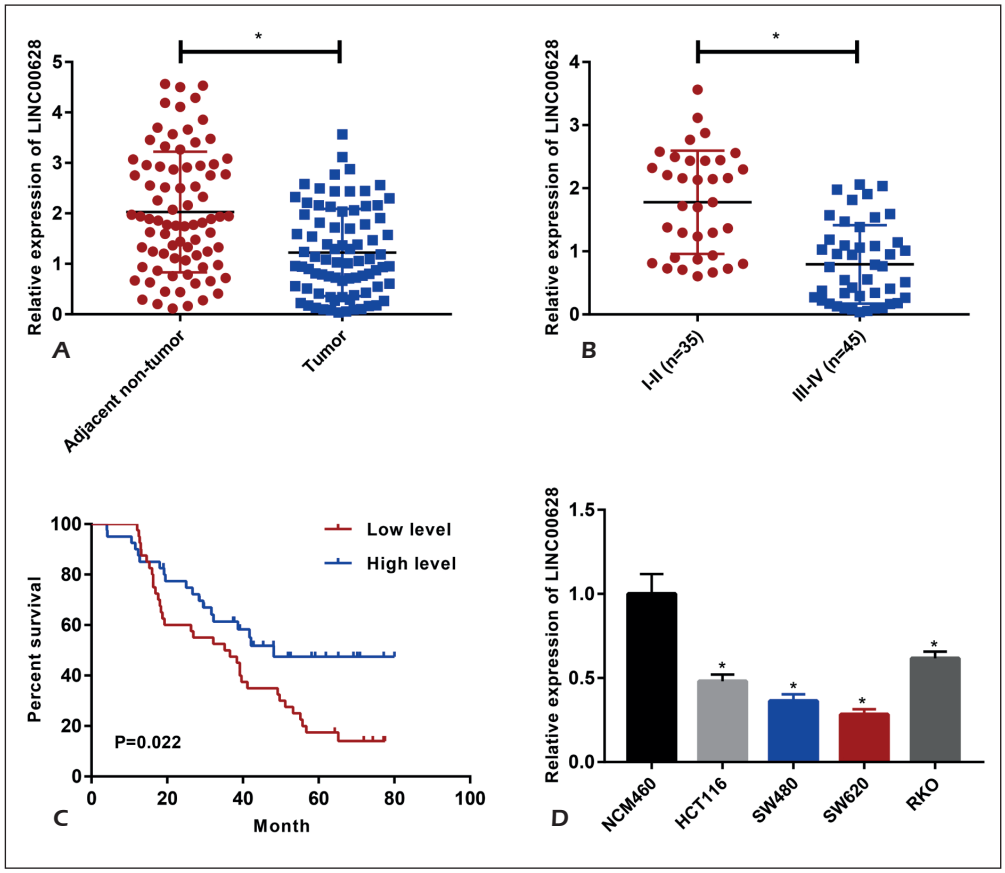

Figure 1. Downregulation of LINC00628 in CRC. **A**, LINC00628 level in adjacent non-tumor tissues and CRC tissues. **B**, LINC00628 level in CRC patients with stage I-II or stage III-IV. **C**, Overall survival in CRC patients with high level or low level of LINC00628. **D**, LINC00628 level in normal colon epithelial cells NCM460 and CRC cells HCT116, SW480, SW620, and RKO.

with stage I-II relative to those with stage III-IV (Figure 1B). Survival analysis uncovered worse prognosis in CRC patients expressing a low level of LINC00628 relative to those with high level (Figure 1C). Subsequently, we determined LINC00628 level in normal colon epithelial cells and CRC cells. LINC00628 was identically downregulated in CRC cell lines (Figure 1D). Among the four selected CRC cell lines, SW620 and SW480 cells expressed the lowest level of LINC00628, and were chosen for the following investigations.

LINC00628 Influenced Proliferation, Cell Cycle, and Apoptosis of CRC

We constructed two LINC00628 siRNAs and tested their transfection efficacy. Transfection of either si-LINC00628-1 or si-LINC00628-2 could markedly downregulate the LINC00628 level in SW480 and SW620 cells, which was much more pronounced in the latter one (Figure 2A). During the following experiments, si-LINC00628-2 was utilized. Viability was markedly elevated after the transfection of si-LINC00628-2 in CRC cells (Figure 2B, 2C). Similarly, the EdU-positive ratio increased after silence of LINC00628 (Figure 2D). Flow cytometry data revealed a decline in cell ratio of G0/G1 phase after the transfection of si-LINC00628-2 (Figure 2E, 2F). Furthermore, the apoptotic percentage decreased due to silence of LINC00628 in CRC (Figure 2G).

