Downregulated Circular RNA hsa_circ_0000291 Suppresses Migration And Proliferation Of Gastric Cancer Via Targeting The miR-183/ITGB1 Axis

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Background: Circular RNAs are implicated in a variety of cancers. This investigation found that hsa_circ_0000291 expression was upregulated in gastric cancer (GC) cell lines, yet its role in GC has not yet been reported.

Objective: To explore the effects of hsa_circ_0000291 on GC cell proliferation and invasion.

Materials and methods: In the current research, we used the gastric cancer cell lines MGC803 and MKN-28 to study hsa_circ_0000291 function. The relationship between hsa_circ_0000291, miR-183 and ITGB1 was analyzed by firefly luciferase analysis and Western blots, and qRT-PCR approaches were used for protein and gene expression analysis, respectively. Tumor growth and metastasis were determined in nude mice xenografts using MKN-28 cells, with or without hsa_circ_0000291 downregulation.

Results: Our data showed that hsa_circ_0000291 was upregulated in GC cell lines, whereas hsa_circ_0000291 silencing suppressed cell metastasis and proliferation in in vivo and in vitro studies. Our results showed that the downregulation of hsa_circ_0000291 suppressed integrin beta 1 (ITGB1) expression via miR-183 “sponging,” which was validated by rescue experiments using the luciferase reporter assay. Our observations suggested that hsa_circ_0000291 silencing suppressed the aggressive, metastatic GC phenotype.

Conclusion: Taken together, hsa_circ_0000291 knockdown inhibited GC cell metastasis and growth by regulating the miR-183/ITGB1 axis. Importantly, this approach could provide a therapy target and potential biomarker for the diagnosis and treatment of GC.

Keywords: hsa_circ_0000291, miR-183, integrin beta 1, proliferation and migration, gastric cancer

Introduction
Gastric cancer (GC) is the main causal factor of mortality among Asian populations.1 While there have been improvements in the diagnostics and treatment of GC, a large proportion of patients have poor prognoses.2 Radical gastric surgery is often associated with high recurrence rates that result in a 5-year overall survival rate of 20%–40%.3,4 Therefore, it is imperative to seek appropriate markers for therapy and the early diagnosis of GC.

Increasing evidence has found that endogenous noncoding RNAs such as circular RNAs (circRNAs), long noncoding RNAs (lncRNAs), and microRNAs (miRNAs), function in the pathological regulation of different diseases, including cancer.5,6 Various from linear RNAs, circRNAs are circular structures, having no 5'
Caps and 3′ tails. Recent reports have illustrated that circRNAs function in the development and progression of GC. Several circRNAs appear to regulate gene function in tumorigenesis via sponge miRNA mechanisms. Nevertheless, circRNA regulatory functions in GC are unclear.

In this research, hsa_circ_0000291 had abnormal expression. Similarly, miR-183 negatively correlated with hsa_circ_0000291 levels. Therefore, our aim was to examine the function and biological roles of hsa_circ_0000291 during GC. The data suggested that hsa_circ_0000291 was upregulated in GC cell lines, and that downregulated hsa_circ_0000291 suppressed GC cell migration and proliferation by targeting the miR-183/ITGB1 axis. We believe these data will provide new therapeutic targets for GC treatment going forward.

Materials And Methods
Animal And Ethics Statement
BALB/c nude mice aged 4 weeks, weighing 15~20 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All procedures were approved by and conducted following the guidelines of the Ethics Committee of the Shanghai Tenth People’s Hospital of China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering. Mice were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital.

