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Knockdown of long non-coding RNA LINC00324 inhibits proliferation, migration and invasion of colorectal cancer cell *via* targeting miR-214-3p

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Abstract. – OBJECTIVE: Increasing evidence demonstrated that long non-coding RNA (lncRNA) played a vital role in human tumorigenesis and progression, including colorectal cancer (CRC). However, the underlying mechanisms are still largely unknown. The aim of this study was to investigate the function of lncRNA LINC00324 on the development of CRC and explore the possible mechanisms.

MATERIALS AND METHODS: The expression level of LINC00324 and miR-214-3p was measured by quantitative real time polymerase chain reaction (qRT-PCR) in CRC cells. The effects on cell proliferation, migration and invasion were assessed by MTT and transwell assays, respectively. In addition, the protein levels of cyclin D1, p21, p27 and three MMPs were detected by Western blot analysis. The target of LINC00324 was predicted by online software and confirmed by luciferase reporter assay.

RESULTS: We first detected the expression of LIN00324 was increased while miR-214-3p was decreased in CRC cells. Knockdown of LIN00324 suppressed proliferation, migration and invasion in SW620 and HCT15 cells. Moreover, overexpression of miR-214-3p also inhibited CRC cell proliferation, migration and invasion. Then, we identified miR-214-3p as directly target of LINC00324 and the expression of miR-214-3p was downregulated by LINC00324. In addition, inhibiting miR-214-3p reversed the effects of LINC00324 on CRC cell proliferation, migration and invasion.

CONCLUSIONS: Our results proved that LINC00324 regulated CRC cell proliferation, migration and invasion by sponging miR-214-3p, suggesting that it might be a potential therapeutic target for CRC therapy.

Key Words:
Colorectal cancer, LncRNA LINC00324, MiR-214-3p, Proliferation, Migration, Invasion.

Introduction

Colorectal cancer (CRC) is one of the most prevalent malignant tumors worldwide, which has a high incidence of 6.1%[1]. The occurrence of CRC is associated with the environment, lifestyle and genetic predispositions. Although much work and effort in diagnosis and therapeutic strategies of CRC have been made, owing to the lack of obvious early symptoms of CRC, many patients are diagnosed in advanced stages, which is the major cause of treatment failure and poor post-surgical survival. Therefore, it is pivotal to understand the pathological mechanisms of the tumorigenesis for the development of effective biomarkers for CRC.

Long noncoding RNAs (lncRNAs) are made up of more than 200 nucleotides, and they are some non-protein coding transcripts[2,3]. Studies have shown that lncRNAs play a crucial role in many complex life activities, including the cell cycle, epigenetic regulation as well as differentiation and regulation[4]. Transcriptome data showed that many lncRNAs were aberrantly expressed in various cancers[5], indicating that lncRNAs played a key role in tumorigenesis and metastasis[6]. LINC00324 has been reported to be a tumor promoter in lung cancer[7]. In addition, previous studies demonstrated that LINC00324 promoted cell proliferation and migration in osteosarcoma[8] and gastric cancer[9]. However, the functions of LINC00324 in CRC have not reported up to now.

Emerging research suggested that lncRNA exerted its roles *via* endogenous binding microRNAs (miRNAs). MiRNAs are noncoding RNAs, which consist of 18-25 nucleotides, and they regulate gene expression through binding to the target mRNAs[10,11]. It is reported that miRNA

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played a tumor-promoting or suppression function in various cancers[12-14]. MiR-214-3p has been shown to suppress proliferation, meanwhile inducing apoptosis in hepatocellular carcinoma[15]. Moreover, miR-214-3p inhibits the development of muscle-invasive bladder cancer[16]. Further, it has been found that miR-214-3p plays a tumor suppressor function in oral squamous cell carcinoma, endometrial carcinoma and colorectal cancer[17-19].

In this study, we first measured the levels of LINC00324 and miR-214-3p in CRC cells. Subsequently, the roles of LINC00324 and miR-214-3p in CRC cells were investigated using gain- and loss-of-function experiments. Furthermore, we researched the relationship between LINC00324 and miR-214-3p and explored the regulatory mechanisms of LINC00324 in CRC cells.

Materials and Methods

Cell Culture and Transfection

Human normal colorectal cell NCM460 and CRC cell lines SW620, HCT15, SW480 and HCT116 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) added with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Then, they were placed in a humidified incubator with 5% CO_2 and a stationary temperature of 37°C.

Si-LINC00324, miR-214-3p mimics, anti-miR-214-3p and the corresponding control fragments were obtained from Genechem (Shanghai, China). The full-length complementary DNA of LINC00324 was amplified to construct an overexpression vector with the pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The cells were seeded on the 6-well plate, and then transfection was done in accordance with the instructions of Lipofectamine 2000 (Invitrogen). The cells were collected after 48 h for follow-up experiments.

qRT-PCR Analysis

Total RNA was isolated from cells by RNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Next, the RNA was used to obtain cDNA by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. SYBR Premix Ex Taq (TaKaRa, Dalian, China) was employed for the qPCR experiments. GAPDH or U6 was used to normalize LINC00324 or miR-214-3p. Primer sequences were listed as follows: LINC00324-F: TGTGGATGACAGTGTTCGGG, LINC00324-R: ACGCTGACCAGAAACCGTAG; miR-214-3p-F: GCATCCTGCCTCCACATGCAT, miR-214-3p-R: GCGCTGAGGAATAATAGAGTATGTAT; GAPDH-F: CCACTCCTCCACCTTTGAC, GAPDH-R: ACCCTGTTGCTGTAGCCA; U6-F: CTTCGGCAGCACATATACT, U6-R: AAAATATGGAACGCTTCACG.

