European Review for Medical and Pharmacological Sciences 2019; 23: 6105-6118

STAT3-induced upregulation of lncRNA DUXAP8 functions as ceRNA for miR-577 to promote the migration and invasion in colorectal cancer through the regulation of RAB14

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Abstract. – OBJECTIVE: Previous reports have shown that long non-coding RNAs (lncRNAs) are involved in a series of biological processes and cancer in humans. Recently, lncRNA double homeobox A pseudogene 8 (DUXAP8) was frequently reported to be aberrantly expressed in multiple cancers and play a functional role. However, the exact expression, function, and mechanism of DUXAP8 in colorectal cancer (CRC) remain uncovered.

PATIENTS AND METHODS: The expression levels of DUXAP8 were detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The clinical influence of DUXAP8 in HCC patients was statistically analyzed. Luciferase reporter and ChIP assays were carried out for the exploration of whether STAT3 was able to bind to the promoter of DUXAP8. Lost-of-function experiments were carried out for the determination of possible cellular function in CRC cells. The modulating associations between DUXAP8 and miR-577 and RAB14 were further studied in CRC cells.

RESULTS: In this study, we first provided evidence that DUXAP8 was overexpressed in CRC and increasing expression of DUXAP8 indicates advanced clinical progression and poor survival of CRC patients. Then, transcription factor STAT3 was demonstrated to upregulate DUXAP8 in CRC cells. Functional assays *via in vitro* assays revealed that DUXAP8 knockdown through shRNA in HCT116 and LOVO cells inhibited cell proliferation, migration and invasion, and promoted apoptosis. Furthermore, an inverse relationship between DUXAP8 and miR-577 was found. In addition, we confirmed that DUXAP8 served as competing endogenous RNA to modulate miR-577, which can modulate RAB14, a well-studied oncogene.

CONCLUSIONS: Our study revealed that the STAT3-induced up-regulation of DUXAP8 might provide a new perspective for CRC therapy.

Key Words:
LncRNAs, DUXAP8, Colorectal cancer, MiR-577, RAB14, Prognosis, Metastasis.

Introduction

AColorectal cancer (CRC) is the 3rd most malignant tumor worldwide and a major cause of morbidity and mortality throughout the world, with 1,420,000 new cases and 720,000 deaths annually[1,2]. The tumorigenesis of CRC is a complex, multi-step and multi-factorial process and its mortality is going up year by year[3,4]. Although the clinical prognosis of CRC patients has been developed with surgical resection followed by system clinical management (radiation, systemic chemotherapy and targeted therapy) in the early stages, the survival time of CRC patients with metastasis remains very poor[5,6]. Up to date, there are no effective methods for these patients, since the traditional treatments are generally unsuccessful in prolonging their survival[7]. Consequently, understanding of the molecular mechanisms underlying CRC metastasis is vital to the strategy of the clinical intervention of CRC.

The research of non-coding RNAs has recently gained prominence in the field of molecular biology[8]. Long non-coding RNAs (lncRNAs) are one class of non-coding RNAs and defined as transcripts longer than 200 nucleotides with the limited coding potential of < 100 amino acids[9]. Growing clinical and basic evidence from various studies show that lncRNAs, especially several disease-related lncRNAs, have crucial roles in diverse biological processes, such as cell growth,

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differentiation and programmed cell death[10,11]. Recently, the emerging roles of several dysregulated lncRNAs in tumor progression attracted increasing attention from scientists of almost every countries[12,13]. Although the exact roles of lncRNAs are only beginning to be elucidated, their basic effects of acting as tumor suppressors or oncogenes have been confirmed using a series of cells experiments[14,15]. On the other hand, lncRNAs are extensively studied as a novel source of cancer markers for diagnosis and prognosis in tumor patients, and the fact that it is relatively easy for them to be detected in tissues and blood makes lncRNAs suitable for this clinical aim[16,17].

Previously, a recently discovered lncRNA named DUXAP8, located in 22q11.1 and a 2107-bp RNA, was reported to be dysregulated in several tumors[18-20]. In addition, several studies discussed the possible clinical significances and the special biological functions of DUXAP8 in some tumors, and the fact that DUXAP8 served as a tumor promoter was also confirmed *in vitro* assays[21,22]. However, the roles of lncRNAs in CRC have not been well studied. In this work, we utilized a series of methods to explore the expression pattern, clinical significances and biological functions of DUXAP8 in CRC, which helps develop individualized follow-up and the therapeutic strategies for CRC patients.

Materials and Methods

Patients' Samples Collection

A total of 127 colorectal and matched normal samples were collected from patients with colorectal cancer between April 2009 to May 2012 at The Affiliated Hospital of Inner Mongolia Medical University. Before surgery, none of the patients were given anti-tumor therapy. Samples were immediately preserved at -80°C after surgery. The patients all signed the written informed consents and the study was approved by the Ethics Committee of The Affiliated Hospital of Inner Mongolia Medical University.

Cell Transfection

The cell lines used in this study were obtained from Zeping Biological corporation (Haidian, Beijing, China). The culture media was Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) with 10% fetal bovine serum. The culture condition was: 37°C, 5% CO_2. The siRNAs used in this study were obtained from HuaNuo corporation (Changsha, Hunan, China). MiRNA mimics and inhibitors were bought from Ribobio biological corporation (Nantong, Jiangsu, China). The plasmids were all cloned by Biowit Biological corporation (Shenzhen, Guangdong, China). Lipofectamine 3000 reagent (Genetown, Binhai, Tianjin, China) was employed to conduct the siRNAs, miRNA mimics or plasmids transfection.

Real Time-Polymerase Chain Reaction Assays

Total RNAs were isolated by TRIzol kits (XinFan, Minhang, Shanghai, China). Subsequently, the isolated RNA was converted to cDNA using a Promega GoScript^{RT} Mix kit (Mengze, Baoshan, Shanghai, China). The quantitative Real Time-Polymerase Chain Reaction (qT-PCR) analysis of DUXAP8, STAT3 and RAB14 was then performed using an Instant Fluorescent qPCR kit (ZeYe, Songjiang, Shanghai, China). A KAPA one-Step miRNA qRT-PCR kit (Yingchuang, Xi'an, Shanxi, China) was used to detect the expression of miR-577. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 served as the endogenous control. Table I summarized the primer sequences.

Cell Proliferation Detection

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) kits were applied to determine the growth curves of treated cells. In short, 3000 cells (per well) were plated into 96-well plates. After incubation with the CCK-8 solution, the growth curves at 450 nm absorbance were examined at indicated time points (24, 48, 72 and 96 h) by a microplate reader machine.

Table I. Primer sequences for PCR.

