

Upregulation of miR-324-5p Inhibits Proliferation and Invasion of Colorectal Cancer Cells by Targeting ELAVL1

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Colorectal cancer (CRC) is a common clinical cancer that remains incurable in most cases. miRNAs are reported to play a part in the development of various tumors. In the present study, we found that miR-324-5p was downregulated in CRC cells, while ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like protein 1 (ELAVL1) showed a higher expression. miR-324-5p transfection significantly inhibited the proliferation as well as invasion in both SW620 and SW480 cells. miR-324-5p mimic transfection markedly decreased the expression of ELAVL1. Luciferase reporter gene assay confirmed that ELAVL1 is a direct target of miR-324-5p. Furthermore, cancer invasion factors uPA, uPAR, and MMP-9 were found to drop significantly in miR-324-5p-transfected groups. To conclude, our findings indicate that miR-324-5p may play a suppressive role in colorectal cell viability and invasion, at least in part, through directly targeting ELAVL1. Therefore, miR-324-5p might function as a promising candidate for CRC treatment and deserves deeper research.

Key words: Colorectal cancer (CRC); miR-324-5p; Proliferation; Invasion; (Embryonic lethal, abnormal vision, *Drosophila*)-like protein 1 (ELAVL1)

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant digestive tract tumors¹ worldwide. This cancer risk usually rises after the age of 50. Development of CRC involves multiple factors, including old age, genetic disorders, and lifestyle factors like diet and obesity. The current treatment of CRC primarily consists of surgery, chemotherapy, and radiation. Although great progress has been made to increase the survival of patients²⁻⁴, in most cases the disease remains incurable. So studies searching for novel targets or therapies are still in urgent need.

Over the recent decades, a type of non-protein-coding RNA molecule known as microRNA (miRNA) has been involved in cancer development via functioning as tumor suppressors or oncogenes⁵. miRNAs can participate in a variety of cellular biological processes including cell survival, apoptosis, proliferation, differentiation, and cell cycle progression^{6,7}. Cao et al.⁸ found that miR-324-5p could suppress hepatocellular carcinoma cell invasion via inhibiting extracellular matrix (ECM) degradation. In addition, miR-324-5p was recently reported

to have essential biological functions in breast cancer and CRC⁹.

ELAVL1, also known as human antigen R (HuR), belongs to a family of proteins analogous to the *Drosophila* embryonic-lethal abnormal vision (ELAV)¹⁰. This is an RNA-binding protein (RBP) containing an AU-rich element (ARE) and a U-rich element (URE). ELAVL1 increases the stability of mRNAs, thereby promoting gene expression¹¹. A previous study revealed that ELAVL1 overexpression was observed in 76% of colorectal adenomas and 94% of colorectal adenocarcinomas¹². Furthermore, a therapeutic strategy to target ELAVL1 in CRC was shown to be highly promising¹³. However, no reports were observed to research the connection between miR-324-5p and ELAVL1 and their applicative potential in suppressing CRC.

In this study, we predicted that miR-324-5p can directly target ELAVL1 and investigated whether miR-324-5p can influence CRC cells via targeting ELAVL1. The final aim was to elucidate the therapeutic potential of miR-324-5p/ELAVL1 in CRC and bring hope to the suffering patients.

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MATERIALS AND METHODS

Cell Culture and Transfection

Two colon cancer cell lines, SW620 and SW480 (ATCC, Manassas, VA, USA)¹⁴, were studied along with the normal human colon mucosal epithelial cell line NCM-460, which was used as a control. All cell lines were incubated with RPMI-1640 (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a 5% CO₂ atmosphere. For transfection, SW620 and SW480 cells were seeded in six-well plates at a cell density of 70%–90% and transfected with Lipofectamine™ 3000 reagent (Thermo Fisher) and miR-324-5p mimic (5'-UGUGGU-UACGGG AUCCCUACGC-3)¹⁵ or miR-324-5p inhibitor (5'-ACACCAAUGCCCUAGGGGAUGCG-3)¹⁶ according to the manufacturer's instructions. Nonhomologous miRNA mimic control was used as the negative control (NC).