Western Blot Analysis

Cells were added with RIPA buffer and statically placed in an ice bath for 15 min; next, they were centrifuged for 20 min at 12,000 rpm. The supernate containing protein was collected. Equivalent proteins were added on each line for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were separated, they were transferred to a polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Billerica, MA, USA). Subsequently, the membrane was sealed with 5% non-fat milk at room temperature for 1 h, and it was later incubated with primary antibodies as follows: anti-Cyclin D1, anti-p21, anti-p27, anti-MMP-2, anti-MMP-9, anti-MMP-14 and anti-DAPDH (Abcam, Cambridge, UK). The membrane was washed with TBST for 3 times, and secondary antibody (Abcam) was used to incubate the membrane for 1 h. Protein signals were detected by using an enhanced ECL system (MilliporeSigma, Burlington, MA, USA).

MTT Assay

For the cell proliferation detection, cells were cultured in a 96-well plate and 20 μL of MTT (5 mg/mL) was added to each well. After the cells were incubated for 4 h at 37°C, they were collected and 50 μL dimethyl sulfoxide (DMSO) was added for the dissolution of formazan crystals in cells. The proliferation was assessed *via* the absorbance at 490 nm wavelength, which was measured with a microplate reader (Molecular Devices, San Jose, CA, USA).

Transwell Assay

For the detection of cell migration, 100 μL of serum-free medium contained cells was added to the apical chamber. Otherwise, for the invasion

detection, the upper chamber was added with extra Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Cell medium containing 10% serum was added into the basolateral chamber. After incubation for 24 h, the cells invaded through the membrane were fixed with 4% paraformaldehyde and dyed with 0.5% crystal violet solution. Then the cells were imaged and counted under a microscope.

Dual-Luciferase Assay

The target gene of LINC00324 was predicted by starBase v2.0. The wild type LINC00324 (WT-LINC00324) containing the putative binding site of miR-214-3p and the mutant LINC00324 (MUT-LINC00324) was constructed into the pGL3-control luciferase reporter vectors, respectively. SW620 and HCT15 cells were co-transfected with WT-LINC00324 or MUT-LINC00324 and miR-214-3p. The Luciferase activity was measured 48 hrs post-transfection. Renilla luciferase acted as internal control.

Statistical Analysis

Data were shown as mean ± standard deviation (SD) with at least 3 repeats. Statistical analysis was carried out by GraphPad Prism 7.0 (San Diego, CA, USA). The comparisons between two or multiple groups were analyzed using Student's *t*-test and one-way analysis of variance (ANOVA) with the Tukey HSD post-hoc test, respectively. $p < 0.05$ was considered as statistically significant.

Results

LINC00324 Was Upregulated and MiR-214-3p Was Downregulated in CRC Cells

The expression levels of LINC00324 and miR-214-3p were first detected in CRC cells and human normal colorectal cells by qRT-PCR, and the result showed that LINC00324 level was significantly increased in CRC cells compared with normal colorectal cell (Figure 1A). On the contrary, miR-214-3p expression was significantly downregulated in CRC cells (Figure 1B). The aberrant expression of LINC00324 and miR-214-3p in CRC cells suggested that they might play a potential role in CRC.

Knockdown of LINC00324 Suppressed Proliferation of CRC Cells

To investigate the role of LINC00324 on cell proliferation in CRC, si-LINC00324 was transfected into SW620 and HCT15 cells to silence LINC00324. In the si-LINC00324 group, the expression of LINC00324 was significantly decreased in both SW620 and HCT15 cells (Figure 2A). Subsequently, MTT assay was performed to detect cell proliferation. The result revealed that knockdown of LINc00324 inhibited SW620 and HCT15 cell proliferation (Figure 2B and C). In addition, the expression levels of cell proliferation-related protein Cyclin D1, p21 and p27 were detected by using western blot (Figure 2D and E). In SW620 cells, the levels of Cyclin D1 were de-