Primer name	Sequences
DUXAP8: Forward	GAGAAGCAGTGGTGGGTTCC
DUXAP8: Reverse	GAGCAACACAGATGAACCGC
STAT3: Forward	GGAGGAGGCATTCGGAAAG
STAT3: Reverse	TCGTTGGTGTCACACAGAT
miR-577: Forward	TGCGGTAGATAAAATATTGG
miR-577: Reverse	CCAGTGCAGGGTCCGAGGT
RAB14: Forward	TATGGCTGATTGTCCTCACACA
RAB14: Reverse	CTGTCCTGCCGTATCCCAAAT
GAPDH: Forward	TGTAGTTGAGGTCAATGAAGGG
GAPDH: Reverse	ACATCGCTCAGACACCATG
U6: Forward	CTCGCTTCGGCAGCACA
U6: Reverse	AACGCTTCACGAATTTGCGT

Cell Colony Formation Detection

The cells were maintained using 6-wells plates (about 800 cells/well) in an incubator for 15 days. Then, the cell colonies were immobilized using paraformaldehyde (4%) and treated by the use of crystal violet. After washing, the colonies were photographed by a microscope.

EdU (5-Ethynyl-2'-Deoxyuridine) Assays

Thermo Fisher Click-iT EdU assay kits (Thermo Fisher Scientific, Waltham, MA, USA) were utilized to conduct the EdU assays. The cells were plated into 48-well plates for 24 h. Then, the EdU solution (final concentration: 10 μM) was incubated with the cells for 2 h. After being washed, the cells were permeabilized using Triton X-100 solution. Then, the Click-iT reaction cocktail was added into the cells and incubated for 20-30 min. After the cellular nuclei were stained using the 2-(4-Amidinophenyl)-6-indolecarbamidine (DAPI) solution, the cells were observed using a fluorescence microscope (Leica DM2500; Leica, Buffalo Grove, IL, USA).

Cell Apoptosis Analysis

The cells were treated using designate siRNAs. Forty-eight hours later, cells were trypsinized and collected in Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) buffer. Then, Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) apoptosis assay kits ((Vazyme, Nanjing, China) was employed to stain the cells. Subsequently, the cellular apoptosis was measured by flow cytometry.

Wound Healing Assays

When the cultured cells reached about 100% confluence, the wounded areas were generated by scraping the cells using pipette tips (200 μl). After washing out the cell debris, the wounded areas were photographed at 0 h and 48 h using a microscope.

Transwell Assays

The transwell assays were conducted using twenty-four well transwell plates containing inserts (8 μm pore sizes; pre-coated with Matrigel; BD Biosciences, Franklin Lakes, NJ, USA). In brief, the treated cells (1×10^5 cells/well) were suspended in medium without serum (200 μl) and sequentially placed into the inserts. Meanwhile, 650 μl medium (with 15% fetal bovine serum) was placed into the receivers of the transwell plates. After 24 h incubation, the cells invaded through the membranes were fixed before crystal violet (0.2%) staining. Finally, the cells were observed and counted by a microscope.

Western Blot Assays

The proteins extracted from cells were separated by dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE; 10%). Subsequently, the proteins were electroblotted onto polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. After being blocked using bovine serum albumin (BSA) solution (5%) for 1 h, the primary antibodies anti-RAB14 (Protein Tech Group, Wuhan, Hubei, China), anti-caspase 3 (Sino, Haidian, Beijing, China) and anti-caspase 9 (AVIVA, Daxing, Beijing, China), were separately employed to incubate with the membranes. After incubation for 12 h (at 4°C), Tris, Tris-Buffered Saline containing 0.2-0.4% Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) was used to wash the membranes. After being incubated with matched secondary antibodies, the RAB14, caspase 3/9 were examined by an ECL assay kit (Mengze, Baoshan, Shanghai, China). GAPDH was used as an endogenous control.

Chromatin Immunoprecipitation (ChIP) Assays

After HCT116 cells were fixed using formaldehyde (1%), the cells were lysed and sonicated (300-500 bp fragments). Subsequently, anti-STAT3 antibody (Cell Signaling Technology, Danvers, MA, USA) was added into the lysates. Then, Thermo Fisher Protein A agarose beads (Genowa, Nanchang, Jiangxi, China) were used to precipitate the DNA fragments. Finally, the qPCR assay was used to analyze the purified DNA fragments.

RNA Pulldown Assays

The biotin-labeled RNAs (Biotin-DUXAP8, Biotin-NC) were obtained from MBL Beijing Biotech corporation (Haidian, Beijing, China). The Biotin-DUXAP8 or Biotin-NC (2.5 μg) was placed into HCT116 cellular lysates, followed by incubation for 1.5 h. Subsequently, Dynabeads M-280 Streptavidin kits (HUST Life, Wuhan, Hubei, China) was employed to precipitate the RNA. Finally, the pulldown mixture was analyzed by qRT-PCR assays.

Luciferase Activity Detection

Predicted binding site 1 (P1), P2 or P3 of DUXAP8 promoter sequence, DUXAP8 wild-

type (DUXAP8 wt), DUXAP8 mutant (DUXAP8 mut), RAB14 wild-type (RAB14 wt) or RAB14 mutant (RAB14 mut) sequence was separately constructed into a Luciferase reporter plasmid by Biowit Biological corporation (Shenzhen, Guangdong, China). Then, the cells were respectively treated using these Luciferase reporters, and cells lysis buffer (Mindi, Changsha, Hunan, China) was placed into the cells 48 h post-incubation. Finally, the Luciferase activity was determined using a Promega Luciferase detection kit (Dongcheng, Beijing, China).

Statistical Analysis

SPSS software (SPSS version 18.0 Chicago, IL, USA) was used in this study. Student's *t*-test or one-way ANOVA was employed to examine the statistical differences. The correlation-ships between clinicopathological features and DUXAP8 levels were evaluated using the chi-square test. Kaplan-Meier methods (with log-rank tests) were utilized to evaluate survival curves. Prognostic relevance of each variable to overall survival (OS) and disease-free survival (DFS) was analyzed by the Cox regression model. A p-value < 0.05 was considered statistically significant.

Results

DUXAP8 is Upregulated in CRC Tissues and Cell Lines

To screen the possible functional lncRNAs that may be involved in colorectal tumorigenesis, we performed an integrative analysis of RNA sequencing data from TCGA datasets. As shown in Figures 1A and B, our observation identified 452 up-regulated lncRNAs and 387 down-regulated lncRNAs shown using hierarchical clustering and volcano plots. Among these dysregulated lncRNAs, DUXAP8 was shown to be most upregulated in CRC specimens ($p < 0.01$, Figure 1C). For demonstrating the above results, we carried out qPCR to measure DUXAP8 expression in 127 pair of CRC tissues and matched normal colorectal tissues. We discovered that DUXAP8 levels were markedly upregulated in CRC samples than that in the corresponding adjacent normal specimens (Figure 1D). In addition, the expression of DUXAP8 showed a higher level in CRC cell lines than that in normal colorectal cells (Figure 1E). All the above results indicated that DUXAP8 was frequently down-regulated in CRC. Because of the relatively higher expression of DUXAP8 in HCT116 and LOVO cell lines, we chose them for the subsequent cells experiments.

