

CircRNA_0023642 promotes migration and invasion of gastric cancer cells by regulating EMT

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Abstract. – OBJECTIVE: Non-coding circular RNAs (circRNAs) have displayed dysregulated expression in various tumor tissues. However, their role in the progression of cancers remains largely unknown. We aimed at examining the expression, functions, and molecular mechanisms of a new circRNA (circRNA_0023642) in gastric cancer (GC).

PATIENTS AND METHODS: We evaluated the expression levels of circRNA_0023642 in GC tissues, adjacent normal tissues and cells lines using qRT-PCR. The functional roles of circRNA_0023642 in GC were determined by Cell Counting Kit-8 (CCK-8) assay, colony formation assay, transwell assay, and flow cytometric analysis. Western blot was used to analyze the effect of circRNA_0023642 on the expression of EMT-related proteins.

RESULTS: We found that circRNA_0023642 was upregulated in GC tissues and cell lines. Functionally, down-regulation of circRNA_0023642 displayed the tumor-inhibitory effects by suppressing cell proliferation, migration, and invasion as well as inducing apoptosis. Mechanically, our results revealed that the abnormal expression of circRNA_0023642 could influence the EMT signaling pathway, which was demonstrated by measuring the expression levels of N-cadherin, vimentin snail, and E-cadherin.

CONCLUSIONS: Our findings suggest that circRNA_0023642 serves as a metastasis activator by promoting EMT and may represent a novel molecular therapeutic target for GC.

Key Words:

CircRNA_0023642, EMT, Gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common malignancies and the second leading cause of

cancer-related deaths worldwide, with estimated 950 thousand new cases and 720 thousand deaths in 2012^{1,2}. The burden of GC for China continues to increase largely, with nearly 47% of worldwide GC cases from China³. Although various improvements have been made on the diagnosis and treatment of GC, the patients diagnosed at advanced stage have a poor long-term survival rate⁴. Unfortunately, due to lacking typical symptoms in the early stages, the first diagnosis for most patients is at advanced stage⁵. A critical problem for GC treatment is the metastasis of cancer cells. Therefore, it is important to investigate the molecular mechanisms underlying the growth and metastasis of GC cells, which also help to identify novel biomarkers for diagnosis of GC patients.

Circular RNAs (circRNAs), a newly-identified non-coding RNA that do not encode proteins, are formed from the covalent linkage of the 3' and 5' ends to form a closed loop⁶. The study of high-throughput RNA sequencing and bioinformatics suggested circRNAs as a regulator in eukaryotic cell⁷. The abnormal expressions of circRNAs are observed in various tumors, suggesting that circRNAs are involved in tumorigenesis and cancer progression^{8,9}. Unlike miRNAs, only a few studies about the biological function in tumors are reported. Current researches^{10,11} indicated that circRNAs functioned as tumor suppressors or oncogenes in tumors by serving as miRNA sponge and regulating tumor-related gene expression. Several circRNAs have already been reported to be important factors in tumors, such as circ-TTBK2 in glioma, circ-ZKSCAN1 in hepatocellular carcinoma and circRNA_100269 in GC¹²⁻¹⁴. However, the expression and functions of most circRNAs remain largely unknown.

Circ_0023642 was a newly identified circRNA. Huang et al¹⁵ reported that circ_0023642 was one of the top 5 upregulated circRNAs by microarray validation by RT-qPCR. However, to our best knowledge, the biological function and the mechanism of circ_0023642 in tumors including GC have not yet been reported. We examined circ_0023642 expression in GC tissues and cell lines. Moreover, we identified the function of circ_0023642 in GC cells by applying loss-of-function approaches *in vitro*.

Patients and Methods

Clinical Samples

A total of 27 pairs of GC tissues and matched non-tumor tissues were collected at the Department of Digestive Endoscopy Center, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital between June 2015 and April 2016. All of the patients were diagnosed with GC according to the criteria of the American Joint Commission on Cancer. None of the patients had received any preoperative therapy. All protocols of this investigation were approved by the Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. All patients provided the written informed consent.