RNA Isolation and RT-qPCR

Cells were transferred into six-well plates, and the total RNA was isolated with Invitrogen TRIzol reagent (Thermo Fisher). Total RNA was then reverse transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa, Dalian, P.R. China). RT-qPCR was performed at 95°C for an initial 10 min followed by 40 cycles of denaturation for 30 s at 95°C, annealing at 55°C for 30 s, and extension at 72°C for 30s with the SYBR Premix Ex Taq (Takara). The mRNA relative expression levels were then calculated with the 2^{-Ct} method after normalization by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). U6 was used as a control for miRNA expression. Sequences (Thermo Fisher) of the PCR primers are as follows: U6, 5'-GCTTCGGCAGCACATATACT-3 (forward) and 5'-AACGCTTCACGAATTTGCGT-3 (reverse); GAPDH, 5'-GGAGCGAGATCCCTCCAAAAT-3 (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3 (reverse); miR-324-5p, 5'-GAGGCCAAGCCCTGGTATG-3 (forward) and 5'-CGGGCCGATTGATCTCAGC-3 (reverse); ELAVL1,

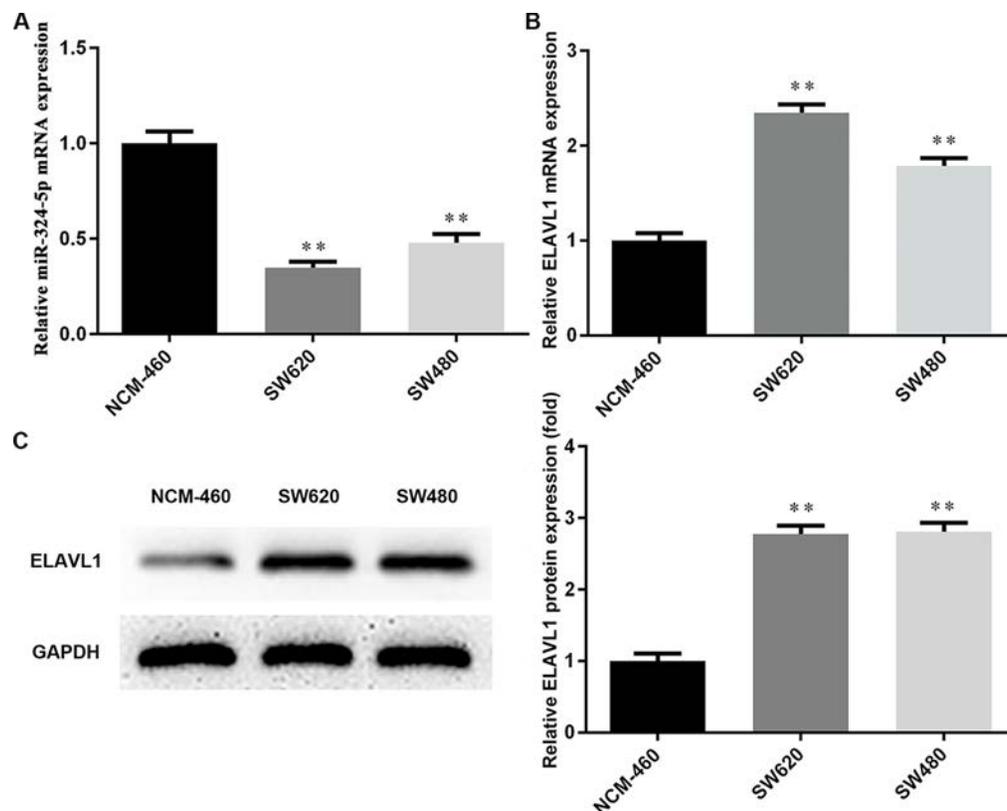


Figure 1. Expression of microRNA (miR)-324-5p and ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like protein 1 (ELAVL1) in colorectal cells. (A) The expression levels of miR-324-5p in colorectal cancer (CRC) cells were detected using RT-qPCR. (B) The expression of ELAVL1 in SW620 and SW480 cells was detected by RT-qPCR. (C) The protein expression levels of ELAVL1 in SW620 and SW480 cells were detected by Western blotting. ** $p < 0.01$ versus NCM-460 group.

5'-AGCTACGAATCTCCGACCAC-3 (forward) and 5'-CGTTATCCCATGTGTCGAAGAA-3 (reverse).

MTT Assay

Forty-eight hours after transfection, SW620 and SW480 cells were cultured at a density of 5×10^3 cells/well in 96-well plates¹⁷. Then cells were incubated with 20 μ l of MTT solution (0.5 mg/ml; Sigma-Aldrich) for a further 4 h at 37°C. The formazan crystals were solubilized in 100 μ l of dimethyl sulfoxide (DMSO; Sigma-Aldrich). Absorbance of each well was measured by a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at a wavelength of 490 nm.

Western Blot

Briefly, total protein was extracted with RIPA buffer containing 1% protease inhibitor PMSF, and a BCA Protein Assay Kit (Beyotime, Shanghai, P.R. China)

was applied to determine the protein concentration. Ten percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate the proteins, and the proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA), and then the membranes were incubated with primary antibodies ELAVL1 (1:1,000; ab200342; Abcam, Cambridge, MA, USA), matrix metalloproteinase 9 (MMP-9; 1:1,000; ab73734; Abcam), urokinase-type plasminogen activator (uPA; 1:1,000; ab169754; Abcam), uPA receptor (uPAR; 1:500; ab103791; Abcam), and GAPDH (1:5,000; ab8245; Abcam), respectively, at 4°C overnight. After washing, corresponding horse radish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; Abcam) were used, and then chemiluminescence (Beyotime) was detected.