Association Between the DUXAP8 Expression and Patients' Survival

To further analyze the possible associations between the DUXAP8 expression and clinicopathological parameters in CRC patients, we used a median level of DUXAP8 expression as a cutoff value to divide 127 patients into high-group (n = 64, expression Ct value > 2.76) and low-group (n = 63, expression Ct value < 2.76). As shown in Table II, the results of the chi-square analysis indicated that increased expression of DUXAP8 positively correlated with lymph nodes metastasis (LNM; $p = 0.010$) and TNM stage ($p = 0.016$). Nevertheless, no marked difference was observed between DUXAP8 expression levels and other parameters, such as gender and age ($p > 0.05$). Then, we explored the prognosis values of DUXAP8 in CRC patients. Kaplan-Meier methods were utilized for investigating the influence of DUXAP8 in the survival of CRC patients and the data proved that high DUXAP8 level was correlated with shorter OS (Figure 1F, $p = 0.009$) and DFS times (Figure 1G, $p = 0.0058$) than low DUXAP8 level. More importantly, Univariate Cox regression analyses validated that LNM, TNM stage, as well as DUXAP8 levels were notably associated with both OS and DFS (Table III). Finally, our multivariate analysis detected that lymph nodes metastasis (OS: HR = 2.984, 95% CI = 1.216-4.215, $p = 0.008$; DFS: RR=3.016, 95% CI = 1.215-4.326, $p = 0.014$), TNM stage (OS: HR = 2.847, 95% CI=1.216-4.642, $p = 0.006$; DFS: HR =2.756, 95% CI=1.219-4.019, $p = 0.017$) and high DUXAP8 expression (OS: HR = 3.26, 95% CI = 1.34-5.13, $p = 0.019$; DFS: HR = 2.783, 95% CI = 1.128-3.787, $p = 0.014$) were independent prognostic factors for CRC (Table III).

STAT3 Positively Orchestrated DUXAP8 Expression via Directly Interacting With its Promoter

Accumulating evidence had indicated that transcriptional factors might contribute to the dysregulation of lncRNAs in cancers. Therefore, we next attempted to discover the transcriptional factor, which was able to regulate the expression of DUXAP8. To achieve that, we searched "JASPAR" bioinformatics tool and found that STAT3, a well-studied transcriptional factor that was capable of inducing lncRNAs dysregulation

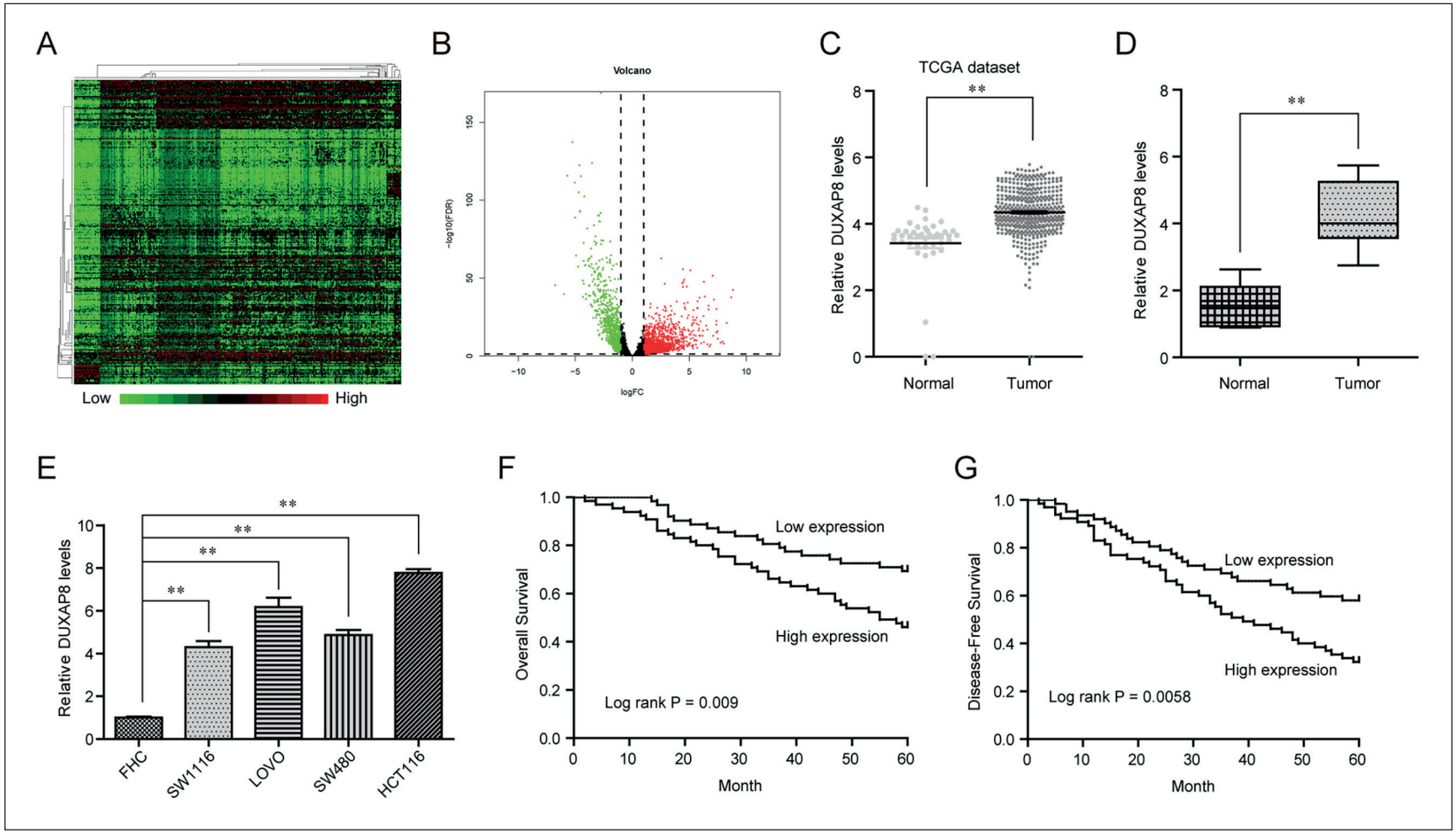

Figure 1. Relative DUXAP8 expression in CRC and its clinical prognosis. ***A,*** Heatmap of the differentially expressed lncRNAs in CRC tumor specimens and normal colorectal tissues based on the TCGA data. ***B,*** Volcano map of aberrantly expressed lncRNAs between CRC and adjacent normal samples using the TCGA data analyses. ***C,*** Expression of DUXAP8 in the TCGA database. ***D,*** Relative levels of DUXAP8 in 127 pairs of CRC tissues and adjacent non-tumor tissues. DUXAP8 expression was normalized to GAPDH levels. ***E,*** The levels of DUXAP8 were increased in the CRC cell lines compared with normal colorectal cells line (FHC). ***F, G,*** Associations of DUXAP8 expression with OS ***(F)*** and DFS ***(G)*** analysis of 127 CRC patients. *p*-values are given on the figures. **p*<0.05, ***p*<0.01.