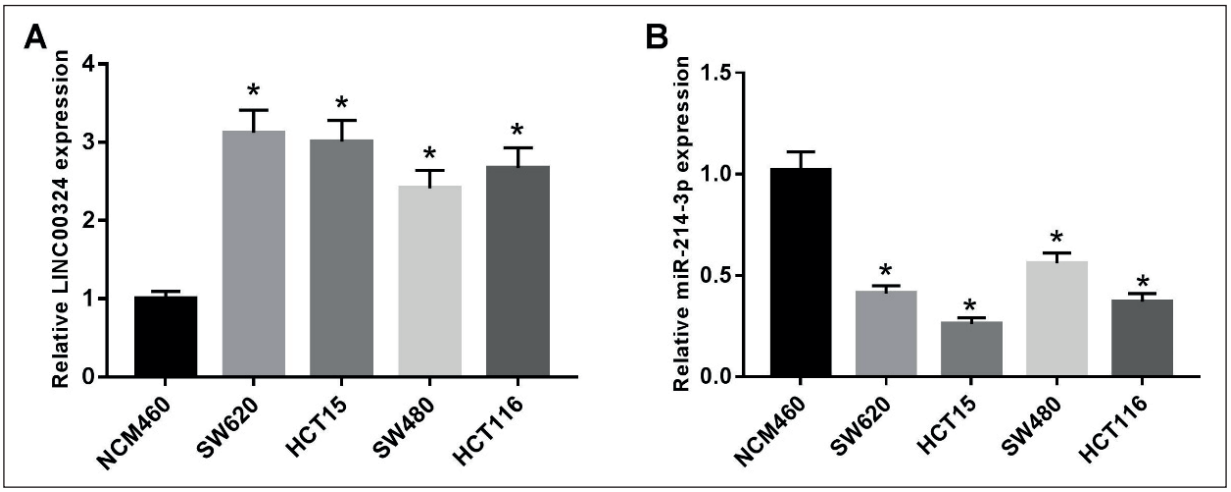

Figure 1. The expression levels of LINC00324 and miR-214-3p in CRC cells. **A**, The expression of LINC00324 in CRC cells was detected by qRT-PCR. **B**, The expression of miR-214-3p in CRC cells was detected by qRT-PCR.

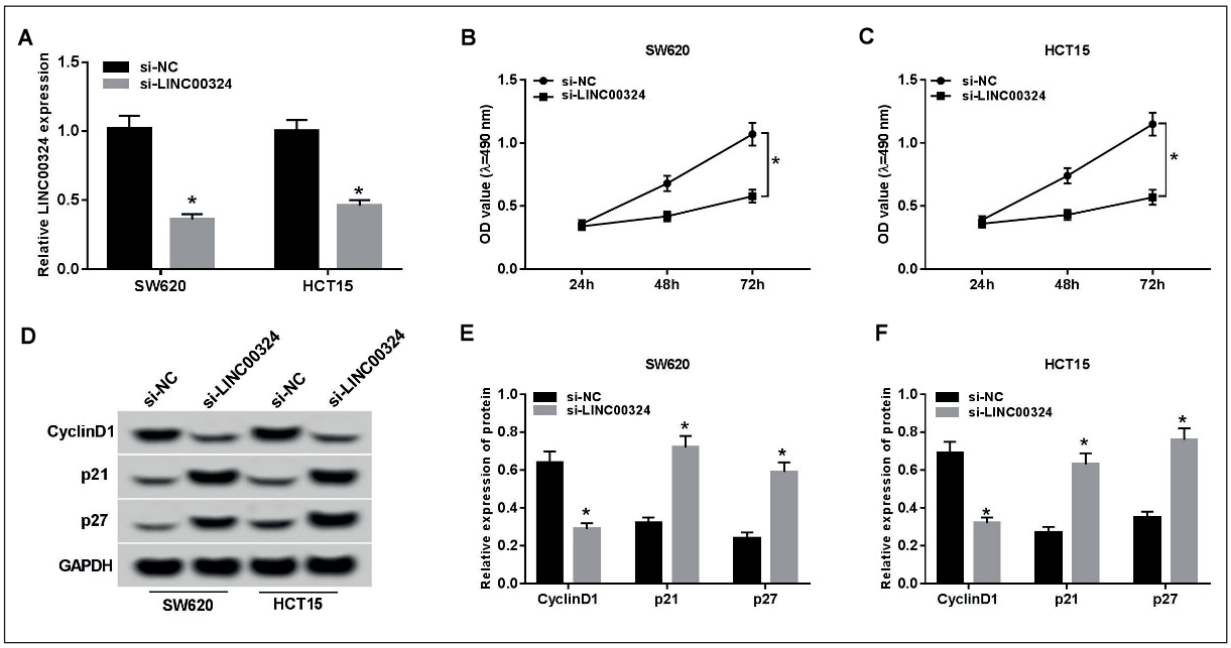

Figure 2. Knockdown of LINC00324 inhibited CRC cell proliferation. **A**, After knockdown of LINC00324 in SW620 and HCT15 cells, the expression of LINC00324 was detected by qRT-PCR. **B** and **C**, Cell proliferation of SW620 and HCT15 cells was measured by MTT assay after knockdown of LINC00324. **D-F**, The levels of proliferation-related proteins were detected by Western blot in SW620 and HCT15 cells transfected with si-LINC00324.

creased while p21 and p27 were increased in the si-LINC00324 group compared with the si-NC group. Also, there was a similar result in HCT15 cells (Figure 2D and F). These data indicated that knockdown of LINC00324 inhibited CRC cell proliferation *in vitro*.

Knockdown of LINC00324 Inhibited Migration and Invasion in CRC Cells

To further explore the effects of LINC00324 knockdown on migration and invasion in CRC cells, transwell migration and invasion assay were performed. Results showed that knockdown of LINC00324 significantly suppressed SW620 as well as HCT15 cell migration and invasion (Figure 3A-D). Moreover, western blot result showed that the protein levels of MMP-2, MMP-9 and MMP-14 were decreased in both SW620 and HCT15 cells transfected with si-LINC00324 compared with the si-NC group (Figure 3E-G). MMP-2, MMP-9 and MMP-14 promote cell migration and invasion; this result indicated cell migration and invasion were suppressed in Si-LINC00324 group, which was consistent with the transwell assay result.