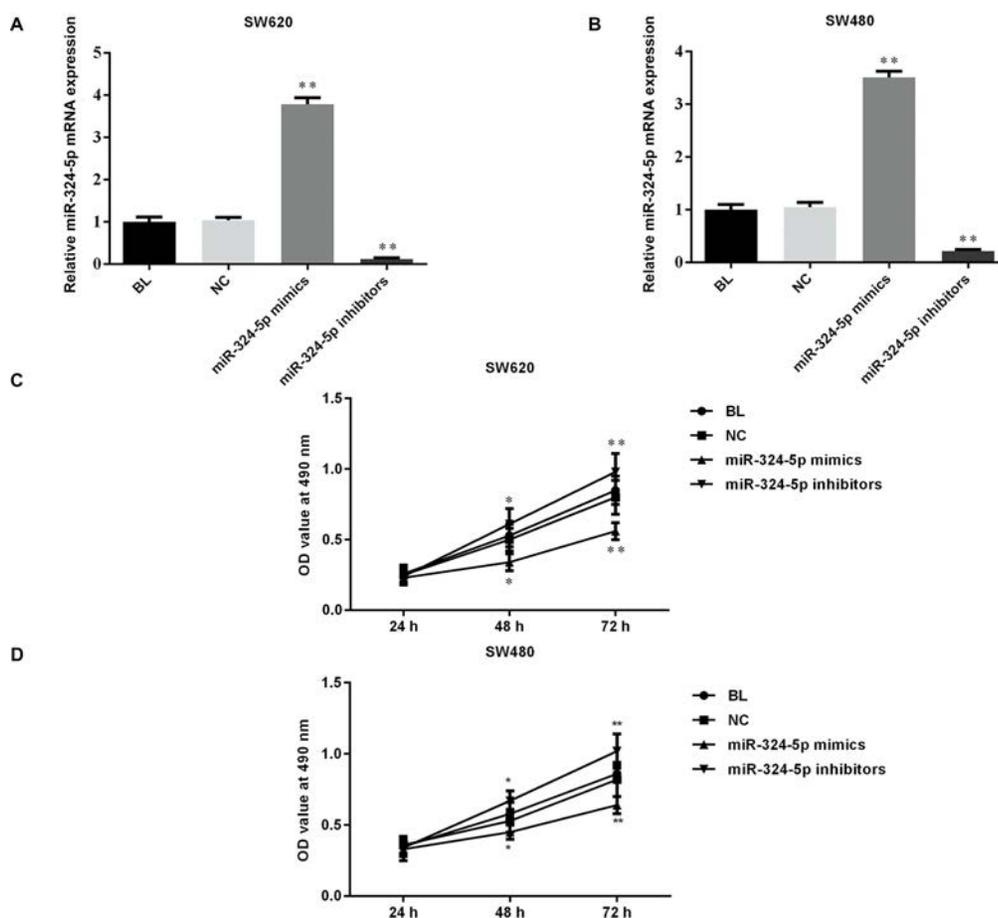


Figure 2. miR-324-5p suppresses proliferation of colorectal cells. RT-qPCR was used to verify the expression of miR-324-5p in SW620 (A) and SW480 (B) cells. MTT assay was used to determine the proliferation of SW620 (C) and SW480 (D) cells. OD values between miR-324-5p mimics and control groups were significantly different. ** $p < 0.01$ versus negative control (NC) group, * $p < 0.05$ versus NC group.