Table II. Relationship between the expression of DUXAP8 and clinicopathological parameters in CRC.

Parameters	Group	Total	DUXAP8 expression		p-value
			High	Low	
Gender	Male	77	41	36	0.425
	Female	50	23	27	
Age (years)	< 60	63	30	33	0.535
	≥ 60	64	34	30	
Tumor size (cm)	< 5	78	35	43	0.116
	≥ 5	49	29	20	
Local invasion	T1-T2	83	35	45	0.153
	T3-T4	44	26	18	
Histological grade	Well and moderately	76	34	42	0.120
	Poorly	51	30	21	
Lymph nodes metastasis	Negative	85	36	49	0.010
	Positive	42	28	14	
TNM stage	I-II	80	34	46	0.016
	III-IV	48	31	17	

in multiple cancer types, had several binding sites (P1, P2, and P3) with high prediction scores in the promoter region of DUXAP8 (Figure 2A). The siRNA targeting STAT3 (si-STAT3) and pcDNA3.1-STAT3 (which was able to consistently express STAT3) were then respectively treated with CRC cells, and qPCR assays demonstrated that si-STAT3 notably decreased STAT3 levels, and pcDNA3.1-STAT3 was able to significantly increase the STAT3 levels in HCT116 and LOVO cells (Figure 2B and C). In addition, Real Time-PCR was carried out to measure the levels of DUXAP8 in CRC cells when their STAT3 was silenced or elevated. The data suggested that knockdown of STAT3 resulted in remarkably decreased DUXAP8 levels, while enhancing STAT3 expression dramatically promoted the levels of DUXAP8 (Figures 2D, 2E). Moreover, the ChIP assays revealed that STAT3 was capable of directly binding to the P3 site of DUXAP8

Table III. Univariate and multivariate analysis of survival in CRC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Univariate and multivariate analysis of disease-free survival in CRC patients (n = 127)						
Gender	1.231	0.562-1.835	0.549	–	–	–
Age	0.783	0.426-1.527	0.492	–	–	–
Tumor size	1.342	0.576-1.963	0.215	–	–	–
Local invasion	1.426	0.739-2.215	0.172	–	–	–
Histological grade	1.556	0.893-2.452	0.124	–	–	–
Lymph nodes metastasis	3.218	1.326-4.783	0.003	2.984	1.216-4.215	0.008
TNM stage	3.452	1.452-5.321	0.001	2.847	1.216-4.642	0.006
DUXAP8 expression	2.986	1.396-4.261	0.009	2.783	1.128-3.787	0.014
Univariate and multivariate analysis of overall survival in CRC patients (n = 127)						
Gender	1.162	0.768-1.952	0.354	–	–	–
Age	0.963	0.557-2.153	0.156	–	–	–
Tumor size	1.321	0.678-1.951	0.211	–	–	–
Local invasion	1.456	0.873-2.331	0.114	–	–	–
Histological grade	1.442	0.962-2.142	0.169	–	–	–
Lymph nodes metastasis	3.521	1.456-5.321	0.001	3.016	1.215-4.326	0.014
TNM stage	2.985	1.328-4.557	0.007	2.756	1.219-4.019	0.017
DUXAP8 expression	3.214	1.442-4.672	0.006	2.895	1.219-4.258	0.011