Overexpression of MiR-214-3p Inhibited Proliferation, Migration and Invasion in CRC Cells

The downregulated expression of miR-214-3p implied it might play an antitumor effect in CRC cells. To confirm that, miR-214-3p was expressed in SW620 and HCT15 cells to explore its effects on cell proliferation, migration and invasion. An increased expression level of miR-214-3p was observed in cells transfected with miR-214-3p mimics (Figure 4A). Overexpression of miR-214-3p inhibited proliferation in both SW620 and HCT15 cells (Figure 4B and C). In addition, the protein level of Cyclin D1 was decreased and p21 was increased in SW620 and HCT15 cells when miR-214-3p was overexpressed (Figure 4D-F). Then, transwell assay results demonstrated that miR-214-3p overexpression impeded migration and invasion in SW620 and HCT15 cells (Figure 4G and H). Alternatively, MMP-2 and MMP-9 were downregulated by miR-214-3p (Figure 4I-K). These results indicated that miR-214-3p overexpression inhibited CRC cell proliferation, migration and invasion.

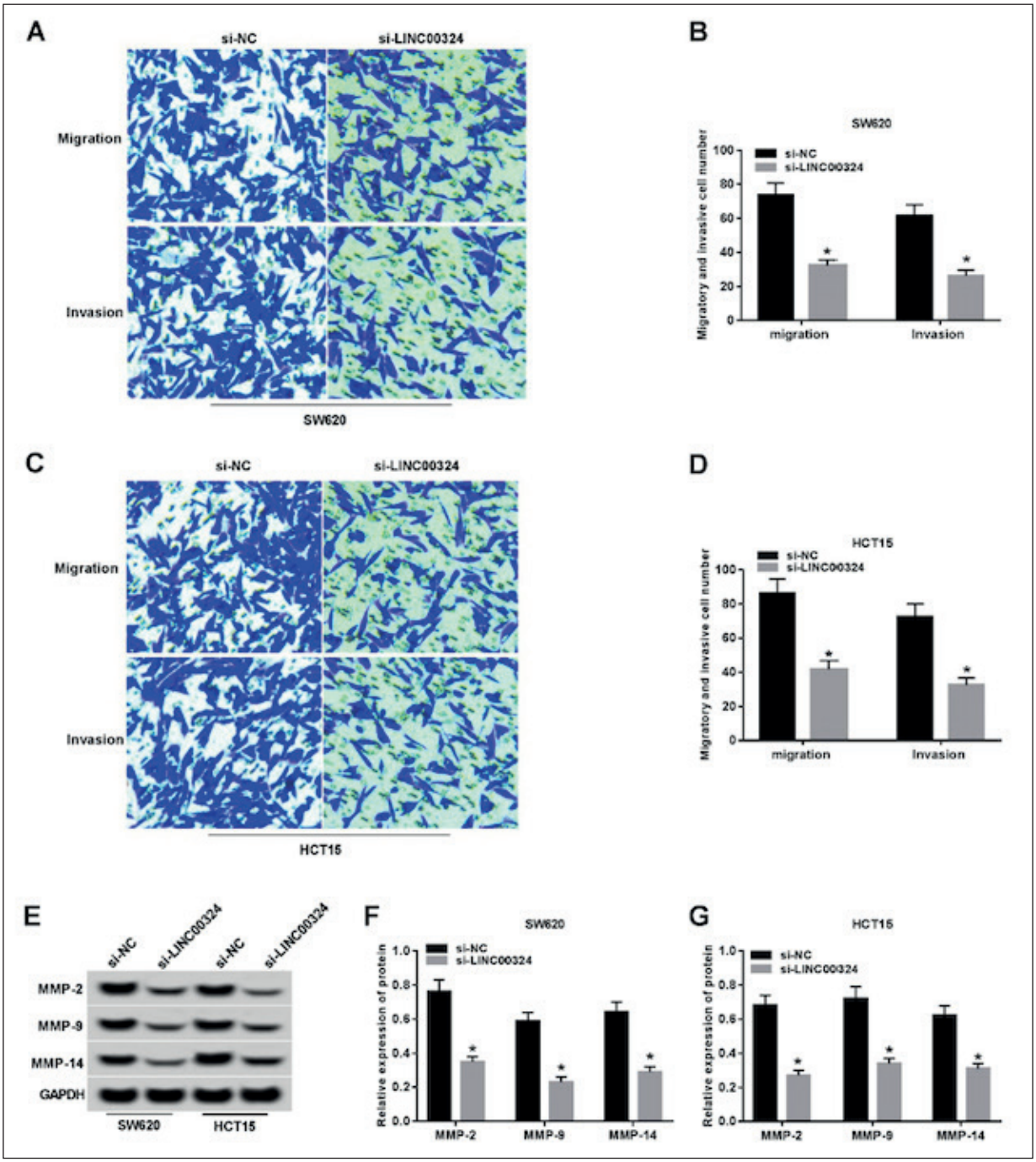

Figure 3. Knockdown of LINC00324 inhibited CRC cell migration and invasion. **A** and **B**, Migration and invasion of SW620 cells transfected with si-LINC00324 were measured by transwell assay (100×). **C** and **D** Migration and invasion of HCT15 cells transfected with si-LINC00324 were measured by transwell assay (100×). **E-G**, The protein levels of MMP-2, MPP-9 and MPP-14 were detected by Western blot in SW620 and HCT15 cells transfected with si-LINC00324.