LINC00628 Regulated p57 Level by Binding to EZH2

To uncover the molecular mechanism of LINC00628 in CRC, we first analyzed the subcellular distribution of LINC00628. As data showed,

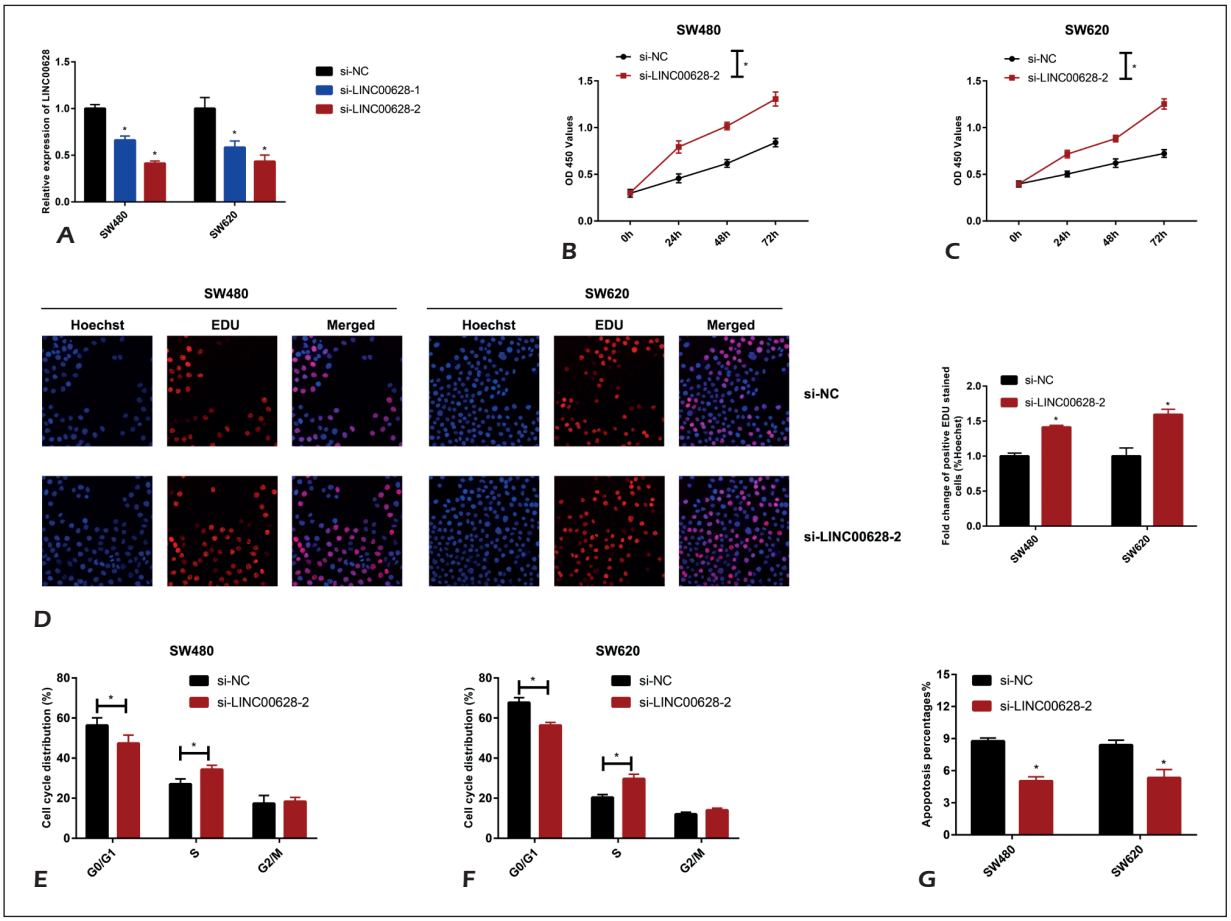

Figure 2. Silence of LINC00628 influenced proliferation, cell cycle, and apoptosis of CRC. **A**, Transfection efficacy of si-LINC00628-1 and si-LINC00628-2 in SW480 and SW620 cells. **B**-**C**, Viability at 0, 24, 48 and 72 h in SW480 and SW620 cells transfected with si-NC or si-LINC00628-2. **D**, EdU-positive ratio in SW480 and SW620 cells transfected with si-NC or si-LINC00628-2 (magnification: 40×). **E**-**F**, Cell cycle distribution in SW480 and SW620 cells transfected with si-NC or si-LINC00628-2. **G**, Apoptosis percentage in SW480 and SW620 cells transfected with si-NC or si-LINC00628-2.

