



CircRNA-0008717 promotes cell proliferation, migration, and invasion by regulating miR-203/Slug in esophageal cancer cells

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Background: Esophageal cancer (EC) is one of the deadliest cancers worldwide. Circular RNAs (circRNAs) have been implicated in the regulation of multiple human diseases, including cancer. In particular, the dysregulation of circRNA-0008717 has been linked to multiple types of cancer. However, the clinical significance and the molecular mechanisms of circRNA-0008717 in EC need to be further investigated. Therefore, this study aimed to prove the role of circRNA-0008717 in EC and its underlying molecular mechanism of action.

Methods: The expression of circRNA-0008717, miR-203, and the Slug was measured in two EC cell lines (EC109 and KYSE-150) by qRT-PCR. EC109 and KYSE-150 cells were first transfected with circRNA-0008717 siRNA (si-circRNA). After that, the proliferation, apoptosis, migration, and invasion of EC109 and KYSE-150 cells were measured. The western blot detected Slug, Vimentin, and E-cadherin protein levels. A dual-luciferase reporter gene assay was used to set up the interactions among circRNA-0008717, miR-203, and Slug.

Results: circRNA-0008717 expression was significantly upregulated in EC cells, and miR-203 expression was decreased. Moreover, si-circRNA-0008717 or si-Slug inhibited the proliferation, migration, and invasion of EC cells. We found that circRNA-0008717 functioned as a sponge of miR-203, resulting in increased expression of Slug. We also reversed the effect of circRNA-0008717 knockdown on the EC progression by co-transfecting EC cells with a miR-203 inhibitor or Slug.

Conclusions: The proliferation, invasion, and migration of EC cells were enhanced by circRNA-0008717 sponging the miR-203 to increase Slug expression.

Keywords: Esophageal cancer (EC); circRNA-0008717; miR-203; Slug; cell proliferation; apoptosis; migration and invasion

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Introduction

Esophageal cancer (EC) is one of the most common causes of cancer-related deaths worldwide (1). Although the treatment of EC has been dramatically improved in recent years, the prognosis of EC remains unsatisfactory (2).

Radiation therapy (RT) is a vital part of multimodal EC therapy (3), and according to several meta-analyses, patients treated with neoadjuvant chemotherapy or combined chemoradiotherapy before EC surgery have a higher rate of survival than those treated with surgery alone(4).

However, the response to these treatments varies from individual to individual, and the treatment for patients with inadequate response to chemotherapy may be ineffective (5,6). Furthermore, radiotherapy resistance often leads to subsequent recurrence and metastasis (5,6). Therefore, it is vital to set up the mechanisms that underlie the progression of EC and develop improved therapeutic strategies.

Circular RNAs (circRNAs) form a covalently closed loop structure and constitute a new class of conserved, stable non-coding RNAs (ncRNAs) that are widely expressed in eukaryotes (7,8). Recently, considerable efforts have been made to understand the role of circRNAs in tumor development (9). Many circRNAs can act as sponges that inhibit the activity of small ncRNAs, microRNAs (miRNAs), which are involved in the post-transcriptional regulation of gene expression (10). CircRNAs have been linked to the progression of multiple diseases, including gastric cancer (11), breast cancer (12), colorectal cancer (13), and malignant melanoma (2). CircRNA-0008717 dysregulation has been implicated in several types of cancer; however, its role in EC progression still is unclear.

Gene expression can be modulated through complementary base-pairing between miRNAs and the 3'-untranslated regions (UTRs) of their target mRNAs, which destabilizes mRNA and prevents translation (14). MiRNAs have increasingly been linked to processes involved in tumorigenesis, including cell growth, apoptosis, migration, and carcinogenesis (15). Therefore, characterizing the role of the large miRNA family is essential for cancer studies (16). Large numbers of miRNAs have been shown as tumor suppressors. In particular, miR-203 has been implicated in several human cancers by targeting the Slug transcription factor (17).

Epithelial-mesenchymal transition (EMT) is a necessary process in tumor development that is regulated by multiple transcription factors, including the Snail family (18). This Slug is known to promote the suppression of E-cadherin, which acts as a crucial tumor suppressor by regulating EMT. The loss of E-cadherin results in dysfunctional cell-to-cell adhesion leading to increased cancer invasion (19,20). In addition, Slug has been reported to involve in a diverse number of processes ranging from tumor cell invasion and metastasis to cell survival and proliferation (21). miR-429 suppresses the cell migration and invasion by targeting Slug in esophageal squamous cell carcinoma (22). Slug inhibition may represent a novel strategy for treatment of esophageal adenocarcinoma (23). However, the effects of miR-203 on cell migration and invasion by targeting Slug in EC remain

unclear.