LINC00324 Directly Interacted With MiR-214-3p

Emerging research suggests that lncRNAs inhibit miRNA-mediated functions *via* directly sponging various miRNAs. LINC00324 was predicted to hold a potential binding site for miR-214-3p by bioinformatics software starBase v2.0 (Figure 5A). To confirm the relationship between LINC00324 and miR-214-3p, dual luciferase reporter assay was performed. The result

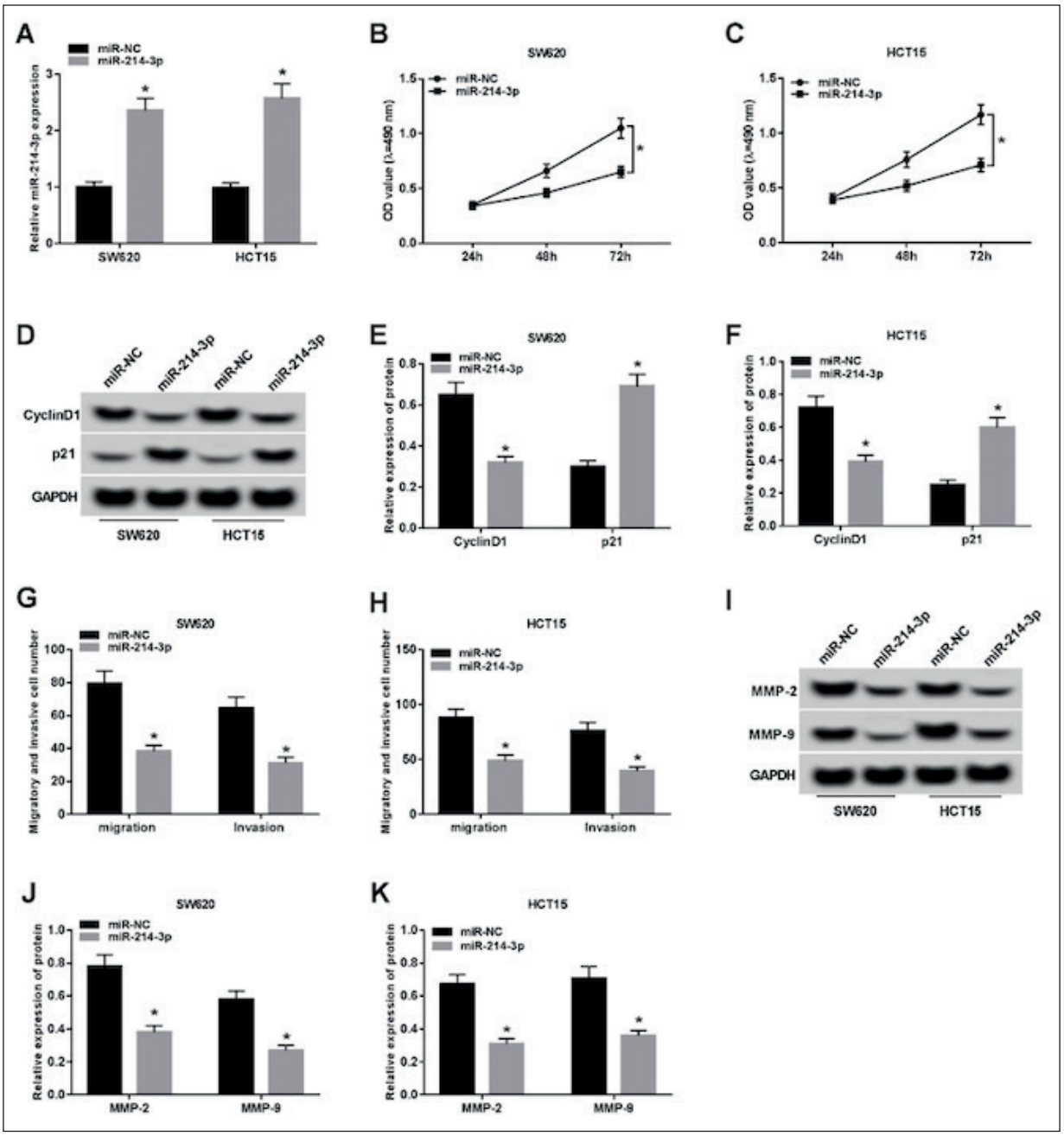

Figure 4. Overexpression of miR214-3p suppressed CRC cell proliferation, migration and invasion. **A**, The expression of miR-214-3p was detected by qRT-PCR in SW620 and HCT15 cells transfected with miR-214-3p mimic. **B** and **C**, Proliferation of SW620 and HCT15 cells transfected with miR-214-3p mimic was measured by MTT assay. **D-F**, The levels of proliferation-related proteins were detected by Western blot in SW620 and HCT15 cells transfected with miR-214-3p mimic. **G** and **H**, Migration and invasion of SW620 and HCT15 cells transfected with miR-214-3p mimic were measured by transwell assay. **I-K**, The protein levels of MMP-2 and MPP-9 were detected by Western blot in SW620 and HCT15 cells transfected with miR-214-3p.