Cell Lines and Transfection

Human GC cell lines (MGC-803, MNK-45, SGC-7901 and HGC-27) and one normal gastric mucosa cell line (GES) were obtained from the Cell Bank of the Chinese Academy of Sciences (Xuhui, Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, 1% penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco, Shenhe, Shenyang, China) in a humidified incubator containing 5% CO₂ at 37°C.

HGC-27 cells cultured on the six-well plate were transfected with siRNA or negative control

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. CircRNA_0023642 siRNA plasmids (50 nM) were purchased from the RiboBio Company (Beijing, China). After 48 h transfection with siRNA, qRT-PCR was performed to determine the transfection efficiency.

RNA Isolation and Quantitative Real-time PCR

RNA was isolated from GC tissue samples or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNAs using a Prime-Script™ one-step RT-PCR kit (TaKaRa, Hangzhou, Zhejiang, China). CircRNA_0023642 or EMT-related genes mRNA expression levels were determined by quantitative RT-PCR with the iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction of PCR was set at 95°C for 10 min for pre-denaturation and then at 95°C for 10 s and at 60°C for 60 s repeating 40 cycles. GAPDH was an internal control. The threshold cycle (Ct) value for each sample was calculated using the ABI analytical thermal cycler. The primers used in q-PCR were shown in Table I.

Cell Viability Assay

Cell proliferation was quantified using the Cell Counting Kit-8. Briefly, the transfected cells were plated into 96-well plates at a density of 1.0×10^3 /well/100 µL and cultured for 24, 48, 72, and 96 h, respectively. Every 24 h, 10 ml CCK-8 reagents (Dojindo, Xuhui, Shanghai, China) were added to each plate. Absorbance was measured at 490 nm with a Thermo Varioskan Flash reader.

Colony Formation Assay

2000 GC cells were seeded on 6-well plates. After incubation for 12 days at 37°C, the cells were washed twice with PBS and stained with crystal violet. Only colonies with more than 50 cells were counted under a microscope.

Table I. The primer sequences included in this study.

Name	primer sequences (5'-3')
CircRNA_0023642: forward	ATGACAAACTGACGGAAAAGGAG
CircRNA_0023642: reverse	AACCAAGGGCAACAGCAATG
GAPDH: forward	GAAGGTGAAGGTCGGAGTC
GAPDH: reverse	GAAGATGGTGTATGGGATTC

Flow Cytometric Cell Cycle and Apoptosis Assay

For cell cycle assay, cells were collected after trypsinization into a single-cell suspension, fixed with cold ethanol at 4°C for 1 h. Then, cells were treated with RNase A for 30 min and labeled for 15 min with PI. For the apoptosis assay, 48 h after transfection, the cells from each well were harvested and stained with Annexin V-APC and 7-AAD using an Apoptosis kit (BD Pharmingen, Xicheng, Beijing, China). Cells in both assays were evaluated by flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Cell Quest Software version 3.3 was used to analyze the data.

Cell Migration and Invasion Assay In Vitro

3×10^4 cells per well in 250 μ l of serum-free medium were pretreated with lycorine and added to the upper chamber. Then, fresh medium with 10% fetal bovine serum (FBS) was placed in the lower chambers. The plates were incubated for 24 h. For the invasion assay, procedures were the same as above, except that the inserts were coated with 200 mg/ml Matrigel. After incubation for 48 h, migrated and invasive cells of the lower chamber were stained with 0.1% crystal violet. Images of HGC-27 cells were taken under a phase-contrast microscope.

Western Blot

Proteins were extracted using RIPA lysis buffer, and equal amounts of protein were se-

parated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA) at 50 V for 3 h at 4°C. After being blocked with 5% fat-free milk, the membrane was probed with primary antibodies including rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-vimentin and rabbit anti-snail. β -actin was used as the internal control. Then, the membranes were washed three times and incubated with goat anti-mouse IgG horseradish-peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO, USA) or goat anti-rabbit IgG HRP (Sigma-Aldrich, St. Louis, MO, USA) for 90 min. Proteins were visualized with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Statistical Analysis

Statistical analysis was performed using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). The two-tailed Student's t-test was used to compare the differences between two groups. A value of $p < 0.05$ indicated a significant difference.