Cell Culture And Transfection
The human GC cell lines MGC-803, BGC823, SGC-7901, MKN-28, and gastric epithelial cell GES-1 were bought from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultivated in DMEM medium (Gibco, Gaithersburg, USA), which contained 10% fetal bovine serum (FBS, Gibco) at 37°C, in 5% CO2. Small interfering RNAs for hsa_circ_0000291 (sicircRNA) (5′-GGGAAAGUUUAUUUGUAUGUUU-3′), miR-183 mimics, miR-183 inhibitors (5′-AGUGAAUU CUACCAGUCCCAUA-3′), negative controls (NC) and the integrin beta 1 (ITGB1) overexpression vector were transfected into cultured MGC-803 or MKN-28 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). To further identify hsa_circ_0000291 effects on tumor growth in vivo, we constructed a hsa_circ_0000291 knockdown model in lentiviral stabilized MKN-28 cells.

Fluorescence In Situ Hybridization (FISH) Analysis
We used probes to the hsa_circ_0000291 sequence for FISH analysis, as reported previously. Specific FITC-labelled hsa_circ_0000291 probes (GTGCTTGAAAGAGGGC CCTGG) were used to counterstain cells following standard protocols (GenePharma, Shanghai, China).

Bioinformatics Analyses
We used the bioinformatics analysis website Circular RNA Interactome for circRNA and miRNA interaction predictions (https://circinteractome.nia.nih.gov/miRNA_Target_Sites/miRNA_target_sites.html). The target site between miR-183 and the 3′-UTR of ITGB1 was predicted using the TargetScan web-based tool (http://www.targetscan.org/vert_71/). Tools provided at http://gepia.cancer-pku.cn/ were used to predict hsa_circ_0000291 expression in the prognosis of gastric cancer.

Cell Proliferation Assay
Following standard protocols provided by Invitrogen (Carlsbad, CA, USA) we used the cell counting Kit-8 (CCK-8) to assess both MGC803 and MKN-28 cell proliferation. Briefly, 2×10³ cells in 100 μL DMEM were seeded into 96-well plates. We measured cell viability at 0, 24, 48 and 72 h after seeding with 10 μL CCK-8.

For colony formation assays, we seeded MGC803 and MKN-28 cells at a density of 2×10³ cells/well into 6 well plates. After culturing in DMEM medium, plus 10% FBS for 10 days, we washed cells in sterile PBS and fixed them with 4% paraformaldehyde for 30 mins before staining with Crystal Violet and counting.

Western Blots
We extracted protein from tumor cells or tissues using RIPA lysis buffer (Sigma-Aldrich, St. Louis, USA). We standardized protein concentrations using the BCA Protein Assay kit (Vigorous Biotechnology Beijing, China) before resolving proteins on SDS-PAGE and transferring them to nitrocellulose membranes (Millipore, Madison, USA). After blocking in nonfat milk (5%) and incubating with primary antibodies, membranes were incubated with horseradish peroxidase-coupled secondary antibodies ITGB1 (1:500, Santa Cruz Biotechnology, Dallas, TX, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000, China).
Santa Cruz Biotechnology, Dallas, TX, USA). GAPDH was used as an internal control.

**RNA Extraction And qRT-PCR**

We performed RNA extractions using TRIzol reagent (Invitrogen), using a previously published protocol. We used pTRUEscript the First Strand cDNA Synthesis Kit (Aidlab, Beijing, China) for cDNA synthesis. We used 2 × SYBR Green qPCR Mix. Aidlab was used for qRT-PCR detection which was conducted using the ABI 7900HT sequence detection system, (Thermo Fisher Scientific). We used the 2$^{-ΔΔCT}$ method to compute results. The hsa_circ_0000291 primers were: forward, 5′-GCCCTCGCTTTACATGTGC-3′ and reverse, 5′-CTAGAGACTATAGGGAAACC-3′. The miR-183 primers were forward, 5′-CGGTATGGCACTGGTAGAATTCACT-3′ and reverse, 5′-CTCAGTTCGAATTTGCGT-3′. GAPDH forward, 5′-GGAGCGTGTTG-3′ and reverse, 5′-GCCCTCGCTGGAGCGAACGCT-3′. GAPDH and U6 were used as internal references.