In this study, we investigated the role of circRNA-0008717 in EC *in vitro*. We found evidence that circRNA-0008717 decreased Slug expression by sponging the miR-203, which promoted the proliferation, invasion, and migration of EC cells. Therefore, circRNA-0008717 could be a suitable therapeutic target to improve the prognosis of EC.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-5205>).

Methods

Cell cultures

Human normal esophageal epithelial cell line (Het-1A) and human EC cell lines (EC109 and KYSE-150) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 10% FBS and 1% streptomycin/penicillin in a 5% CO₂ incubator at 37 °C.

Cell transfection and grouping

EC109 and KYSE-150 cells at a density of 1×10^5 cells/well were seeded in 6-well plates. After 24 h of cell adherence, the medium was removed. The miR-203 mimics and scrambles negative control RNA (NC-mimics), miR-203 inhibitor and scrambled negative control RNA (NC-inhibitor), the siRNA for circRNA-0008717 (targeted covalent closed junction), Slug were chemically synthesized by GenePharma (GenePharma, Shanghai, China). The si-circRNA-0008717 sequence was as follows: 5'-TAGAAGACCATGGGGGATGTCAAGAGCATCCCCATGGTCTTCTATTTTTT-3'. si-Slug sequence was as follows: 5'-TACATGGAGATGTCGAGCACCAT-3'. The full-length sequences of Slug were respectively synthesized and cloned into pcDNA3.1 (GenePharma, Shanghai, China) plasmid to produce pcDNA3.1/Slug (pcDNA Slug). EC109 and KYSE-150 cells were transfected using Lipofectamine[®] 3000 Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The transfected cells were randomly divided into the si-circRNA group (treated with circRNA-0008717 siRNA), si-NC group (treated with circRNA-0008717 siRNA negative control), miR-203 mimic group (treated with miR-203 mimic),

NC-mimic group (treated with miR-203 mimic negative control), miR-203 inhibitor group (treated with miR-203 inhibitor), NC-inhibitor group (treated with miR-203 inhibitor negative control), si-Slug group (treated with Slug siRNA), si-circRNA + miR-203 inhibitor group (treated with circRNA-0008717 siRNA and miR-203 inhibitor), and si-circRNA + Slug group (treated with circRNA-0008717 siRNA and pcDNA Slug). Finally, all cells were incubated at 37 °C for 48 h.

Quantitative real-time PCR (qRT-PCR) assays

Total RNA from EC tissue, normal tissue, or cell lines was extracted by TRIZOL (Invitrogen). A NanoDrop ND-1000 spectrophotometer (NanoDrop, USA) was used to measure the concentration of total RNA. Then, total RNA (500 ng) was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) and analyzed by qRT-PCR using an SYBR Green PCR kit (TaKaRa) with the following thermocycling parameters: 95 °C for 3 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 30 s. The following primer sequences are used: circRNA-0008717: Forward: 5'-CTAAGGAGTCACAGGAAGACATC-3'; Reverse: 5'-GTAGAATCTCTCAGACTCAAGGTTG-3'; *Slug*: Forward: 5'-GCTGTAGGAACCGCCGCGGTGTC-3'; Reverse: 5'-ATTTGTCATTTGGCTTCGGAGTG-3'; *GAPDH*: Forward: 5'-GAAGGTGAAGGTCGGAGTC-3'; Reverse: 5'-GAAGATGGTGATGGGATTC-3'. miR-203 (Lot No. 91126N22) and U6 (Lot No. 8300871042) primers were ordered from GenePharma (GenePharma, Shanghai, China). The transcript level of U6 was used to normalize miR-203 expression. *GAPDH* was used to normalize the transcript levels of circRNA-0008717 and *Slug*. Relative expression is calculated using the $2^{-\Delta\Delta C_t}$ method (24).

Western blot analysis

Total protein was extracted from EC109 and KYSE-150 cells using RIPA lysis buffer (Sigma, USA). Total protein (50 µg per sample) is separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk for 2 hours and incubated with primary antibodies anti-GAPDH (1:1,000, ab181602, Abcam, UK), anti-Slug (1:1,000, ab51772, Abcam, UK), anti-Vimentin (1:1,000, ab92547, Abcam, UK), or anti-E-cadherin (1:1,000, ab40772, Abcam, UK) at 4 °C overnight. After washing

three times, the membranes were incubated with a peroxidase-labeled secondary antibody (anti-rabbit IgG, 1:2,000, ab6721, Abcam, UK) for 2 hours. Enhanced chemiluminescence (ECL) (ThermoFisher, USA) was used to visualize protein bands followed by analysis with Image Lab™ Software (Bio-Rad, USA).