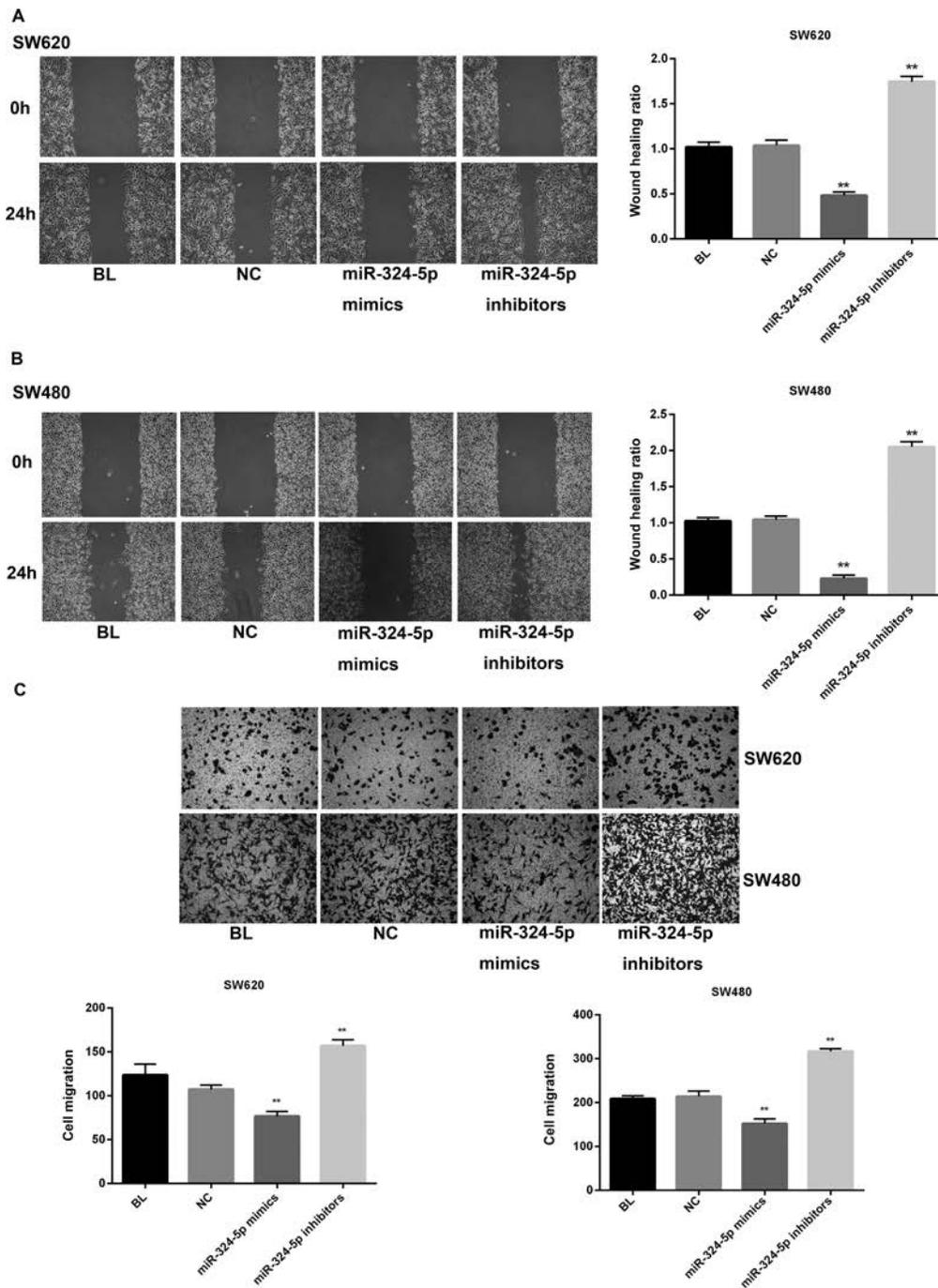


Figure 3. miR-324-5p suppresses migration and invasion of colorectal cells. Wound healing assay verified the effect of miR-324-5p on cell migration in SW620 (A) and SW480 (B) cells. (C) Transwell invasion assay was used to measure the invasion of SW620 and SW480 cells. $**p < 0.01$ versus NC group.

Wound Healing Assay

Cells were seeded into six-well dishes at a density of 1×10^5 cells per well and incubated in a humidified incubator at 37°C with 5% CO_2 . A wound was generated using a sterilized 1-ml pipette in the cell monolayer. The debris was washed with PBS and incubated with serum-free medium. Photos were taken at 0 and 24 h after the scraping, respectively. Experiments were carried out in triplicate at least three times.

Transwell Assay

Cells were serum starved for 24 h and then plated at the density of 1×10^5 cells per well in the serum-free medium. RPMI-1640 medium containing 10% FBS was added into the bottom chamber (8- μm pore size; BD

Pharmingen, San Diego, CA, USA) as a chemoattractant. After incubation for 24 h¹⁸, nonmigrating cells were removed from the upper chamber with a cotton swab, and the cells that migrated through the membrane were fixed with 4% formaldehyde (Sigma-Aldrich) and stained with crystal violet staining solutions (Sigma-Aldrich)¹⁹.