**Migration Assay**

For cell migration analysis, MGC803 and MKN-28 cells were placed into a transwell upper chamber at a density of 1 × 10^5 cells (8 μm pore membrane, BD Biosciences, Franklin Lakes, USA) in 200 μL serum-free medium. Complete medium (500 μL) was added to the bottom chamber. After 24 h culture, cell numbers in the bottom chamber were calculated after fixing with 4% paraformaldehyde and staining with 0.1% crystal violet.

**Tumor Xenograft Formation And Metastasis Assays**

A total of 2 × 10^7 MKN-28 cells with or without hsa_circ_0000291 silencing was injected into the right flanks of nude mice, as reported previously. Tumor sizes (volume = 0.5 × length × width^2) were measured by a vernier calipers every five days for 30 days before mice were euthanized.

For metastasis analyses, 2 × 10^5 MKN-28 cells transfected with luciferase expression vectors, with or without hsa_circ_0000291 silencing, were intravenously injected into mice tails. MKN-28 cell metastasis was analyzed using bioluminescence imaging after intravenously injected for 30 day by luciferin intravenous injection (150 mg luciferin/kg body weight).

**Dual Luciferase Reporter Assay**

Reporter plasmids were constructed by inserting wild-type/mutated-type circRNA, or the ITGB1 3′-UTR sequence into a pGL3 vector (Promega, Madison, USA). Then lipofectamine 2000 was used and miR-183 mimics combined with reporter plasmids co-transfected into 239T cells. Firefly and Renilla luciferase activities were captured by a Dual Luciferase Reporter Assay System (Promega, Sunnyvale, USA) after culturing for 48 h.

**Statistics Analyses**

We utilized GraphPad Prism (GraphPad, La Jolla, USA) for data analyses. The data were calculated as the mean ± SEM. p value ≤ 0.05 reflected significant differences.

**Results**

**Hsa_circ_0000291 Downregulation Suppresses Tumor Progression In Vivo**

We observed that hsa_circ_0000291 expression was increased in gastric cancer tissues when compared with adjacent normal tissues (Figure 1A). The RT-qPCR detection method also found that hsa_circ_0000291 expression in GC cell lines increased when compared to GES1 cells (Figure 1B). Hsa_circ_0000291 was derived from a CD44 gene exon. A fluorescence in situ hybridization assay showed that hsa_circ_0000291 localized to the cytoplasm (Figure 1C). To identify if hsa_circ_0000291 participated in the progress of GC, lentiviral stable strains of hsa_circ_0000291 knockdown (sh-circRNA) in MKN-28 cells were constructed. Our data showed that hsa_circ_0000291 expression in sh-circRNA MKN-28 cells was significantly downregulated, when compared to control or negative control (NC) cells (Figure 2A). The lentiviral-stabilized circRNA silenced MKN-28 cells or NCs were used for subcutaneous tumorigenesis analysis. These data indicated that hsa_circ_0000291 knockdown suppressed tumor growth (weight and volume) when compared to the NC group (Figure 2B–D). Bioluminescence imaging showed that hsa_circ_0000291 silencing suppressed MKN-28 cell metastasis (majority in lung tissue) in mice (Figure 2E). Using qRT-PCR, we found that miR-183 expression was upregulated following hsa_circ_0000291 silencing in mouse tumor tissues (Figure 2F). Western blot detection revealed that ITGB1 expression was downregulated after hsa_circ_0000291 knockdown (Figure 2G and H). These results suggested that hsa_circ_0000291 silencing suppressed tumor metastasis and growth in vivo. The results also showed that both miR-183 and ITGB1 participated in GC progression.
Figure 1 The expression of hsa_circ_0000291 and sub-cellular localization. (A) The qRT-PCR assay shows the expression of hsa_circ_0000291 in gastric cancer tissues and adjacent normal tissues. Data are denoted by the mean ± SD, ***p < 0.001 versus normal group. (B) The qRT-PCR assay shows the expression of hsa_circ_0000291 in GC cell lines (MGC803, MKN-28, SGC7901 and BGC823) and normal human gastric epithelial cell GES1. Data are denoted by the mean ± SD, ***p < 0.001 versus GES1 group. (C) Fluorescence in situ hybridization was performed to capture the subcellular localization of hsa_circ_0000291. DAPI = nuclear staining (bottom, left); hsa_circ_0000291 = green fluorescent-tagged hsa_circ_0000291 (top, left). Merged images are plotted at right.