Dual-luciferase reporter gene assay

TargetScan (<http://www.targetscan.org/>) was used to predict the interaction between circRNA-0008717 and miR-203 and the exact target binding sites. The predicted interaction was examined using a dual-luciferase assay. The wild-type *Slug* reporter (*Slug*-Wt) and wild type circRNA-0008717 reporter (circRNA-Wt) were constructed by cloning the 3' UTR of the *Slug* containing the miR-203 binding site and full-length circRNA-0008717 sequence each into a pGL3 vector (Promega, Madison, WI, USA). GeneArt™ The Site-Directed Mutagenesis System (Thermo Fisher Scientific) was used to generate a mutated circRNA-0008717 reporter (circRNA-0008717-Mut) and a mutated *Slug* reporter (*Slug*-mut). Each reporter vector is co-transfected with the miR-203 mimics or miR-203 mimics NC into EC109 and KYSE-150 cells using Lipofectamine 3000. After 48 h, luciferase activity was measured using a dual-luciferase kit (Promega, USA).

Cell counting kit-8 (CCK-8) assay

A cell counting kit-8 (CCK-8) kit (Sigma, USA) was used to measure the cell proliferation of EC109 and KYSE-150 cells in 96-well plates (2×10^4 cells/well). In brief, 10 µL of CCK-8 reagent was added into each well at 24, 48, 72, and 96 hours, and cells were incubated for 1 hour at room temperature. A microplate reader (Bio-Rad, USA) at 450 nm was used to analyze the results.

Transwell assay

Transwell chambers (Corning, USA) were used to detect cell invasion. Briefly, 200 µL of cell suspension (0.1×10^6 cells) was added to an upper chamber pre-coated with Matrigel (Corning, USA), and the lower chamber contained 600 µL of DMEM with 10% FBS. Cells were incubated for 24 hours at 37 °C. Cells that had migrated to the lower chamber were fixed for 20 minutes in 1% formaldehyde and stained for 20 minutes in crystal violet (0.1%). Stained cells were visualized with a microscope (Olympus), and five

randomly selected fields were used to count the number of invading cells.

The scratch wound assay

Transfected EC109 and KYSE-150 cells were seeded into 6-well plates, and a scratch wound assay was used to detect the cell. A wound was introduced to the cell layers using a 200 μ L pipette tip, and cells were cultured in 10% FBS-supplemented DMEM. Cell migration was measured at 0 and 48 hours with an inverted microscope.

Cell apoptosis assay

Cell apoptosis was assayed by the flow cytometry (BD Biosciences, USA). After transfection for 24 hours, EC109 and KYSE-150 cells were harvested through trypsinization and then resuspended with PBS buffer. Subsequently, cells were double stained with Annexin V-Alexa Fluor 647 and propidium iodide (PI). Finally, the apoptotic rate was then analyzed using flow cytometer (BD Biosciences, USA).

RNA pull down assay

RNA pull-down were performed as described previously (25). In brief, circRNA-0008717-Wt, circRNA-0008717-Mut and NC were biotinylated to be Bio-circRNA-Wt, Bio-circRNA-Mut and Bio-NC by GenePharma (GenePharma, Shanghai, China). miR-203-Wt, miR-203-Mut and miR-NC were transcribed using TranscriptAid T7 High Yield Transcription Kit (ThermoFisher Scientific, USA). Biotin RNA labeling mix (GenePharma, China) was used to produce Bio-miR-203-Wt, Bio-miR-203-Mut and Bio-miR-NC. EC109 or KYSE-150 cells were transfected and incubated for 48 hours. After the incubation, cells were lysed with lysis buffer. Then, cell lysate was incubated with Dynabeads M-280 Streptavidin (Invitrogen, CA). RT-qPCR was used to measure purified RNA complex.

Statistical analysis

All data are the mean \pm SD from at least 3 independent experiments. A Student's *t*-test was used to find significant differences between the two groups, and a one-way ANOVA with Tukey's post hoc test was used to compare the means of more than two groups. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 22.0 (Chicago, IL, USA) and GraphPad Prism

7.0 (GraphPad, San Diego, CA, USA).

Results

The circRNA-0008717 knockdown inhibited the malignant evolution of esophageal cancer cells