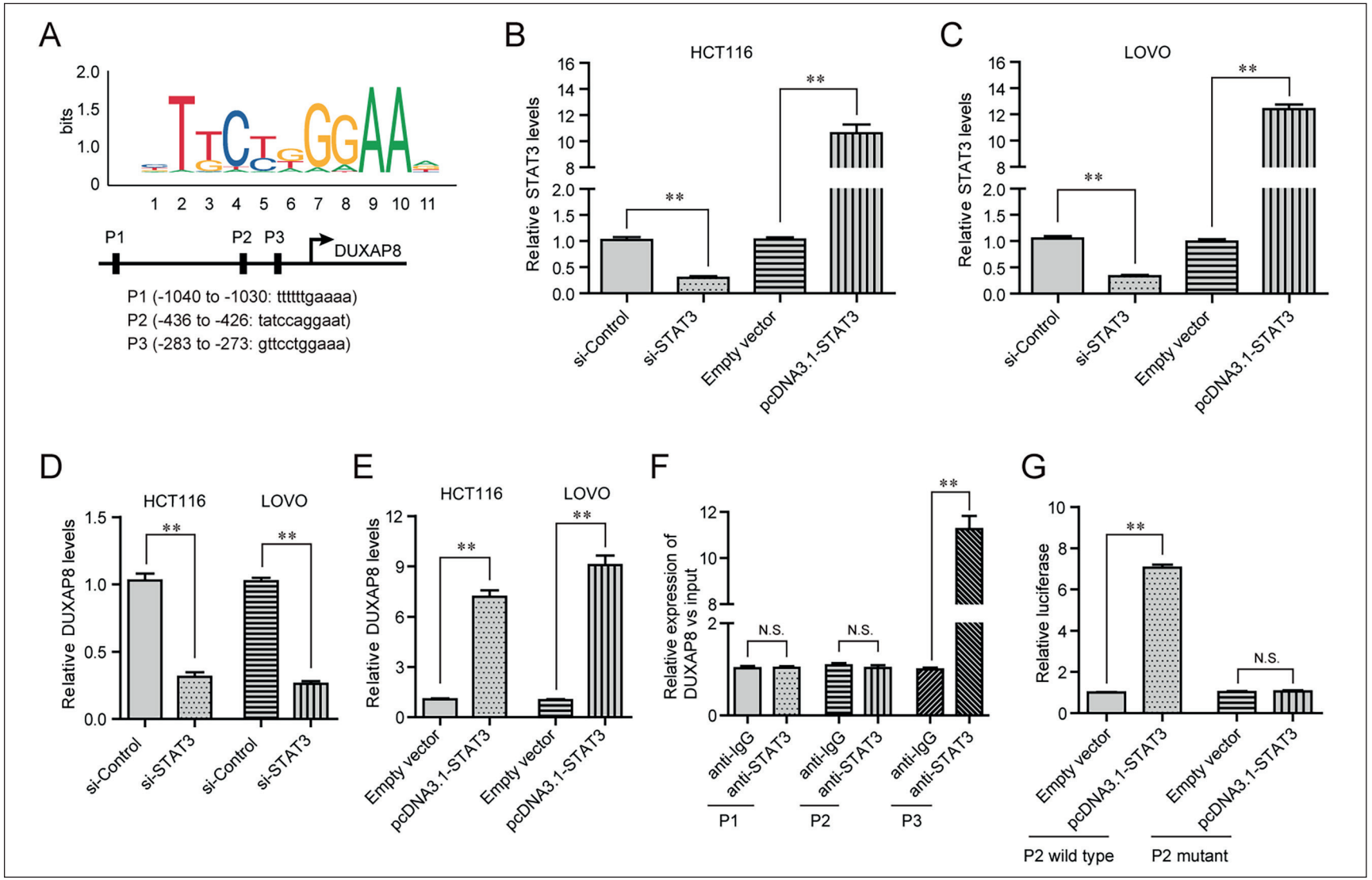

Figure 2. STAT3 activated DUXAP8 expression in CRC cells. ***A,*** JASPR predicted the binding sites in DUXAP8 promoter regions. ***B, C,*** The qPCR detection of STAT3 expression in the HCT116 and LOVO cells. ***D, E,*** The qPCR detected DUXAP8 levels. ***F,*** ChIP-qPCR analysis of STAT3 occupancy in the DUXAP8 promoter regions. ***G,*** Luciferase reporter activity analysis of HCT116 cells after co-transfection with P2 wild type or mutant Luciferase reporters with pcDNA3.1-STAT3 vectors. *$p<0.05$, **$p<0.01$.

promoter (Figure 2F). Therefore, we respectively cloned wild-type or mutant-type P3 site into pGL3 vector (P3 wild-type or P3 mutant), and sequentially performed Dual-Luciferase reporter assays for further demonstrating that STAT3 was capable of interacting with DUXAP8 promoter. The results indicated that forced STAT3 expression increased the Luciferase activities in cells after treatment with P3 wild-type reporters, whereas the activities of Luciferase were not altered in HCT116 cells after co-transfection with pcDNA3.1-STAT3 and P3 mutant vectors (Figure 2G). Our findings clarified that STAT3 induced the aberrant expression of DUXAP8 in CRC.

Knockdown of DUXAP8 Suppressed the CRC Cell Growth and Promoted Apoptosis

To determine the biological functions of DUXAP8 in CRC cells, si-RNA-1, si-RNA-2 (targeting DUXAP8) were first synthesized to repress the expression of DUXAP8. Subsequently, qRT-PCR assays were carried out and the silencing efficiencies of these siRNAs were more than 65% in HCT116 and LOVO cells (Figure 3A). Afterward, the effects of DUXAP8 on cellular growth were explored using CCK-8 assays. The results indicated that silence of DUXAP8 in HCT116 and LOVO cells remarkably depressed the cell proliferation at 48, 72, and 96 h post-transfection (Figure 3B). Moreover, depletion of DUXAP8 led to a markedly decreased colony number of HCT116 and LOVO cells (Figure 3C). In addition, EdU assay was carried out to evaluate the impact of DUXAP8 depletion on cellular proliferation. As the results presented in Figures 4D and 4E, knockdown of DUXAP8 by DUXAP8 siRNAs transfection dramatically attenuated the cell proliferation of HCT116 and LOVO cells. To assess whether the effects of DUXAP8 on colorectal cellular proliferation were dependent on the modulation of cell apoptosis, we next performed flow cytometry. The data suggested that transfection of DUXAP8 siRNAs induced remarkable cell apoptosis of HCT116 and LOVO cells (Figure 3F). Western blot analysis was performed and we found that repressing the expression of DUXAP8 notably elevated the protein levels of caspase 3/9 (Figure 3G). Our results validated that DUXAP8 could affect CRC development.

DUXAP8 Modulated the Metastatic Potentials of CRC Cells

To investigate whether silencing the expression of DUXAP8 had an impact on the oncogenic behavior of CRC cells, the HCT116 and LOVO cell metastasis was further evaluated using wound-healing and transwell assays. Wound-healing detection revealed that the scratching gaps in DUXAP8 silenced-cells were significantly wider than that of the controls, suggesting that knockdown of DUXAP8 markedly reduced the migration of CRC cells (Figures 4A and B). Meanwhile, transwell invasion assays certified that depression of DUXAP8 resulted in a marked reduction of cellular invasion of HCT116 and LOVO cells (Figures 4C and D). Overall, all the observations demonstrated that suppression of DUXAP8 was capable of impeding the cellular migration and invasion of CRC cells.

DUXAP8 Regulated RAB14 Expression via Acting as the Sponge of MiR-577 in CRC Cells

Next, we attempted to uncover the mechanisms by which DUXAP8 orchestrated CRC development. Since lncRNAs might serve as miRNA sponges to affect cellular phenotypes, we first utilized qRT-PCR analysis to determine the subcellular location of DUXAP8. As the data shown in Figure 5A, DUXAP8 was mainly expressed in the cytoplasm, indicating that DUXAP8 might act as a miRNA sponge. Hence, we next applied an online bioinformatics tool "StarBase" to predict the possible DUXAP8 targeting miRNAs. Among those predicted miRNAs, miR-577, which acted as tumor suppressor in cancer, was found to be a DUXAP8 possible target (Figure 5B). Bioinformatics analyses using TCGA dataset by "StarBase" discovered that miR-577 expression was negatively correlated with DUXAP8 expression in CRC tissues (Figure 5C). We next employed Luciferase activity detection assays to examine whether miR-577 was the exact target of DUXAP8. The results manifested that forcing miR-577 expression remarkably depressed the Luciferase activities of DUXAP8 wt reporter-transfected HCT116 and LOVO cells, while had no impact on that of DUXAP8 mut reporter-transfected cells (Figure 5D). In addition, RNA pull-down analysis certified that there was a substantial enrichment of miR-577 in the DUXAP8-pulled down pellets compared with the control group, which indicated that DUXAP8 could precipitate miR-577 in CRC cells (Figure 5E). Furthermore, the data from Real-Time analysis validated that miR-577 levels in the CRC samples were notably upregulated when compared with the normal specimens (Figure 5F). Additionally, the expression of miR-