LINC00628 was distributed in the nucleus (Figure 3A, 3B). Previous studies[17] have shown that nuclear lncRNAs could regulate gene expressions by binding to the PRC2 complex. Through the RIP assay, the interaction between LINC00628 and EZH2 was discovered in SW480 and SW620 cells (Figure 3C). In breast cancer and ovarian cancer, EZH2 participates in tumor progression by regulating p57 level[18,19]. Hence, we speculated that LINC00628 may also regulate p57 level by binding to EZH2, thus influencing the CRC progression. P57 level was found to be downregulated in CRC cells (Figure 3D). Also, transfection of si-LINC00628-2 markedly downregulated p57 level in CRC cells (Figure 3E). Furthermore, to clarify the involvement of EZH2 in p57 regulation, si-EZH2 was constructed (Figure 3F). The downregulated p57 in CRC cells transfected with si-LINC00628-2 was partially reversed by the co-transfection of si-EZH2 (Figure 3G).

LINC00628 Influenced Proliferation, Cell Cycle, and Apoptosis of CRC Via Regulating p57 Level

pcDNA3.1-p57 was constructed and its transfection could upregulate p57 level in CRC cells (Figure 4A). The elevated viability in SW480 cells transfected with si-LINC00628-2 was partial-

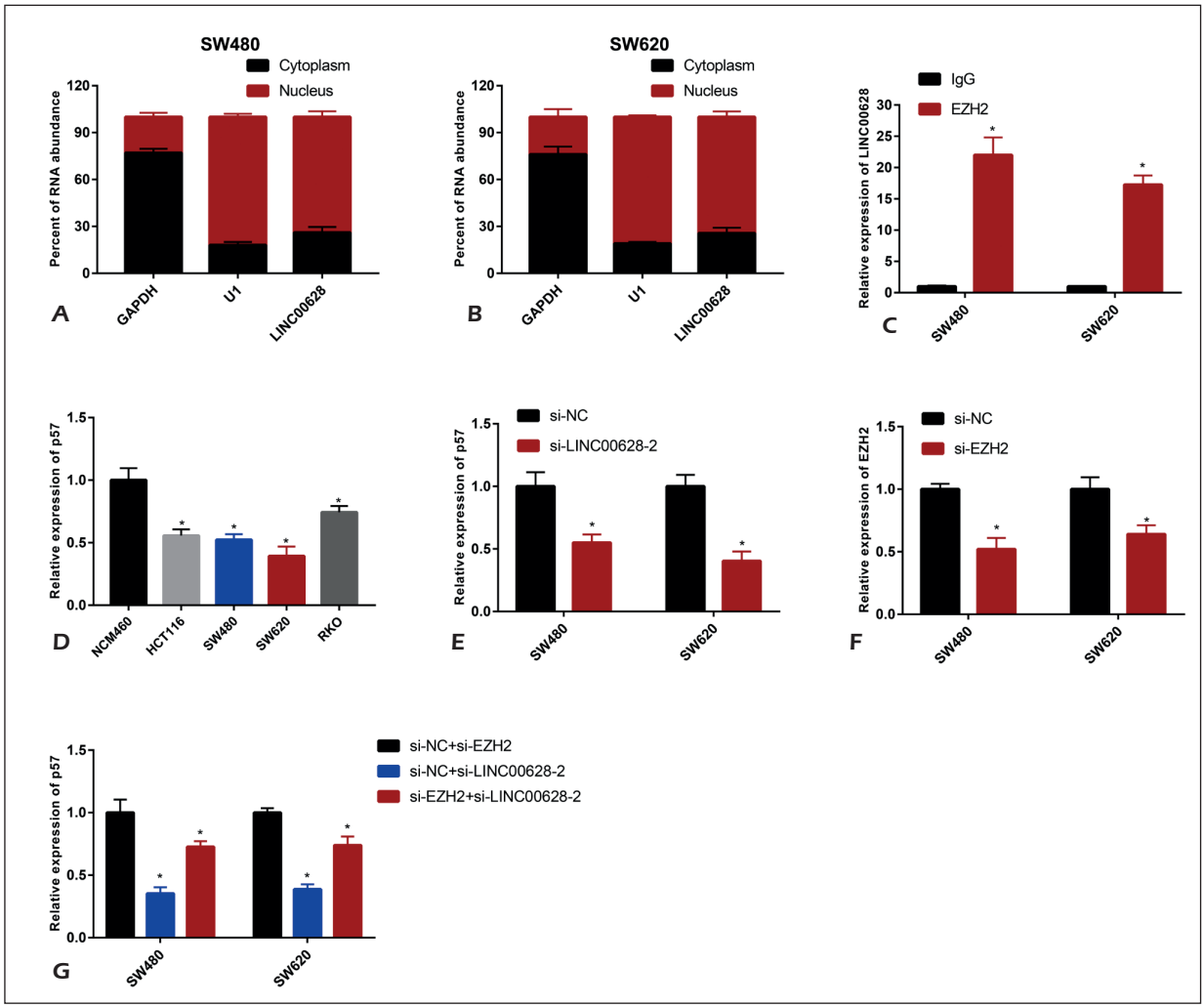

Figure 3. LINC00628 regulated p57 level by binding to EZH2. **A-B**, Cytoplasm and nuclear fractions of LINC00628 in SW480 and SW620 cells. GAPDH and U1 were internal references for cytoplasm and nucleus, respectively. **C**, Immunoprecipitant of LINC00628 in anti-IgG and anti-EZH2. **D**, P57 level in normal colon epithelial cells NCM460 and CRC cells HCT116, SW480, SW620, and RKO. **E**, P57 level in SW480 and SW620 cells transfected with si-NC or si-LINC00628-2. **F**, Transfection efficacy of si-EZH2 in SW480 and SW620 cells. **G**, P57 level in SW480 and SW620 cells transfected with si-NC + si-EZH2, si-NC + si-LINC00628-2 or si-EZH2 + si-LINC00628-2.