Dual-Luciferase Reporter Gene Assay

miR-324-5p targets were predicted with the TargetScan database (<https://www.targetscan.org>). Luciferase reporter vectors for the wild type and mutant type of ELAVL1 3'-untranslated regions (3'-UTRs) were then constructed. Either miR-324-5p mimics or control mimics [negative control (NC)] were cotransfected with the wild-type or mutant-type luciferase reporter vector into SW620 and

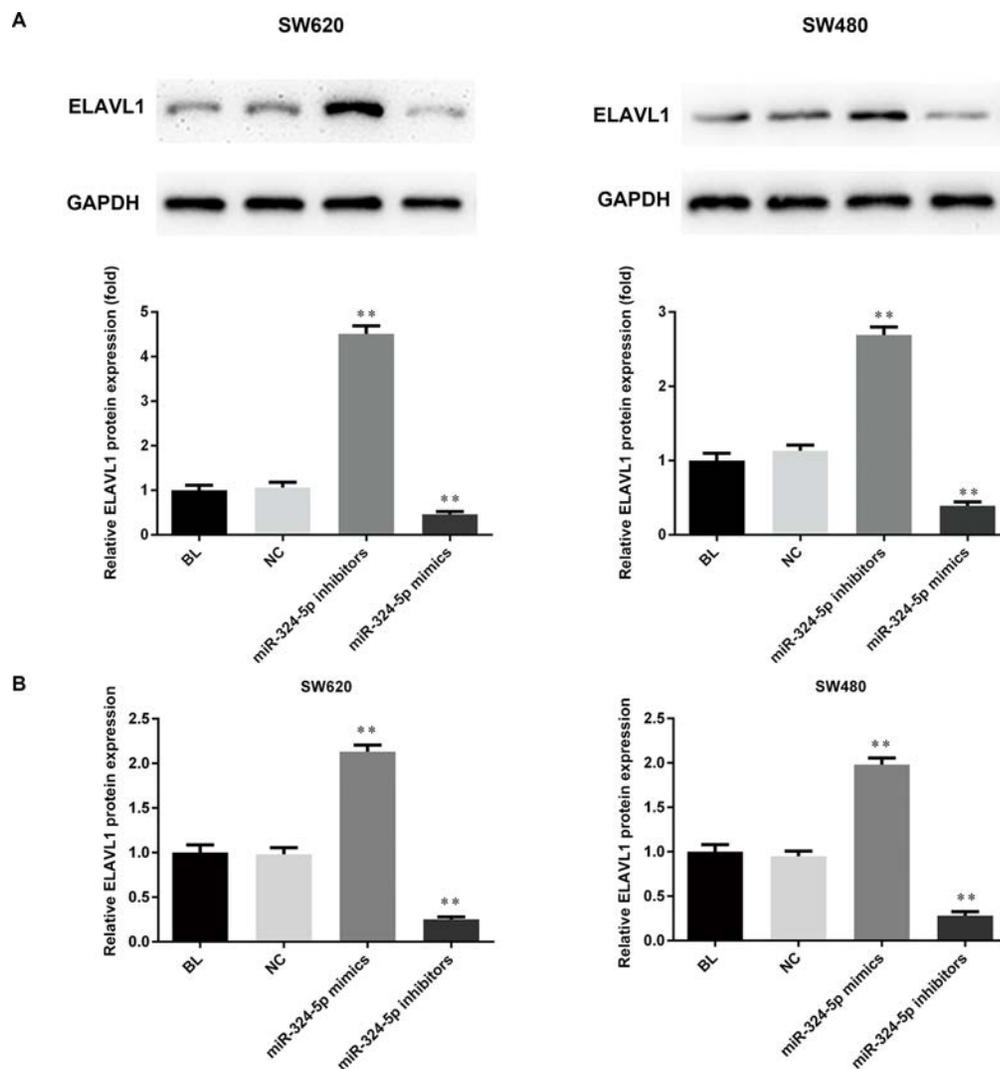


Figure 4. miR-324-5p overexpression suppressed ELAVL1. In SW620 and SW480 cells, ELAVL1 expressions in protein (A) and mRNA (B) were measured by Western blotting. ** $p < 0.01$ versus NC group.

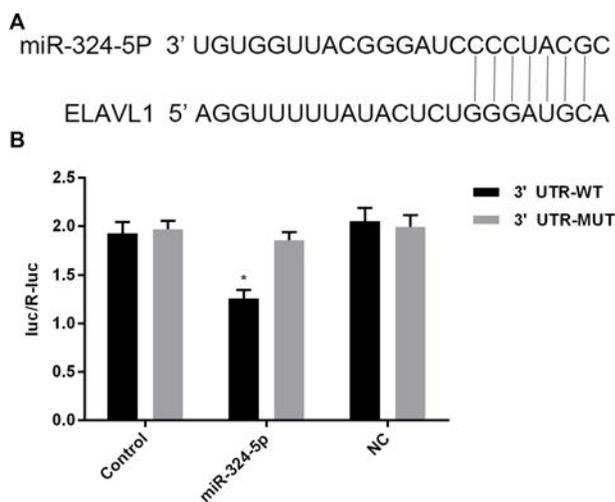


Figure 5. ELAVL1 was a direct target of miR-324-5p. (A) Predicted miR-324-5p target sequences in the 3'-untranslated region (3'-UTR) of ELAVL1. (B) The miR-324-5p target sequence from ELAVL1 was cloned into the 3'-UTR of a luciferase reporter gene. Seed site mut was used to control for binding specificity. Luciferase activity was determined by the Dual-Luciferase Reporter Assay System. * $p < 0.05$ versus NC group.