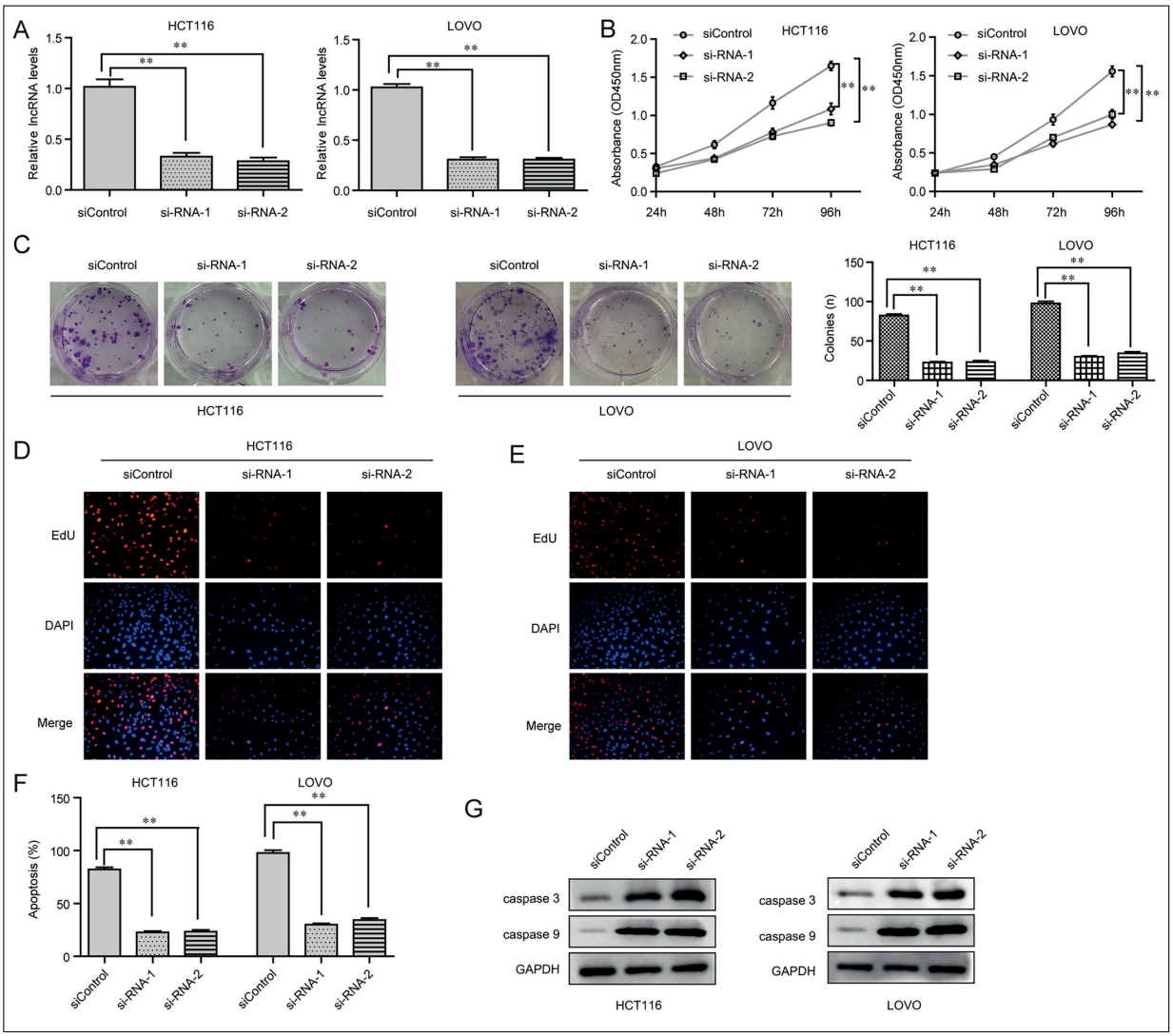

Figure 3. DUXAP8 knockdown inhibited colorectal cell growth *in vitro*. ***A,*** Relative levels of DUXAP8 were detected by qPCR assays. ***B,*** Proliferative curves were evaluated using CCK-8 assays. ***C,*** Colony-forming abilities were assessed by clonogenic assays (Magnification: 10×). ***D,*** and ***E,*** EdU assays measured the proliferation of CRC cells transfected with DUXAP8 siRNAs. Proliferative cells were labeled with EdU (red); nuclear fractions were labeled with DAPI (blue) (Magnification: 100×). ***F,*** Cell apoptosis was determined by flow cytometry. ***G,*** Relative protein levels of caspase 3/9 were examined by Western blot assays. *p<0.05, **p<0.01.

577 was remarkably suppressed in HCT116 and LOVO cells when their DUXAP8 was enhanced expression, while miR-577 levels were markedly increased in cells when their endogenous DUXAP8 was knocked down (Figure 5G). Therefore, these data suggested that DUXAP8 directly targeted miR-577 in CRC cells. The "StarBase" was then employed to discover the possible target genes of miR-577, and RAB14, a widely reported onco-promoter, was discovered to be a possible miR-577 target gene (Figure 5H). Applying Dual-Luciferase reporter assays, we demonstrated that the Luciferase activities in HCT116 and LOVO cells were markedly decreased when they were co-transfected with miR-577 mimics and pcDNA3.1-RAB14 wt reporter plasmids, while notable alteration of Luciferase activities was not found in cells treated with miR-577 mimics and pcDNA3.1-RAB14 mut reporter plasmids (Figure 5I). Subsequently, qPCR was carried out to measure the expressing changes of DUXAP8 and RAB14 in HCT116 cells treated with miR-577 mimics or inhibitors. The results validated that the expression of DUXAP8 and RAB14 signifi-