ly reversed by co-transfection of pcDNA3.1-p57 (Figure 4B). EdU assay showed the same trend as that of the CCK-8 assay revealed (Figure 4C). Cell ratio in S phase increased owing to LINC00628 knockdown, which was slightly reduced by overexpression of p57 (Figure 4D). Moreover, the inhibited apoptosis in CRC cells with LINC00628 knockdown was further stimulated by the p57 overexpression (Figure 4E).

Discussion

LncRNAs are of significance in the tumorigenesis and deterioration of CRC. For example, the silence of MALAT1 blocks nuclear translocation of β-catenin, thereafter downregulating c-Myc and MMP-7, which provides a favorable condition for CRC cells to invade and metastasize[19]. LncRNA CCAT1 is upregulated in CRC cells relative to that of intestinal mucosal epithelial cells. The tissue-specificity of CCAT1 gives the potential to be a tumor biomarker[20]. In addition, the overexpression of CCAT2 mediates Myc transcription through TCF7LZ, thus inducing CRC cells to proliferate and metastasize by activating the Wnt pathway[21]. It is reported that cRNDE is abnormally upregulated in over 90% colorectal adenomas and adenocarcinoma cells. By activating the IGFS pathway, cRNDE contributes to trigger the carcinogenesis of intestinal epithelial cells[22]. In intestinal cancer cells, UCA1 is upregulated. The knockdown of UCA1 greatly reduces proliferative rate and invasiveness of intestinal cancer cells[23].

This research mainly focused on the expression pattern and biological functions of LINC00628 in CRC. Compared with adjacent non-tumor tissues, LINC00628 was markedly downregulated in CRC tissues. Silence of LINC00628 led to proliferation stimulation and apoptosis suppression in CRC cells.

PRC2 is a highly conserved histone methyltransferase, consisting of three key subunits: EZH2, EED, and SUZ. EZH2, a catalytic subunit of PRC, can cause chromatin condensation and inhibition of multiple transcription factors and pathways by catalyzing H3K27me3[24-26]. Increasing evidence has demonstrated the carcinogenic effect of EZH2. It inhibits apoptosis and promotes the proliferative