Figure 2 Downregulation hsa_circ_0000291 suppressed tumor metastasis and growth in nude mice xenografts. (A) The quantitative reverse transcription-polymerase chain reaction assay illustrates the hsa_circ_0000291 expression in adenovirus-transfected cells (sh-circRNA) or negative control (NC) transfected MKN-28 cells. Data are denoted by the mean ± SD, ***p < 0.001 versus NC. (B) Representative photographs of MKN-28 tumor formation in xenografts of nude mice. (C) Tumor volume summary in mice that measured weekly. Data are denoted by the mean ± SD, **p < 0.01, ***p < 0.001 versus NC. (D) Tumor weight was captured 30 days from injection. Data are denoted by the mean ± SD, ***p < 0.001 versus NC. (E) Live imaging demonstrates the hsa_circ_0000291 effects on metastasis of MKN-28 cells 30 day after intravenous tail injection. scale bars, 1 cm. (F) qRT-PCR assay showing the miR-183 expression. Data are denoted by the mean ± SD, ***p < 0.001 versus control. (G and H) Western blot analysis of the integrin beta 1 (ITGB1) expression in tumor tissues. Data are denoted by the mean ± SD, ***p < 0.001 versus NC.
Knockdown Of hsa_circ_0000291 Inhibits Cell Migration And Proliferation By Regulating The miR-183/ITGB1 Axis

To further explore regulatory mechanisms, MGC803 and MKN-28 cells were transfected with a hsa_circ_0000291 silencing vector (sicircRNA), combined with an ITGB1 overexpression vector, or treatment with an miR-183 inhibitor. Data showed that hsa_circ_0000291 expression was downregulated after sicircRNA administration, but downregulated miR-183 or overexpression of ITGB1 could not rescue hsa_circ_0000291 expression in these cells (Figure 3A and B). Our qRT-PCR data illustrated that downregulated hsa_circ_0000291 promoted miR-183 expression. MiR-183 treatment also suppressed miR-183 expression (Figure 3A and B). Overexpressed ITGB1 had no effects on miR-183 expression after hsa_circ_0000291 silencing in both MGC803 and MKN-28 cell lines (Figure 3C and D). Western blot analysis demonstrated that downregulated hsa_circ_0000291 blocked ITGB1 expression. Inhibited miR-183 rescued the expression of ITGB1, and ITGB1 expression levels were increased after transfection with the ITGB1 overexpression vector (Figure 3E and F). These observations suggested that ITGB1 was downstream of miR-183.

The result from the CCK8 assay and cloning formation revealed that hsa_circ_0000291 silencing suppressed MGC803 and MKN-28 cell proliferation, but that miR-183 inhibitor treatment rescued the proliferation of both MGC803 and MKN-28 cells. ITGB1 overexpression further promoted the proliferation of both MGC803 and MKN-28 cell lines (Figure 3G–K). Transwell migration assay data suggested that hsa_circ_0000291 silencing-induced migration inhibition was rescued after treatment with the miR-183 inhibitor. The upregulation of ITGB1 promoted the migration of both MGC803 and MKN-28 cell lines (Figure 3L–N). In summary, these data suggest that hsa_circ_0000291 silencing suppressed tumor cell metastasis and proliferation by enhancing miR-183 expression, while suppressing ITGB1 expression.