Results

CircRNA_0023642 Up-Regulates in GC Tissues and Cell Lines

To identify the role of circRNA_0023642 in gastric carcinogenesis, we analyzed the expres-

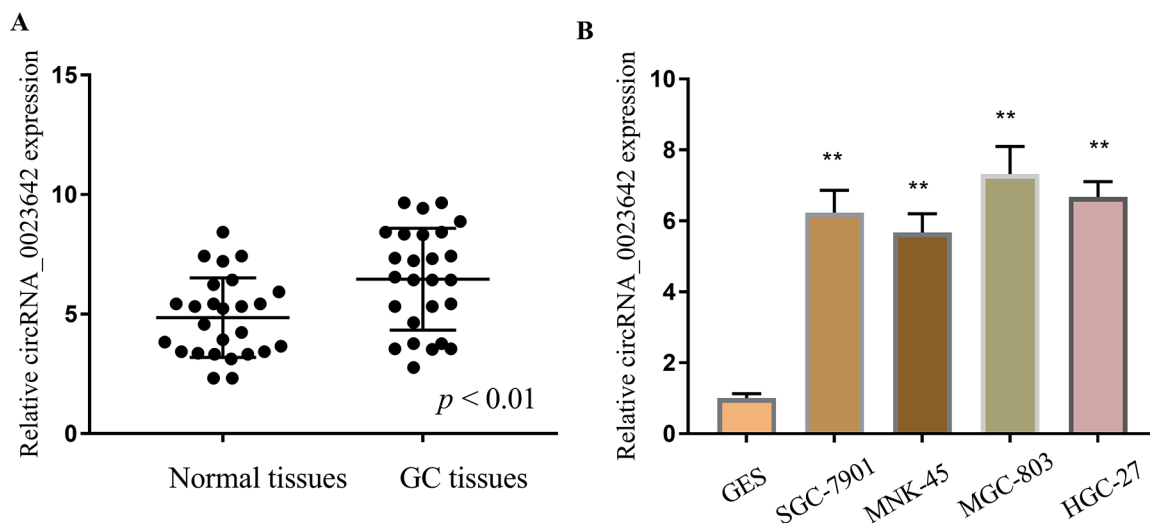


Figure 1. High expression of circRNA_0023642 in both GC tissues and cell lines. (A) CircRNA_0023642 expression in 26 pairs of gastric cancer and adjacent non-tumor tissues detected by qRT-PCR. (B) The qPCR analysis of circRNA_0023642 for a normal gastric mucosa cell line (GES) and for GC cell lines (MGC-803, MNK-45, SGC-7901 and HGC-27). * $p < 0.05$, ** $p < 0.01$.