revealed that the luciferase expression of WT-LINC00324 was markedly decreased by miR-214-3p in SW620 and HCT15 cells, while there was no change when the LINC00324 was mutated (Figure 5B and C), which confirmed miR-214-3p to be a target of LINC00324. Subsequent-

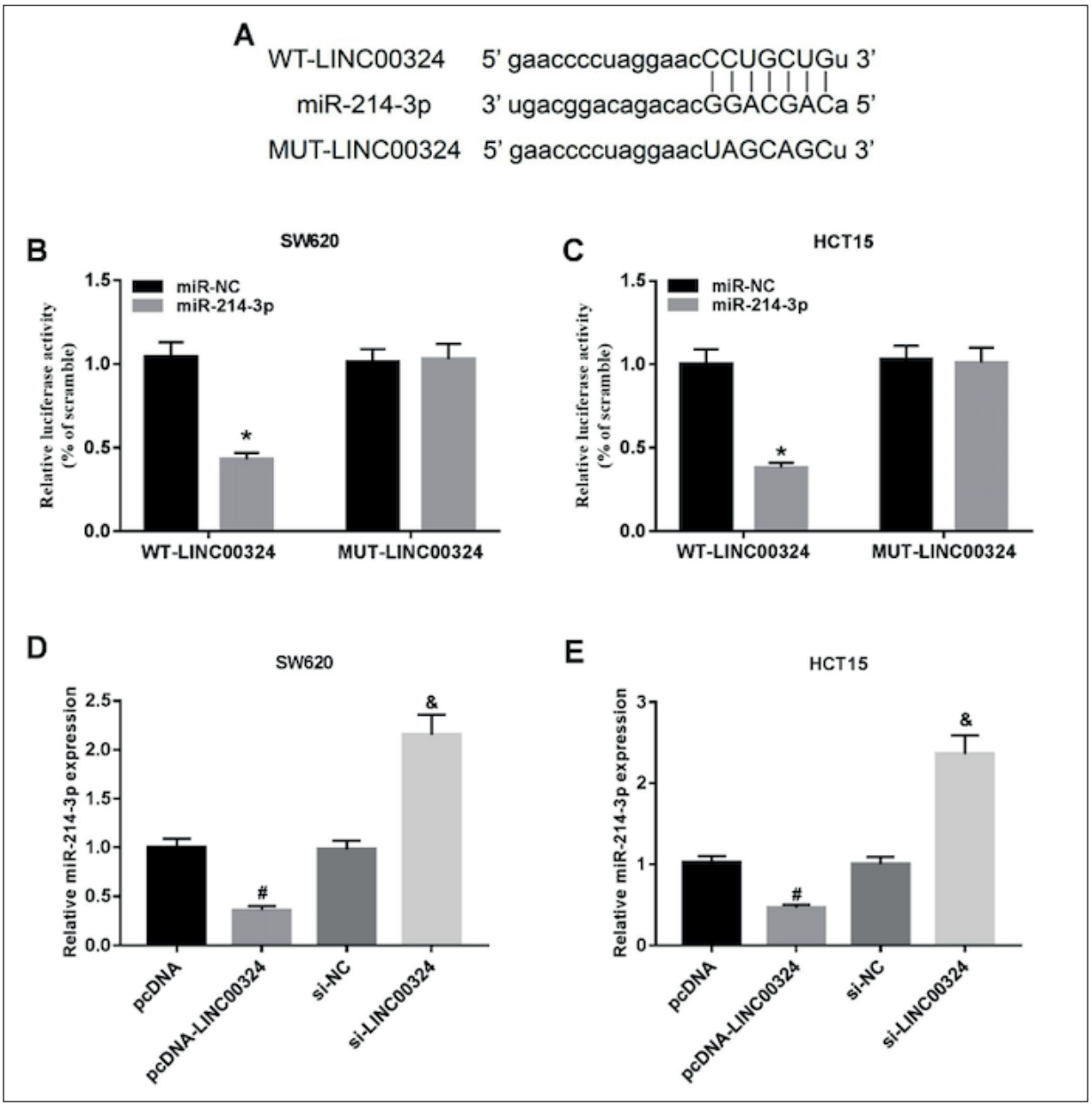

Figure 5. MiR-214-3p was a target of LINC00324 and downregulated by LINC00324. **A**, the binding site between LINC00324 and miR-214-3p was predicated by starBase v2.0. **B** and **C**, The luciferase activity was measured in SW620 and HCT15 cells co-transfected with miR-214-3p and WT-LINC00324 or MUT-LINC00324. **D** and **E**, The expression of miR-214-3p was detected in SW620 and HCT15 cells transfected with pcDNA-LINC00324 or si-LINC00324.

ly, we detected the expression of miR-214-3p in cells transfected with pcDNA-LINC00324 or si-LINC00324. The data showed that miR-214-3p level was decreased when LINC00324 was overexpressed, and knockdown of LINC00324 increased the expression of miR-214-3p in both SW620 and HCT15 cells (Figure 5D and E), indicating miR-213-3p was negatively regulated by LINC00324.