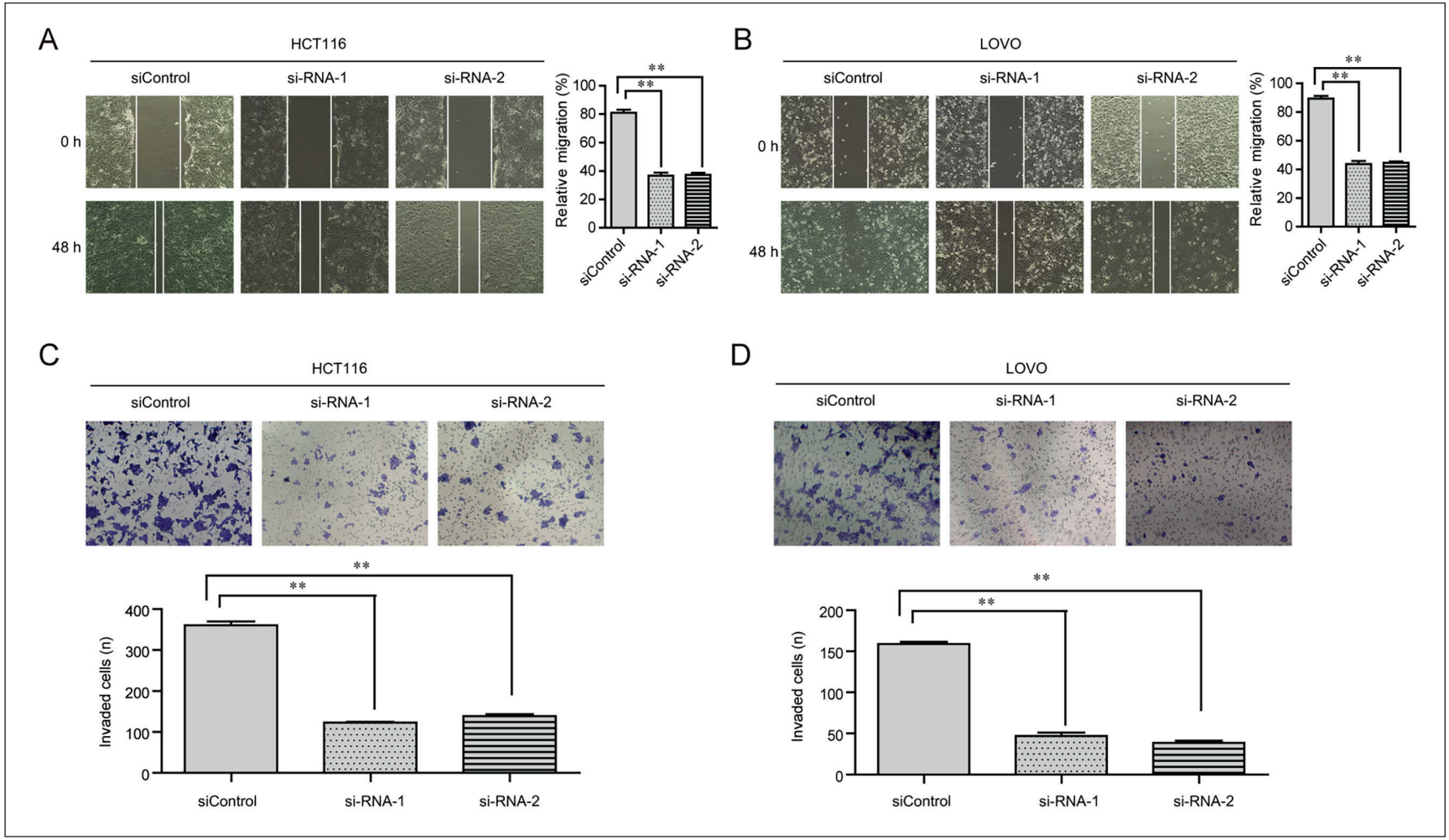

Figure 4. Knockdown of DUXAP8 expression suppressed the migration and invasion of CRC cells. ***A,*** and ***B,*** The effects of DUXAP8 knockdown on cellular migration were measured by wound healing assays (Magnification: 10×). ***C, D,*** The effects of DUXAP8 knockdown on cellular invasion were measured by transwell invasion assays (Magnification: 40×). *$p<0.05$, **$p<0.01$.

cantly declined following enhancing miR-577 expression, while DUXAP8 and RAB14 levels were dramatically elevated following miR-577 knockdown (Figure 5J). The results from Western blot confirmed that enhancing the expression of DUXAP8 was capable of restoring the miR-577