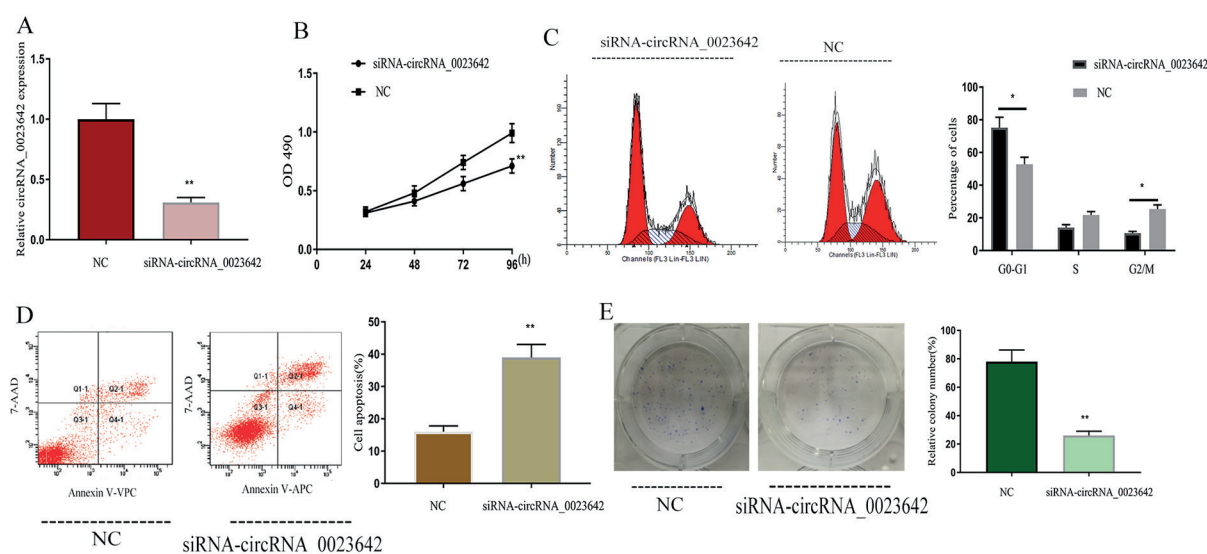


Figure 2. Down-regulation of circRNA_0023642 suppresses cell proliferation and colony formation and promotes cell apoptosis in GC cells. **(A)** The relative expression levels of circRNA_0023642 in HGC-27 cells were determined by RT-qPCR after transfection with si-circRNA_0023642 or si-NC. **(B)** HGC-27 cells after transfection were examined by CCK8 assay after different time periods. **(C)** Flow cytometry assays were performed to analyze the cell cycle progression when HGC-27 cells transfected with si-circRNA_0023642. **(D)** Changes of apoptosis in HGC-27 cells were determined by flow cytometric assay. **(E)** Effects of circRNA_0023642 knockdown on the colony formation of HGC-27 cells were determined. * $p < 0.05$, ** $p < 0.01$.

sion of circRNA_0023642 in 27 pairs of GC tissues and the matched adjacent normal tissues by RT-PCR. As shown in Figure 1A, we observed that expression of circRNA_0023642 was significantly upregulated in GC tissues compared with that in non-tumorous tissues ($p < 0.01$). Then, we further detected the levels of circRNA_0023642 in GC cell lines. It showed that circRNA_0023642 expression levels in GC cell lines were up-regulated significantly compared with GES cell (Figure 1B). These data indicated that increased circRNA_0023642 expression might contribute to GC development.

Down-Regulation of circRNA_0023642 Exerts a Tumor-Promotive Function in GC

To explore the effect of circRNA_0023642 on GC cell growth, HGC-27 cells were infected with siRNA-circRNA_0023642 or si-NC control, respectively. The qRT-PCR assay showed that circRNA_0023642 expression was downregulated by siRNA-circRNA_0023642 transfection, which confirmed effective transfection (Figure 2A). Then, we performed CCK-8 to determine the proliferation of GC cells, and the result revealed that cell proliferations of HGC-27 cell lines were reduced when

circRNA_0023642 was down-regulated compared with the si-NC group (Figure 2B). Then, we performed flow cytometry to analyze the effect of circRNA_0023642 on GC cells cycle progression and apoptosis. PI staining revealed that the siRNA-circRNA_0023642 transfection induced G1 arrest (Figure 3C). Moreover, Figure 4 indicated that down-regulation of circRNA_002364 promoted GC cell apoptosis. In addition, by performing colony formation assays, we observed that down-regulation of circRNA_0023642 dramatically reduced the proliferative capacities of HGC-27 cells (Figure 2E). These findings strongly suggested that circRNA_0023642 functioned as a tumor promoter in GC.

CircRNA_0023642 Inhibition Suppresses HGC-27 Cell Migration and Invasion in vitro

To explore whether circRNA_0023642 influenced the metastasis ability of GC cells, we performed cell migration and invasion assays using transwell assay. Our results showed that down-regulation of circRNA_0023642 in HGC-27 cells effectively decreased the migratory abilities (Figure 3A). The invasive abilities also exerted similar trend (Figure 3B). These data

revealed that circRNA_0023642 inhibition led to an effective inhibition of circRNA_0023642 cells metastasis.

CircRNA_0023642 Can Modulate EMT of GC Cells

Above experiment results highlighted the important role of circRNA_0023642 as a regulator associated with metastasis of GC. Then, we further studied the possible potential mechanism. As one of the most important signaling pathways, EMT plays a critical role in metastasis of tumor cells. Then, we performed Western blot and RT-PCR to explore the effect of circRNA_0023642 inhibition on EMT-related genes. As shown in Figure 4A-B, we found that down-regulation of circRNA_0023642 decreased the expression of N-cadherin, vimentin, and snail, but promoted the expression of E-cadherin at both mRNA and proteins levels. Those data suggested that circRNA_0023642 could affect the activity of EMT signaling pathway in GC progression.