SW480 cells by Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). The pmirGLO Dual-Luciferase miRNA Target Expression vector (Promega, Madison, WI, USA) was transfected and served as the control. Forty-eight hours after cell transfection, the luciferase activity was assessed with the Dual-Luciferase Reporter Assay System (Promega)²⁰.

Statistical Analysis

Data are expressed as mean \pm standard error of the mean (SEM) of triplicate samples. Data significance was measured through Student's *t*-test and one-way analysis of variance. Statistical analysis was performed by IBM SPSS Statistics v20.0 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered as a statistically significant difference.

RESULTS

miR-324-5p Is Downregulated While ELAVL1 Is Overexpressed in CRC Cells

A couple of isogenic human colon cancer cell lines derived from a lymph node metastasis (SW620) or the primary tumor (SW480) was used in this study. First,

the expression of miR-324-5p and ELAVL1 in CRC cells was measured via qRT-PCR and Western blotting. As shown in Figure 1A, compared with the normal human colon mucosal epithelial cell line NCM-460, miR-324-5p was at a significantly lower level in SW620 and SW480 cells. Conversely, ELAVL1 exhibited an obvious increase in both mRNA and protein levels in CRC cells (Fig. 1B and C). Our results provide novel evidence of miR-324-5p downregulation and ELAVL1 upregulation in CRC cells, indicating that miR-324-5p and ELAVL1 may play a part in CRC development.

Overexpression of miR-324-5p in CRC Cells Significantly Inhibits Cell Proliferation

To explore whether miR-324-5p can affect the proliferation in CRC, we stably overexpressed miR-324-5p in SW620 and SW480 cells. Figure 2A and B shows that transfection of miR-324-5p mimics was a success in our experiments. As shown in Figure 2C and D, MTT assays revealed that miR-324-5p overexpression significantly reduced the proliferation, while miR-324-5p inhibitors obviously heightened the proliferation in both SW620 and SW480 cells. Results here indicated that miR-324-5p may help inhibit the viability of CRC cells.

miR-324-5p Transfection Suppresses the Migration and Invasion of CRC Cells In Vitro