First, we measured the expression of circRNA-0008717 in EC cell lines (EC109 and KYSE-150) to show if circRNA-0008717 participated in EC progression. The qRT-PCR results showed that circRNA-0008717 expression was significantly higher in EC109 and KYSE-150 cells than in the normal cell line Het-1A ($P < 0.05$, $P < 0.01$) (Figure 1A). These results suggested that circRNA-0008717 was upregulated in EC cells. Second, circRNA-0008717 siRNA (si-circRNA) and its scramble control (si-NC) were separately transfected into EC109 and KYSE-150 cells. As shown by the qRT-PCR results (Figure 1B), the introduction of si-circRNA-0008717 to EC109 and KYSE-150 cells significantly decreased the level of circRNA-0008717 transcripts compared to the si-NC group ($P < 0.001$, $P < 0.01$). Finally, the CCK-8 assay suggested that the circRNA-0008717 knockdown inhibited the proliferation of EC109 and KYSE-150 cells (Figure 1C). Furthermore, the invasion and migration abilities of EC109 and KYSE-150 cells transfected with si-circRNA-0008717 were suppressed (Figure 1D,E). Additionally, as expected, si-circRNA-0008717 significantly enhanced cell apoptosis in both EC109 and KYSE-150 cells ($P < 0.001$, $P < 0.01$, Figure 1F). Therefore, these findings suggest that knocking down circRNA-0008717 inhibited the proliferation, migration, and invasion of EC cells.

CircRNA-0008717 sponged miR-203 in esophageal cancer cells

The expression of miR-203 in EC cell lines was measured by qRT-PCR. The results showed that the level of miR-203 was significantly lower in EC109 and KYSE-150 cells than in Het-1A cells ($P < 0.001$) (Figure 2A). The expression of miR-203 in EC109 and KYSE-150 cells transfected with the miR-203 mimics or miR-203 inhibitor was measured by qRT-PCR. As shown in Figure 2B, the expression of miR-203 in cells in the miR-203 mimic group was significantly higher than in the NC-mimic group ($P < 0.01$). Meanwhile, the expression of miR-203 in cells in the miR-203 inhibitor group was significantly lower than in the NC-inhibitor group ($P < 0.001$). Therefore,