Discussion

Growing studies have shown and confirmed circRNAs as a critical regulator in the occur-

ce and development of various tumors, as well as in GC^{16,17}. Our attention focused on a novel circRNA, circRNA_0023642. We analyzed circRNA_0023642 expression in GC tissues from our hospital and GC cell lines by RT-PCR and observed circRNA_0023642 upregulation in both GC tissues and cell lines compared with control. Then, we performed *in vitro* assay by silencing circRNA_0023642 and found down-regulation of circRNA_0023642 was able to reduce GC cell proliferation, invasion and migration, and promote cell apoptosis. Our results firstly provided evidence that circRNA_0023642 served as a tumor promoter in the regulation of tumor growth and metastasis.

Recently, several correlations between altered circRNAs and human diseases have been reported, and the function of several circRNAs have been identified in various tumors^{18,19}. For instance, Zhao et al²⁰ showed that circ_0124644 had important diagnostic value in screening coronary artery disease. Wang et al²¹ reported that heart-related circRNA (HRCR) acted as a positive role in the protection of cardiac hypertrophy and heart failure by targeting miR-223. In the progression of tumors, more and more evidence suggested that circRNAs served as tumors suppressor or oncogenes by modula-

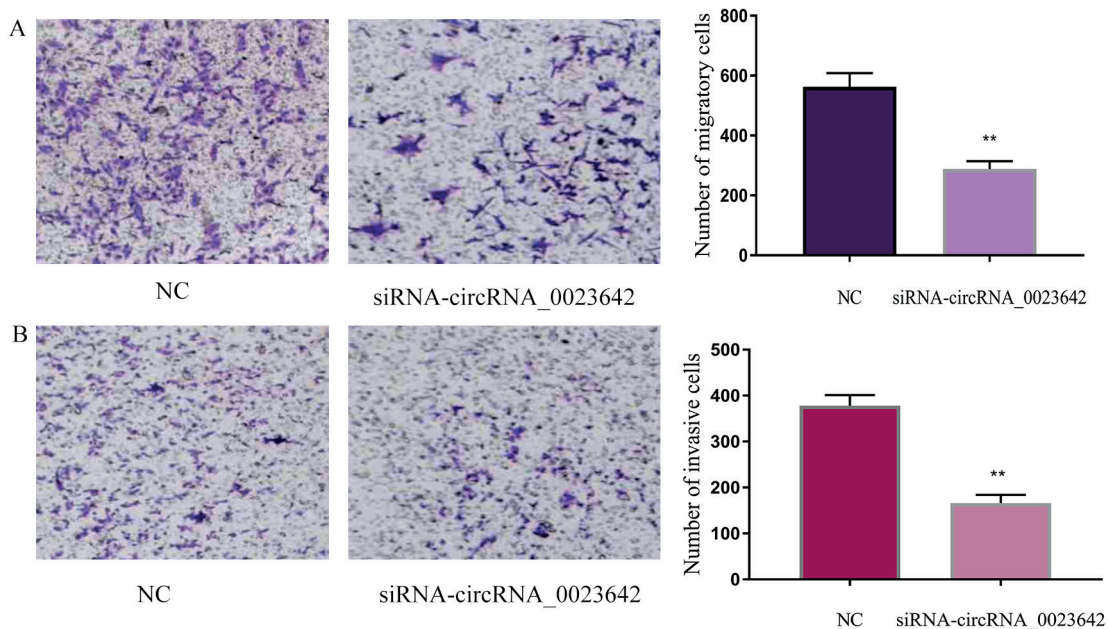


Figure 3. Knockdown of circRNA_0023642 inhibits HGC-27 cells migration and invasion. **(A)** Migratory capacities in HGC-27 cells increased after transfection, as determined by performing transwell-migration assay. **(B)** Invasive capacities in GC cells increased after transfection, as determined by performing and transwell-invasion assays. * $p < 0.05$, ** $p < 0.01$.