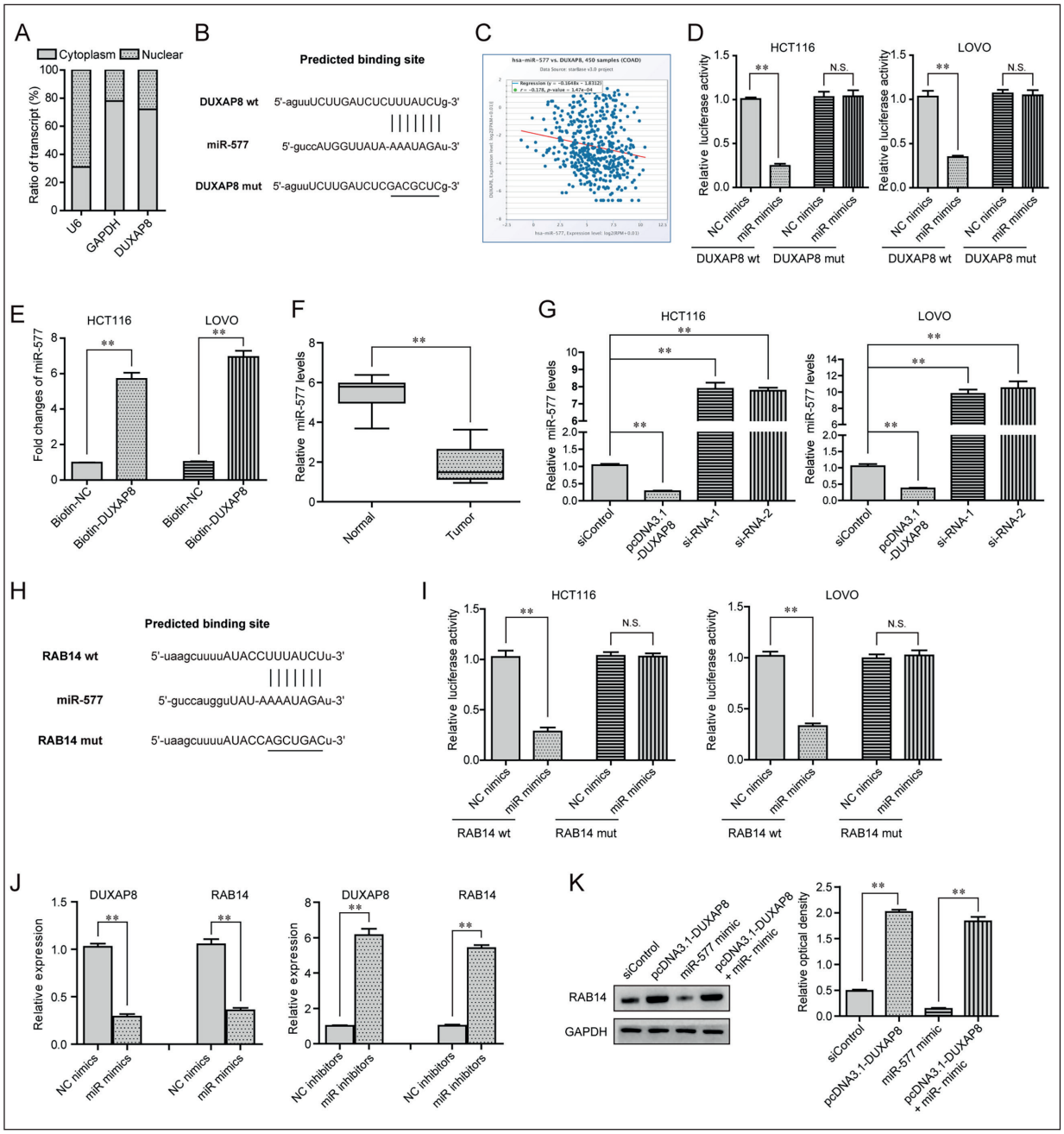

Figure 5. RAB14 acted as the target of miR-577/DUXAP8 in CRC cells. ***A,*** The subcellular location of DUXAP8 in HCT116 cells was determined by qRT-PCR analysis. ***B,*** "StarBase" analyzed the complementary binding sites of miR-577 in DUXAP8 sequence. ***C,*** "StarBase" analyzed the relative expressing correlation between miR-577 and DUXAP8 using TCGA dataset. ***D,*** Luciferase activities detection was carried out to study the binding of miR-577 and DUXAP8. ***E,*** RNA pull-down analyses validated that DUXAP8 could precipitate miR-577. ***F,*** The qRT-PCR assays revealed miR-577 expression in CRC tissue and matched normal tissues. ***G,*** The qPCR assays examined miR-577 expression in cells after transfecting with DUXAP8 siRNAs or pcDNA3.1-DUXAP8 vectors. ***H,*** A binding site of miR-577 in the 3′-UTR of RAB14 was predicted using "StarBase". ***I,*** Luciferase activity in HCT116 and LOVO cells transfected with designated plasmids were assessed by Dual-Luciferase reporter assays. ***J,*** The qRT-PCR assays determined the relative expression of DUXAP8 and RAB14 in cells transfected with miR-577 mimics or inhibitors. ***K,*** RAB14 protein levels in HCT116 cells were detected by Western blot assays after various transfections. *$p<0.05$, **$p<0.01$.

suppressing effects on RAB14 expression (Figure 5K). Therefore, these data proved that DUXAP8 affected the levels of RAB14 by regulating miR-577 expression in CRC cells.

Discussion

CRC is the third most commonly diagnosed cancer worldwide and remains a serious health problem. The prediction of the clinical outcome before clinical treatment is important for the management of treatment methods. Currently, several clinical staging systems, such as TNM system, has been used to predict the outcomes in CRC patients[23]. Unfortunately, some limitations were significant in clinical practice, such as lack of accuracy and linking molecular subclasses. In recent years, several studies involving lncRNAs have been expanded to various categories for various cancer, including CRC, indicating that lncRNAs might be potential diagnostic and prognostic biomarkers for CRC patients[24,25]. In this work, we certified a novel CRC-relevant lncRNA, DUXAP8, which was proved to be highly expressed in both CRC tissues and cells using a series of methods. Then, it was also verified that high DUXAP8 levels were correlated with LNM, TNM stage and overall survival. Further investigations confirmed that the high expression of DUXAP8 was an independent prognostic factor for CRC patients. Overall, our findings may shed light for developing novel CRC biomarkers.

The activation or suppression of gene promoter area using specific promoters play a key role in the modulation of the expression of genes. Recently, growing studies reported that several transcription factors are involved in the regulation of lncRNAs[26]. For instance, SP1-induced lncRNA-ZFAS1 promoted CRC cells proliferation and metastasis[27]. ELK1-induced upregulation of lncRNA HOXA10-AS promotes the migration and invasion of lung cancer cells via regulating the Wnt/β-catenin signaling[28]. To explore the molecular mechanism, which led to the overexpression of DUXAP8 in CRC, the JASPAR CORE database was used to analyze the promoter of DUXAP8 and the results showed that STAT3 may be candidate transcript factor. Previously, STAT3 has been observed to act as a tumor promoter in various malignancies, including CRC. Using Dual-Luciferase assays and CHIP assays, we further confirmed that STAT3 may be a regulator which interacted with the promoter of DUXAP8. Our findings revealed that STAT3 activates DUXAP8 translational expression to lead to up-regulation of DUXAP8 in CRC.

DUXAP8, a pseudogene derived lncRNA, is abnormally expressed in several cancers and act as a positive modulator which contributed to the progression of malignancy[21]. For example, Ma et al[22] found that DUXAP8 was upregulated in gastric cancer and its knockdown lead to the suppression of the ability of tumor cell proliferation and migration by modulating PLEKHO1 expression. Sun et al[29] showed that DUXAP8 levels were dramatically up-regulated in lung cancer and its down-regulating using si-DUXAP8 distinctly inhibited cell proliferation and invasion via the modulation of EGR1 and RHOB. Huang et al[30] discovered that DUXAP8 was highly expressed in renal cell carcinoma and its silencing suppressed tumor progression by functioning as ceRNA sponging miR-126. These findings highlighted the potential of DUXAP8 as a therapeutic target for the above cancers. Nevertheless, the expressing state and functions of DUXAP8 in CRC still need to be elucidated. In our work, for the first time, we reported that DUXAP8 silence inhibited the cell growth and metastasis of CRC cells, and accelerated apoptosis by modulation of Caspase 3 and Caspase 9. Our findings were in line with previous findings that DUXAP8 served as an onco-regulator in tumor progression.

Inspired by the ‘‘competitive endogenous RNAs’’ regulatory mechanism and in increasing data highlighted that several functional lncRNAs may participate in the regulation of this circuitry[31]. To fully explore CRC pathogenesis, we focused on the mechanism of DUXAP8 as a ceRNA to regulate the expression of miRNAs. To study this notion, the result of qRT-PCR analysis firstly suggested that miR-577 expression is decreased in CRC tissues, and the data from bioinformatics software further confirmed that the levels of this miRNA were inversely correlated with DUXAP8 levels. MiR-577 was reported to depress tumor cells growth and mobility by targeting several tumor-related genes transcript[32,33]. Here, we showed that DUXAP8 directly targeted miR-577 with molecular binding at 3’-UTR. Following Luciferase reporter assays demonstrated this online prediction. Besides, we observed using RT-PCR that suppression or up-regulation of DUXAP8 may negatively modulate miR-577 expression. As a more well-known oncogene, RAB14 is widely expressed in various tumor,

including CRC[34,35]. In addition, the regulation of RAB14 was involved in several miRNAs in several tumors. In this work, we used algorithm programs (TargetScan and PicTar software) and found that miR-577 binding sites in the 3'UTR of RAB14. Following Luciferase reporter assays confirmed this prediction. What's more, we performed Western blot to determine the expression of RAB14 protein and our results indicated that RAB14 expression was decreased by DUXAP8 suppression or miR-577 upregulation. Overall, we findings suggested that DUXAP8 may act as a ceRNA to promote DUXAP8 expression by sponging 577 in CRC.

Conclusions

Our study revealed that DUXAP8 overexpression was related to advanced clinical progress and poor prognosis in CRC patients. Functional assays suggested that DUXAP8 accelerated CRC tumorigenesis via inhibition of miR-577 and promotion of RAB14. Thus, targeting the DUXAP8/miR-577/RAB14 axis may help establish new strategies for therapy of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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