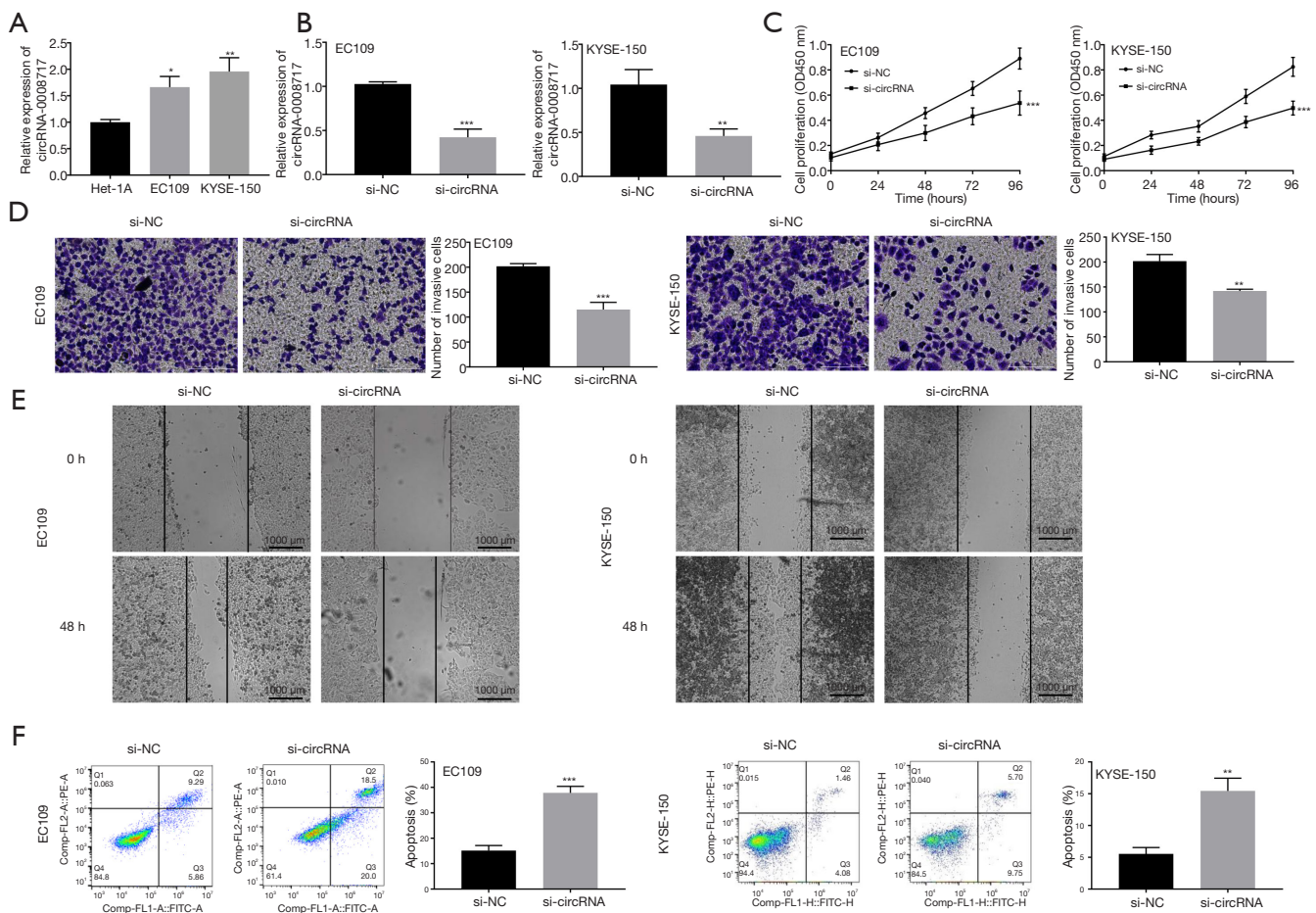


Figure 1 Suppression of circRNA-0008717 inhibited the proliferation, migration, and invasion of EC cells. (A) The abundance of circRNA-0008717 transcripts in Het-1A, EC109, and KYSE-150 cells as measured by qRT-PCR. *, $P < 0.05$, **, $P < 0.01$ vs. Het-1A. (B) qRT-PCR results show the knockdown efficiency of circRNA-0008717 in EC109 and KYSE-150 cells. **, $P < 0.01$, and ***, $P < 0.001$ vs. si-NC. (C) CCK-8 results show the proliferation of transfected cells at 0, 24, 48, 72, and 96 h. ***, $P < 0.001$ vs. si-NC. (D) The transwell assay shows that si-circRNA reduced the invasion of EC109 and KYSE-150 cells (cells were dyed by 0.1% crystal violet, magnification $\times 40$; scale bar: 200 μm). **, $P < 0.01$, and ***, $P < 0.001$ vs. si-NC. (E) Scratch wound assay shows that si-circRNA reduced the migration of EC109 and KYSE-150 cells (magnification $\times 40$; scale bar: 1,000 μm). (F) Apoptosis of EC109 and KYSE-150 cells transfected with si-circRNA-0008717, as detected by Flow Cytometry. **, $P < 0.01$, and ***, $P < 0.001$ vs. si-NC.

the transfection of the miR-203 mimic and miR-203 inhibitor was considered successful. We subsequently used TargetScan to find potential miRNAs that could be targeted by the sponge activity of circRNA-0008717 and found that the seed region of miR-203 contained complementary sequences (Figure 2C). RNA pull-down assay further validated that miR-203 could directly bind with circRNA-0008717 (Figure 2D). The predicted interaction between miR-203 and circRNA-0008717 was confirmed using a luciferase reporter assay. The luciferase

activity of the wild type miR-203 significantly inhibited the circRNA-0008717 reporter; however, the activity of the circRNA-0008717 reporter with mutated binding sites was unaffected (Figure 2E).

Furthermore, cells transfected with si-circRNA have elevated expression of miR-203 (Figure 2F). However, the luciferase activity of the wild type circRNA-0008717 reporter was unaffected in the miR-203 group (Figure 2G). All these results show that circRNA-0008717 could directly sponge miR-203 in EC cells.