The Inhibitory Effects Of miR-183 On Cell Proliferation And Migration Were Reversed After ITGB1 Up-Regulation In Vitro

To identify the relationship between miR-183 and ITGB1, we transfected both MGC803 and MKN-28 cell lines with miR-183 mimics, with/without the ITGB1 overexpression vector. Our qRT-PCR data revealed that miR-183 expression was upregulated in both MGC803 and MKN-28 cell lines after transfection with the miR-183 mimic, and that ITGB1 overexpression did not affect miR-183 expression, (Figure 4A and B). Western blot analysis revealed that ITGB1 expression was downregulated after miR-183 overexpression but was rescued after transfection with the ITGB1 overexpression vector (Figure 4C and D). The results from the CCK8 (Figure 4E and F) and cloning formation assay revealed that cell proliferation was suppressed after miR-183 overexpression, but ITGB1 overexpression rescued and promoted the proliferation of both MGC803 and MKN-28 cell lines (Figure 4G–I). Transwell migration assays revealed that miR-183 expression inhibited cell migration, whereas upregulation of ITGB1 promoted the migration of both MGC803 and MKN-28 cell lines (Figure 4J–L). These results suggested that the overexpression of miR-183 inhibited tumor cell migration and proliferation by targeting ITGB1.

The Relationship Between hsa_circ_0000291, miR-183 And ITGB1

To predict a relationship between hsa_circ_0000291, miR-183 and ITGB1, our bioinformatics analyses showed that hsa_circ_0000291 appeared to target miR-183. To physically validate this observation, a luciferin reporter vector was constructed. The results of this luciferase reporter assay suggested that miR-183 inhibited luciferase activity after transfection with the wild luciferase reporter vector, but that it did not affect luciferase activity after transfection with a mutated luciferase reporter vector, suggesting that miR-183 was the target of hsa_circ_0000291 (Figure 5A and B).

Bioinformatics analysis found that miR-183 directly interacted with the ITGB1 3’-UTR and suppressed post-translational ITGB1 expression (Figure 5C). A luciferase reporter assay showed that miR-183 inhibited luciferase activity, after transfection with a wild-type luciferase reporter vector, but luciferase activity was not affected after transfection with a mutated luciferase reporter vector, suggesting that ITGB1 was the target of miR-183 (Figure 5D). These data indicated that hsa_circ_0000291 knockdown inhibited metastasis and the growth of gastric cancer through targeting the miR-183/ITGB1 axis.

Discussion

Studies have identified that GC is increasingly diagnosed at advanced stages. The prognosis is poor, with a five-year survival rate of 5%~20%, with surgery considered a viable standard option. However, postoperative recurrence and metastasis rates are unsatisfactory.
family of ncRNAs where biological mechanisms are largely unknown. In this study, our data showed that hsa_circ_0000291 expression increased in GC cell lines, in comparison to wild-type cells, suggesting that hsa_circ_0000291 participates in GC progression. We also found that hsa_circ_0000291 silencing suppressed GC metastasis and proliferation in in vitro and in vivo models, suggesting that hsa_circ_0000291 may function as an oncogene.
The silencing of hsa_circ_0000291 promoted miR-183 expression and suppressed ITGB1 expression. Previous studies have suggested that miR-183 upregulation promotes apoptosis and inhibits human endometrial cancer cell invasion, proliferation, migration and epithelial-mesenchymal transition by targeting ezrin.\textsuperscript{19} MiR-183 also promotes canonical Wnt signaling activities and regulates bladder cancer apoptosis and growth by the downregulation of AXIN2.\textsuperscript{20} In this study, the hsa_circ_0000291 silencing effects on GC proliferation and metastasis were abolished by an miR-183 inhibitor, suggesting that hsa_circ_0000291 silencing suppressed GC proliferation and metastasis by absorbing miR-183. This observation appeared to be consistent with a previous report suggesting that circRNAs may function as sponges for microRNAs.\textsuperscript{18} CircRNAs-miRNAs participate in several physiological and pathophysiological processes, including cell differentiation, proliferation and metastasis.\textsuperscript{21-23} Bifluorescein studies have also shown that miR-183 is the hsa_circ_0000291 target.