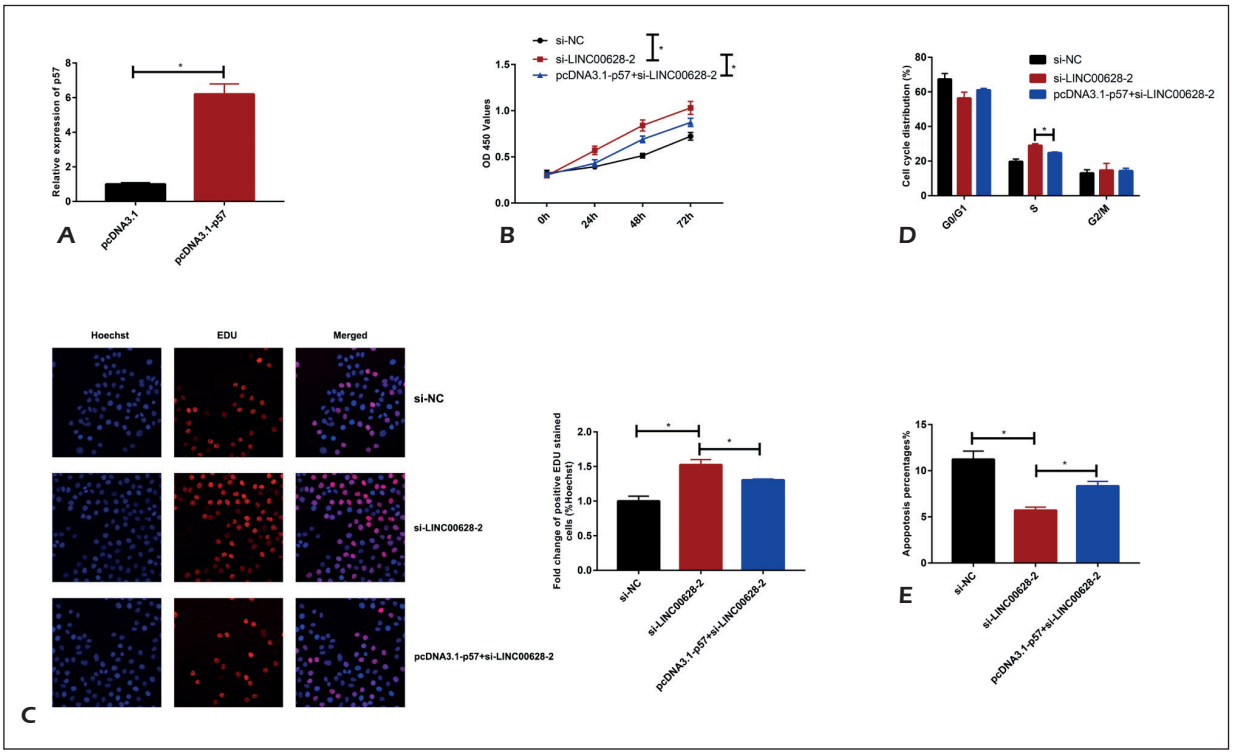

Figure 4. LINC00628 influenced proliferation, cell cycle, and apoptosis of CRC via regulating p57 level. **A**, Transfection efficacy of pcDNA3.1-p57 in SW480 cells. SW480 cells were transfected with si-NC, si-LINC00628-2, or pcDNA3.1-p57 + si-LINC00628-2. Viability (**B**) EdU-positive ratio (**C**) cell cycle distribution (magnification: 40×) (**D**) and apoptotic percentage (**E**) were determined.

ability of tumor cells by regulating oncogenes and tumor-suppressor genes. For instance, the tumor suppressor RUNX3 could be regulated by the EZH2 methylation. EZH2 downregulates the RUNX3 level, thus triggering the proliferative rate of many types of tumor cells[27,28]. In ovarian cancer, EZH2 could downregulate p57, a cell cycle regulator protein[29]. Rap1GAP is able to alleviate head and neck squamous cell carcinoma, thyroid cancer, pancreatic cancer, and malignant melanoma. Through hypermethylation, EZH2 targets the promoter region of Rap1GAP to further downregulate Rap1, thus stimulating tumor progression[30,31]. EZH2 is confirmed to inhibit the transcription of MSMB, thus promoting the growth of prostate cancer cells[32]. Besides, EZH2 negatively mediates the epigenetic inheritance of DAB2IP promoter by silencing Ras, thereafter stimulating metastasis of prostate cancer[33-35]. In undifferentiated thyroid cancer cells, EZH2 directly prevents proliferation inhibition and differentiation by silencing the thyroid-specific transcript PAX8[36]. LINC00628 inhibited p57 level *via* binding to EZH2. The knockdown of LINC00628 could downregulate p57 level, which was partially reversed by the EZH2 knockdown. Moreover, rescue experiments confirmed that the overexpression of p57 could reverse the regulatory effects of downregulated LINC00628 on proliferative and apoptotic abilities of CRC.

Conclusions

Taken together the above data showed that LINC00628 was downregulated in CRC. Also, it aggravates the progression of CRC by binding to EZH2 to further inhibit p57 level.

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Conflict of Interests

The Authors declared that they have no conflict of interests.

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