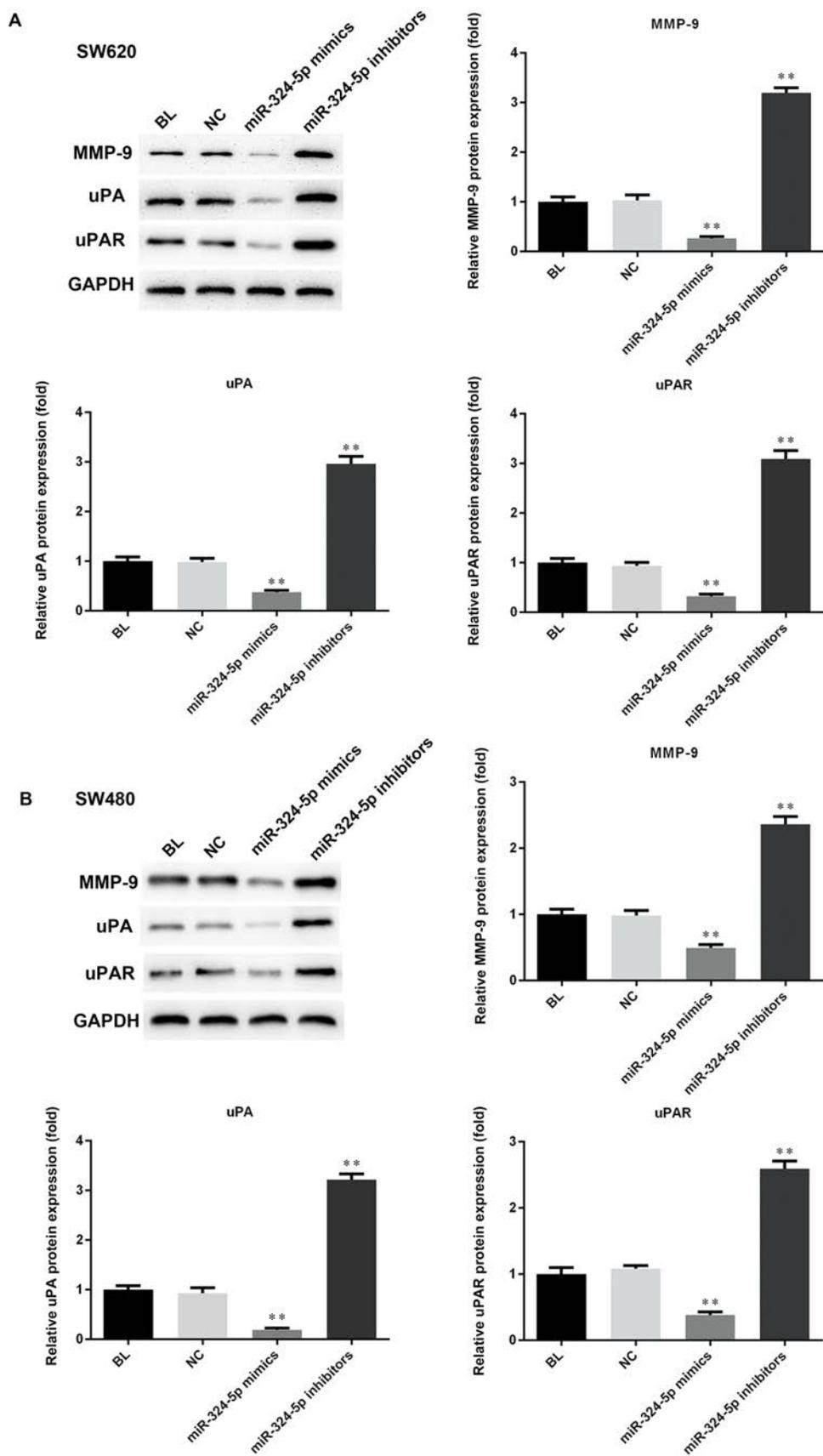
To further observe the effect of miR-324-5p on cell motility in CRC, the wound healing assay and Transwell invasion assay were performed. As exhibited in Figure 3A, wound closure of miR-324-5p-transfected SW620 cells was slower than cells in the blank (BL) or NC group after scratching, while miR-324-5p inhibition significantly promoted wound closure. Likewise, SW480 cells showed similar results (Fig. 3B). Invasive ability was also observed in a parallel invasive assay (Fig. 3C). In both SW620 and SW480 cells, the number of miR-324-5p mimic-transfected cells that penetrated the membrane largely decreased compared with the BL and NC groups. Also, the miR-324-5p inhibitor group showed markedly more penetrating cells than the BL and NC groups. Taken together, our findings demonstrated that miR-324-5p could suppress the migration and invasion of CRC cells.

ELAVL1 Expression in SW620 and SW480 Cells Was Significantly Inhibited by miR-324-5p

We then tried to determine the influence of miR-324-5p on ELAVL1 expression in CRC cells. Figure 4A

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Figure 6. miR-324-5p overexpression suppressed matrix metalloproteinase 9 (MMP-9) and urokinase-type plasminogen activator (uPA) system. In SW620 (A) and SW480 (B) cells, the proinvasion factors MMP-9, uPA, and uPA receptor (uPAR) were tested by Western blotting. ** $p < 0.01$ versus NC group.



shows that in SW620 and SW480 cells, ELAVL1 protein had a much lower expression in the miR-324-5p-transfected group, while miR-324-5p inhibition significantly promoted ELAVL1 expression to a level even higher than in the BL and NC groups. In addition, RT-qPCR was applied to evaluate the mRNA level of ELAVL1. As indicated in Figure 4B, the mRNA level of ELAVL1 in CRC cells showed the same tendency as with protein. miR-324-5p overexpression decreased ELAVL1 expression, and the corresponding inhibitors markedly reversed the low expression of ELAVL1. Results showed that miR-324-5p may negatively regulate ELAVL1 in CRC.

miR-324-5p Directly Binds and Regulates ELAVL1

To further understand the connection of miR-324-5p and ELAVL1 in CRC, the potential target genes of miR-324-5p were analyzed by TargetScan databases. As shown in Figure 5A, ELAVL1 was one of the candidate targets. Bioinformatic analysis indicated that the 3'-UTR of ELAVL1 contains a predicted binding site for miR-324-5p. Luciferase reporter assays were then used to confirm that ELAVL1 is regulated by miR-324-5p. In Figure 5B, the relative activity of luciferase in the reporter containing a wild-type ELAVL1 3'-UTR was significantly decreased after miR-324-5p cotransfection in SW620 cells, thus confirming that ELAVL1 serves as a direct target of miR-324-5p.