We showed that miR-183 overexpression suppressed ITGB1 expression. Previously, it has been shown that ITGB1 plays vital roles in cell growth and motility in non-small cell lung cancer (NSCLC), breast cancer, GC and liver cancer.\textsuperscript{24} In NSCLC, miR-134 inhibits tumor cell mobility and invasions by modulating ITGB1.\textsuperscript{24} In another study, it was found that ITGB1 is the candidate miR-493-5p target and that ITGB1 expression levels were associated with non-small-cell carcinoma prognoses.\textsuperscript{25} In this study, we noted that miR-183 inhibited ITGB1 expression at the post-transcriptional level by interacting with the

Figure 4 ITGB1 overexpression reversed miR-183-induced cell migration and growth inhibition in vitro. MGC803 and MKN-28 cells were transfected with miR-183 mimics combined with or without the ITGB1 overexpression vector. (A and B) qRT-PCR assay showing the expression of miR-183 in both MGC803 (A) and MKN-28 (B) cells. Data are denoted by the mean ± SD. ***P < 0.001 versus NC. (C and D) Western blot showing ITGB1 expression in both MGC803 (C) and MKN-28 (D) cells. Relative protein levels were analyzed, and data are denoted by the mean ± SD. ***P < 0.001 versus NC. ###P < 0.001 versus mimic. (E and F) CCK8 assays were performed to assess cell proliferation in both MGC803 (E) and MKN-28 (F) cells. Data are denoted by the mean ± SD. ***P < 0.001 versus NC. ###P < 0.001 versus mimic. (G–I) Cloning formation assay showing the cell proliferation of MGC803 and MKN-28 cells. Data are denoted by the mean ± SD. ***P < 0.001 versus NC. ###P < 0.001 versus mimic. scale bars, 200 μm. (J–L) Cell invasion and migration were determined in MGC803 and MKN-28 cells by Transwell® assays. Data are denoted by the mean ± SD. ***P < 0.001 versus NC. ###P < 0.001 versus mimic. scale bars, 50 μm.
Conclusions

Our study revealed that hsa_circ_0000291 expression in GC cell lines was increased, and that hsa_circ_0000291 silencing suppressed GC cell metastasis and proliferation both in vivo and in vitro. Our results showed that hsa_circ_0000291 downregulation suppressed cell migration and proliferation by targeting the miR-183/ITGB1 axis. In conclusion, we identified hsa_circ_0000291 as a promising GC therapeutic target, therefore further investigation of the hsa_circ_0000291/miR-183/ITGB1 axis is required and may provide a foundation for developing novel potential therapeutic strategies towards GC treatment.

Ethics Approval

The Animal Research Committee of Shanghai Tenth People’s Hospital, Tongji University approved all experimental protocols and surgical procedures.

Abbreviations

circRNAs, circular RNAs; GC, gastric cancer; ITGB1, integrin beta 1; miRNA, microRNA; CCK-8, Cell Counting Kit-8.

Availability Of Data And Materials

All data analyzed and generated in this research report are enclosed in the article.

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Author Contributions

CC and ML conceived the research and drafted the manuscript with feedback from all authors. SH, YY and YW carried out the experiments and analyzed the data. WL, LP and XZ conducted experiments and corrected the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Figure 5 The interaction relationships among miR-183, hsa_circ_0000291, and ITGB1. (A) The predicted miR-183 binding sites in the hsa_circ_0000291. The hsa_circ_0000291 mutated (Mut) version is also given. (B) The relative luciferase activity was determined 48 h after transfection with miR-183 mimic/normal control (NC) or with the hsa_circ_0000291 wild-type/Mut in HEK293T cells. Data are denoted by the mean ± SD. ***P < 0.001. (C) The predicted miR-183 binding sites with the ITGB1 3’-UTR. The 3’-UTR-ITGB1 mutated version is also provided. (D) Relative luciferase activity was determined 48 h after transfection with miR-183 mimic/normal control or with the 3’-UTR-ITGB1 wild-type/Mut in HEK293T cells. Data are denoted by the mean ± SD. ***P < 0.001.
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