Downregulation of miR-214-3p Reversed Effects of LINC00324 Knockdown on Proliferation, Migration and Invasion of CRC Cells

To further investigate the interaction between LINC00324 and miR-214-3p, the level of miR-214-3p was measured in cells transfected with si-LINC00324 or co-transfected with si-LINC00324 and anti-miR-21-3p. The result

showed that miR-214-3p expression was upregulated by si-LINC00324 but rescued when co-transfected with anti-miR-214-3p (Figure 6A). MTT assay suggested that the inhibiting effect of LINC00324 knockdown on SW620 and HCT15 cell proliferation was reversed by anti-miR-214-3p (Figure 6B and C). The western blot results of Cyclin D1 and p21 further detected this (Figure 6D-F). In addition, cell migration and invasion were observed. The data indicated that inhibiting miR-214-3p expression rescued the effects of LINC00324 knockdown on migration and invasion of SW620 and HCT15 cells (Figure 6G and H). Besides, the protein levels of MMP-2 and MMP-9 were decreased in cells transfected with si-LINC00324 while upregulated by anti-miR-214-3p (Figure 6I-K). All results proved that the downregulation of miR-214-3p rescued the inhibiting effects of LINC00324 knockdown on proliferation, migration and invasion of SW620 and HCT15 cells.

Discussion

Recently, many lncRNAs have been proved to be important regulators in cell growth and tumorigenesis (20). However, their regulatory mechanism is still poorly understood. In this study, we showed that LINC00324 was highly expressed, while miR-214-3p was decreased in CRC cells. Then, we demonstrated that knockdown of LINC00324 and miR-214-3p overexpression both suppressed proliferation, migration and invasion in CRC cells. Furthermore, we researched the relationship between LINC00324 and miR-214-3p and identified miR-214-3p as a target of LINC00324. Additionally, knockdown of miR-214-3p rescued the inhibited effects of LINC00324 knockdown on proliferation, migration and invasion in CRC cells.

Gene mutations and dysregulations were frequently found in cancers. The aberrantly expressed lncRNAs were suggested to have vital functions in the development and progression of cancers[20]. For instance, the upregulated lncRNA CRNDE promoted CRC cell proliferation[21]; inversely, lncRNA KIAA0125 was downregulated and had a tumor-suppressor activity in CRC[22]. Moreover, lncRNAs have been shown as promising diagnostic biomarkers for many cancers[23-25]. In the present study, we detected a high expression level of LINC00324 in CRC cells, implying that LINC00324 might serve as a proto-oncogene in CRC. LINC00324 has been found to be abnormally expressed in various cancers, including osteosarcoma, gastric cancer and lung adenocarcinoma, and the tumor-promoting functions of LINC00324 have been verified in these cancers[7-9]. In addition, Gong et al[26] identified LINC00324 as a feasible prognostic biomarker for thymoma. However, as far as we know, there is no study reporting the effect of LINC00324 in CRC.

We studied the roles of LINC00324 in CRC cells development. Knockdown of LINC00324 inhibited CRC cell proliferation, migration and invasion. In addition, Cyclin D1 and three MMPs levels were decreased and p21 as well as p27 were induced by LINC00324 knockdown. Cyclin D1 (CCND1) is an important regulator in the cell cycle. It was reported that Cyclin D1 was upregulated in CRC and promoted cell proliferation in CRC[27]. P21 and p27 are cyclin-dependent kinase (CDK) inhibitors, which play an inhibitory effect on cell proliferation[28,29]. MMPs were reported to overexpress in many cancers[30]. Also, it has been demonstrated that the levels of MMP-2, MMP-9 and MMP-10 were increased in CRC and promoted cell migration and invasion[31,32].

A number of studies have elucidated that lncRNAs exhibited tumor-suppressive or promoting functions *via* interacting with miRNAs. We found the level of miR-214-3p was reduced in CRC cells. Later experiment revealed that miR-214-3p was a downstream target of LINC00324. Increased evidence indicated that miR-214-3p participated in cancer development. In particular, overexpression of miR-214-3p inhibited CRC cell proliferation by passivating the PI3K/Akt pathway[19]. Also, miR-214 played a suppressing effect in CRC by targeting MED19[33]. In the present study, the tumor-suppressing role of miR-214-3p overexpression was also proved in CRC cells. Combining with the recovery experiment, LINC00324 was considered to play a tumor-promoting action in CRC cells *via* inhibiting miR-214-3p expression.

Although the functions of LINC00324 and its downstream target miR-214-3p have been demonstrated in CRC cells, there are some shortages and deficiencies in the present study. We did not obtain CRC tissue samples and perform functional experiments *in vivo*. Additionally, the downstream of miR-214-3p and the mechanism on regulating CRC development still need further study.