Proinvasion/Metastasis Protein Expression Downstream of ELAVL1 Was Inhibited by miR-324-5p in SW620 and SW480 Cells

To explore deeper the mechanism of miR-324-5p-induced proliferation and invasion inhibition, classic proinvasion/metastasis proteins MMP-9, uPA, and uPAR²¹ were tested by Western blotting. As shown in Figure 6, it was revealed that expression of the above proteins significantly dropped in miR-324-5p-transfected cells compared with BL and NC cells. On the other hand, miR-324-5p inhibition markedly increased the levels of MMP-9, uPA, and uPAR. On this basis, we suspect that miR-324-5p regulates cell invasion and proliferation via targeting ELAVL1 and affecting its downstream proinvasion/metastasis proteins.

DISCUSSION

Taken together, major findings in our current study may be listed as follows: 1) in CRC cell lines SW620 and SW480, miR-324-5p was downregulated in parallel with an overexpression of ELAVL1. 2) miR-324-5p transfection significantly suppressed proliferation ability in both SW620 and SW480 cells. Migration and invasion were also inhibited markedly by miR-324-5p overexpression. 3) miR-324-5p transfection decreased the expression of ELAVL1. 4) ELAVL1 was a direct target

of miR-324-5p, and downstream proinvasion factors of ELAVL1, MMP-9, uPA, and uPAR were inhibited by miR-324-5p overexpression.

miR-324-5p is widely reported to be involved in human diseases. A previous study demonstrated that miR-324-5p attenuates mitochondrial fission and cardiomyocyte apoptosis, thus playing a part in cardiac diseases¹⁶. miR-324-5p was also observed to inhibit glioma cell proliferation by targeting glioma-associated oncogene 1¹⁵. More importantly, this miRNA shows an aberrant expression in almost all human cancers, including breast cancer, esophageal carcinoma, and CRC⁹. In the present study, we mainly explore the role of miR-324-5p in CRC and associated mechanisms. First, this miRNA was confirmed to be low in CRC cells (Fig. 1A). Moreover, existence of miR-324-5p was found to significantly restrain proliferation (Fig. 2C and D), migration, and invasion (Fig. 3) of CRC cells, indicating that miR-324-5p may inhibit CRC development and progression. Earlier evidence provided support that miR-324-5p might function as a therapeutic molecule in CRC^{22,23}, which is consistent with our findings, even though the mechanisms mentioned are quite different.

The RBP ELAVL1 is mainly located in the nucleus but is translocated into the cytoplasm upon cellular stress²⁴. It is generally regarded as an mRNA-stabilizing factor to increase gene expression by binding to the 3'-UTRs of mRNAs. Previous studies show ELAVL1 takes part in cell cycle regulation, cell migration, tumorigenesis, and inflammation²⁵. Furthermore, ELAVL1 aberrant expression is observed in various cancers, like breast, colon, and brain cancers²⁶ and is demonstrated to correlate with poor prognosis²⁷. However, articles focusing on the potential effect of ELAVL1 and relative molecular mechanisms in CRC were not found. In our study, for the first time, an miR-324-5p-ELAVL1 connection was proposed in CRC. We demonstrated that ELAVL1 has an abnormally high expression in CRC (Fig. 1B and C), while miR-324-5p transfection dramatically reduces ELAVL1 (Fig. 4). To confirm that miR-324-5p targets ELAVL1, a luciferase reporter assay was carried out (Fig. 5), which indicated that in CRC cells, ELAVL1 serves as a direct target of miR-324-5p. Therefore, we came to the conclusion that ELAVL1 is directly regulated by miR-324-5p and that miR-324-5p might inhibit cell proliferation and invasion in CRC, at least partially via ELAVL1 targeting.

To confirm our suspicions above and gain a deeper understanding of how miR-324-5p affects CRC, some downstream molecules of ELAVL1 were investigated (Fig. 6). The invasive growth of tumor cells is strongly dependent on ECM reconstruction²⁸. The uPA system (uPA and its receptor uPAR) and matrix metalloproteinases (MMP-2/MMP-9) are well established as being responsible for ECM reconstruction²⁹. Strong clinical and experimental evidence has demonstrated a correlation of

elevated uPA and MMP levels with cancer progression and metastasis^{30,31} in CRC. In addition, ELAVL1 was believed to regulate uPA and MMPs²¹. In the current study, we found that miR-324-5p overexpression significantly reduced the level of uPA, uPAR, and MMP-9, which may happen to explain the invasion- and migration-inhibiting effects of miR-324-5p. However, to be more rigorous, follow-up studies are needed, and ELAVL1 inhibition or knockout should be explored.

To sum up, this study suggested that miR-324-5p inhibits cell proliferation and invasion in CRC at least in part via negatively regulating ELAVL1. Therefore, miR-324-5p might function as an effective tumor-suppressing miRNA in CRC. Our study is the first to describe miR-324-5p-ELAVL1 regulation and provides a novel and promising therapeutic target in CRC.

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