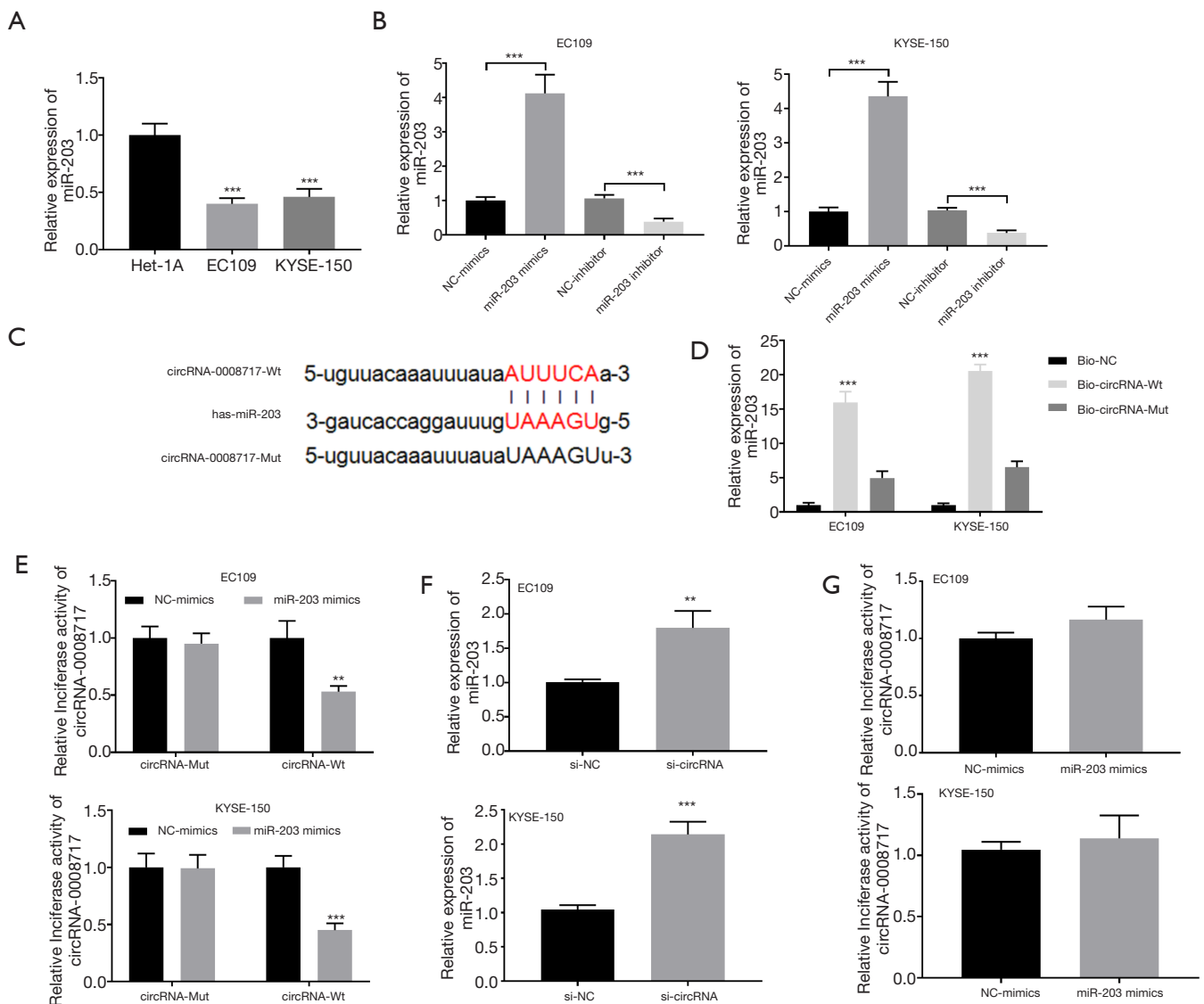


Figure 2 CircRNA-0008717 sponged miR-203 in EC cells. (A) The miR-203 expression is down-regulated in EC lines (EC109 and KYSE-150) as measured by qRT-PCR. ***, $P < 0.001$ vs. Het-1A. (B) A miR-203 mimic and miR-203 inhibitor were transfected into EC109, and KYSE-150 cells and qRT-PCR was used to measure the expression of miR-203. ***, $P < 0.001$ vs. si-NC group. (C) The putative sequences of miR-203 and circRNA-0008717 with the binding sites shown in red. (D) RNA pull down assay was conducted to further confirm the binding ability between circRNA-0008717 and miR-203 in EC109 and KYSE-150 cells. ***, $P < 0.001$ vs. Bio-circRNA-Mut group. (E) miR-203 significantly inhibited the luciferase activity of the wild type reporter for circRNA-0008717; however, miR-203 did not inhibit the luciferase activity of the reporter vector that contained the mutant binding sites of circRNA-0008717. **, $P < 0.01$, ***, $P < 0.001$ vs. NC-mimic group. (F) miR-203 is upregulated in EC cells transfected with si-circRNA. **, $P < 0.01$, ***, $P < 0.001$ vs. si-NC group. (G) miR-203 did not influence the expression level of circRNA-0008717.

miR-203 targeted Slug in esophageal cancer cells

We used Targetscan to find the targets of circRNA-0008717 and miR-203. We found a complementary match between the miR-203 and circRNA-0008717 binding sites and the

3' UTR of Slug (Figure 3A). The interaction between miR-203 and Slug was evaluated using a luciferase reporter assay. In cells transfected with the miR-203 mimic, the luciferase activity of the wild-type Slug vector was significantly lower