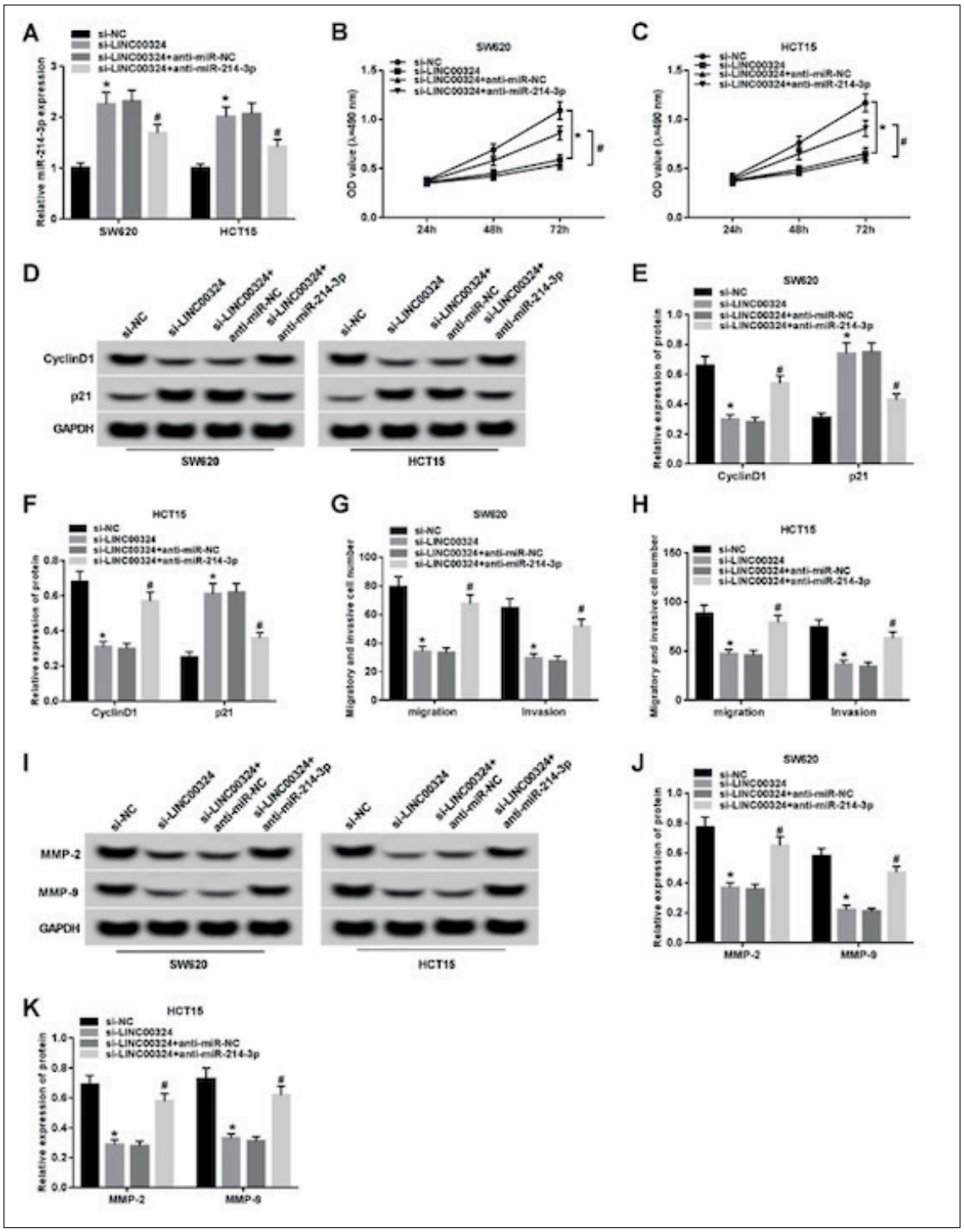

Figure 6. Inhibiting miR-214-3p expression reversed the effects of LINC00324 knockdown on proliferation, migration and invasion of CRC cells. **A**, the expression of miR-214-3p was detected in SW620 and HCT15 cells transfected with si-LINC00324 or si-LINC0032 + anti-miR-214-3p. **B** and **C**, Proliferation of SW620 and HCT15 cells transfected with si-LINC00324 or si-LINC0032 + anti-miR-214-3p was measured by MTT assay. **D-F**, The levels of proliferation-related proteins were detected by Western blot in SW620 and HCT15 cells transfected with si-LINC00324 or si-LINC0032 + anti-miR-214-3p. **G** and **H**, Migration and invasion of SW620 and HCT15 cells transfected with si-LINC00324 or si-LINC0032 + anti-miR-214-3p were measured by transwell assay. **I-K**, The protein levels of MMP-2 and MPP-9 were detected by Western blot in SW620 and HCT15 cells transfected with si-LINC00324 or si-LINC0032 + anti-miR-214-3p.

Conclusions

These results showed that LINC00324 was increased and miR-214-3p was decreased in CRC cells. Subsequently, we demonstrated LINC00324 played a positive role in CRC cell proliferation, migration and invasion by targeting miR-214-3p. This is the first study to report the effect of LINC00324 on CRC progress and the underlying mechanism, which may provide a theoretical basis for the diagnosis and treatment of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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