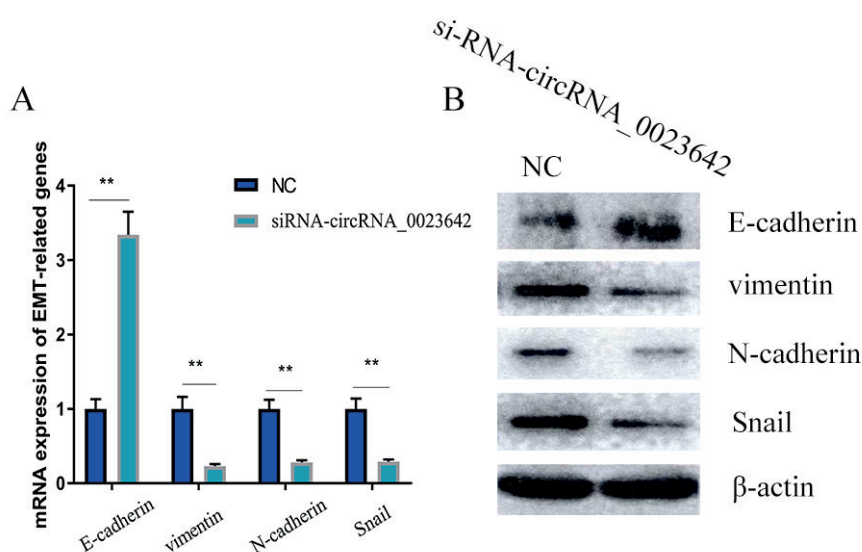


Figure 4. Down-regulation of circRNA_0023642 suppresses the EMT process in GC cells. **(A)** N-cadherin, Vimentin, E-cadherin and Snail mRNA expression levels in HGC-27 cells transfected with NC or si-circRNA_0023642 were analyzed by using RT-PCR. **(B)** N-cadherin, Vimentin, E-cadherin and Snail proteins expression levels in HGC-27 cells transfected with NC or si-circRNA_0023642 were analyzed by using Western blot. * $p < 0.05$, ** $p < 0.01$.

ting tumor-related genes²². For instance, Jin et al²³ reported that the expression levels of circRNA-0016347 were up-regulated in osteosarcoma and its knockdown suppressed the cellular proliferation, invasion, and metastasis in osteosarcoma by targeting miR-214. Fang et al²⁴ identified a novel circRNA named circular RNA ITCH, which could suppress proliferation of esophageal squamous cell carcinoma cells by the Wnt/ β -catenin pathway. Of note, previously Zhang et al¹⁴ reported that circRNA_100269 was lower expressed in GC and served as a tumor suppressor by inhibiting GC cell growth by targeting miR-630. Those results highlighted the potential of circRNA_100269 as a therapeutic target for tumors, including GC. However, the function of most circRNAs in GC has not been identified. Huang et al¹⁵ reported several dysregulation circRNAs in GC using a microarray analysis. They found that circRNA_0023642 expression was significantly up-regulated in GC¹⁵. However, up to date, whether circRNA_0023642 was involved in the progression of GC and its potential mechanism, have not been investigated. By *in vitro* assay, our results supported circRNA_0023642 as a stimulative regulator in GC development.

The epithelial-mesenchymal transition (EMT) is an important cellular mechanism in embryonic development, tissue repair, and disease²⁵. It is known to us that cells that have undergone

EMT gain a higher migrative and invasive potential²⁶. Recent studies^{27,28} have highlighted the importance of circRNAs in the regulation of the EMT. In GC, forced circ-104916 expression was reported to inhibit the metastasis of GC cells by altering the EMT process²⁹. In this investigation, by Western blot, we observed that circRNA_0023642 knockdown resulted in increased E-cadherin expression and decreased fibronectin, vimentin, and snail expression, suggesting that circRNA_0023642 promoted the activation of EMT signaling in GC. This promotion enhanced migration and invasion of GC cells. However, the detailed mechanisms of how circRNA_0023642 regulated EMT needed further investigation.

Conclusions

We firstly reported the function and mechanism of circRNA_0023642 in GC development. Our findings discover a new role of circRNA_0023642, modulating EMT signaling in GC tumorigenesis, further revealing that circRNA_0023642 may function as a potential diagnostic and therapeutic marker for GC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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