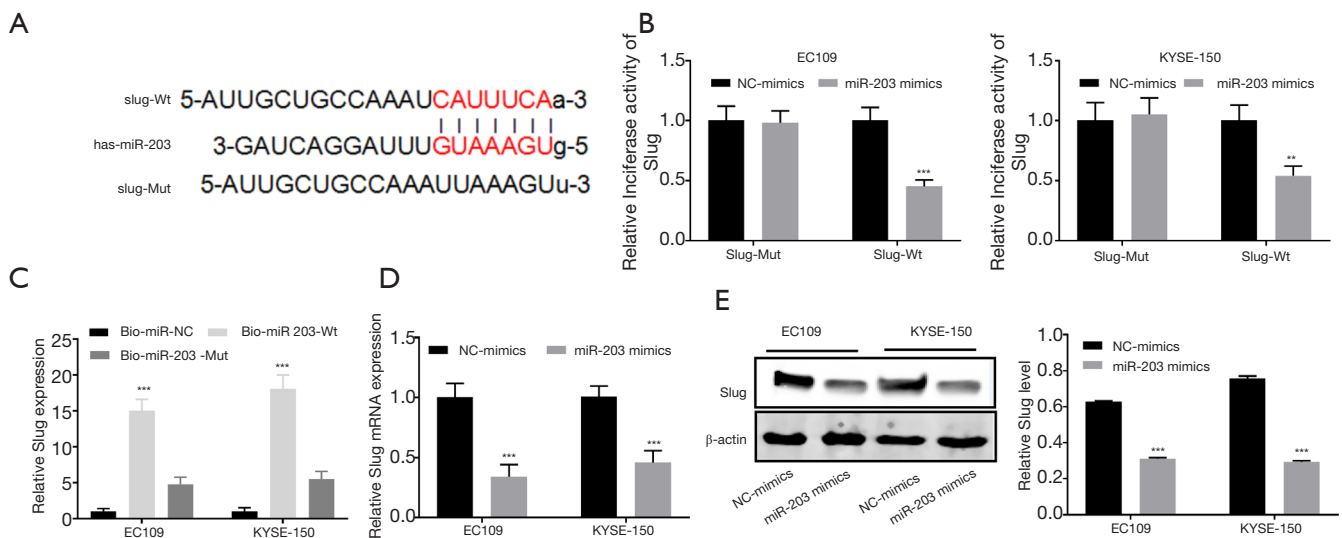


Figure 3 The MiR-203 targeted the Slug transcription factor in EC cells. (A) The putative sequence of miR-203 and Slug with binding sites shown in red. (B) miR-203 significantly inhibited the luciferase activity of the wild type Slug reporter; however, miR-203 did not inhibit the luciferase activity of the reporter vector containing the mutated binding sites of Slugs in EC109 and KYSE-150 cells. **, $P < 0.01$, ***, $P < 0.001$ vs. NC-mimics group. (C) RNA pull down assay was conducted to further confirm the binding ability between Slug and miR-203 in EC109 and KYSE-150 cells. ***, $P < 0.001$ vs. Bio-miR-203-Mut group. (D) Slug mRNA expression was down-regulated by the miR-203 mimic. ***, $P < 0.001$ vs. NC-mimics group. (E) The Slug protein level was down-regulated by the miR-203 mimic. ***, $P < 0.001$ vs. NC-mimic group.

than the reporter vector with mutated binding sites of Slug (Figure 3B). RNA pull-down assay further validated that miR-203 could directly bind with Slug (Figure 3C). We then examined how miR-203 affected Slug at the transcript and protein levels using qRT-PCR and Western blot. The expression of Slug in EC109 and KYSE-150 cells was inhibited by the miR-203 mimic (Figure 3D,E). These findings suggested that miR-203 directly targeted Slug in EC cells.

CircRNA-0008717 upregulated Slug expression by sponging miR-203

Next sought to set up if sponging of miR-203 by circRNA-0008717 increased the expression of Slug. The qRT-PCR and Western blot results showed that knocking down circRNA-0008717 in EC109 and KYSE-150 cells significantly decreased the levels of Slug mRNA and protein ($P < 0.001$) (Figure 4A,B). Furthermore, co-transfection of the miR-203 inhibitor increased Slug expression in EC cells (Figure 4C). These findings supply evidence for a mechanism by which circRNA-0008717 competitively binds miR-203 and increases the expression of Slug in EC cells. Later, the abundance of two EMT marker proteins

was determined by Western blot further to characterize the invasive capabilities of circRNA-0008717 knockdown cells. Knocking down circRNA-0008717 reduced the amount of the mesenchymal marker vimentin and increased the amount of E-cadherin, which it uses as an epithelial marker. However, cells co-transfected with the miR-203 inhibitor had a reverse effect on vimentin and E-cadherin (Figure 4D).

Slug siRNA inhibited the malignant evolution of esophageal cancer cells

To assess the effect of Slug on EC cell phenotype, we performed gain-of-function investigations by transfecting the si-Slug in EC109 and KYSE-150 cells. The CCK-8 assay suggested that si-Slug inhibited the proliferation of EC109 and KYSE-150 cells ($P < 0.001$, Figure 5A). Furthermore, the invasion and migration abilities of EC109 and KYSE-150 were suppressed by si-Slug ($P < 0.001$, $P < 0.01$, Figure 5B,C). Moreover, si-Slug highly enhanced the cell apoptosis of EC109 and KYSE-150 cells ($P < 0.01$, Figure 5D). Therefore, these data reveal that Slug siRNA inhibited the proliferation, migration, and invasion of EC cells.

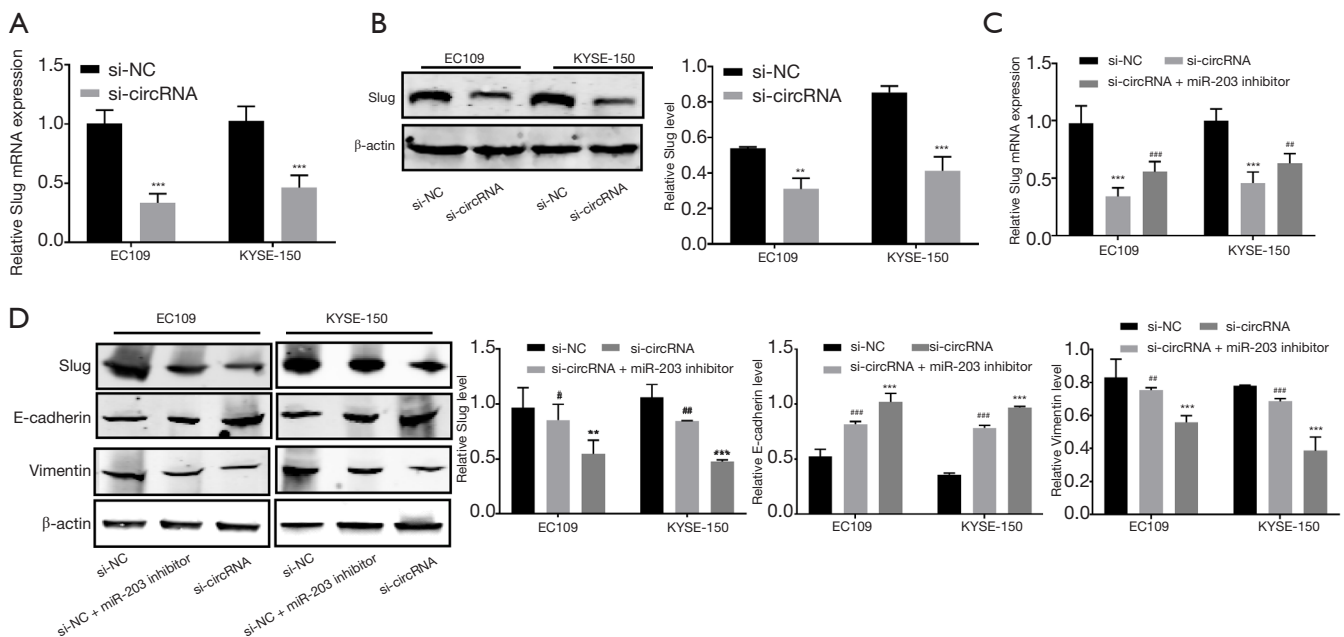


Figure 4 CircRNA-0008717 upregulated the expression of Slugs by sponging miR-203. (A) The abundance of Slug mRNA in circRNA-0008717, knockdown EC109 and KYSE-150 cells as measured by RT-qPCR. (B) The Slug protein level in circRNA-0008717, knockdown EC109 and KYSE-150 cells determined by the Western blot. (C) Expression of Slug mRNA in EC109 and KYSE-150 cells co-transfected with miR-203 inhibitor and si-circRNA-0008717 as measured by qRT-PCR. (D) The abundance of Slug, vimentin, and E-cadherin proteins in EC109 and KYSE-150 cells co-transfected with miR-203 inhibitor and si-circRNA-0008717 as determined by Western blot. **, $P < 0.01$, ***, $P < 0.001$ vs. si-NC group; #, $P < 0.01$, ###, $P < 0.001$ vs. si-circRNA group.

CircRNA-0008717 promoted the progression of esophageal cancer progression by sponging miR-203 and upregulating Slug

Finally, we investigated if the sponging of miR-203 circRNA-0008717 and increased Slug expression affected the proliferation and invasion of EC cells. We co-transfected the miR-203 inhibitor and circRNA-0008717 siRNA, or co-transfected pcDNA Slug and circRNA-0008717 siRNA into EC109 and KYSE-150 cells to establish if circRNA-0008717 exerted its oncogenic effect through miR-203 to liberate Slug transcripts. The proliferation of EC109 and KYSE-150 was significantly repressed by circRNA-0008717 siRNA. However, the miR-203 inhibitor or pcDNA Slug reduced this effect (Figure 6A). Similarly, the miR-203 inhibitor or pcDNA Slug reduced the migration and invasion of circRNA-0008717 knockdown cells (Figure 6B,C). In addition, the flow cytometry assay showed that the apoptosis of EC109 and KYSE-150 was significantly increased by circRNA-0008717 siRNA. By contrast, the miR-203 inhibitor or pcDNA Slug could

rescue it (Figure 6D).

Discussion

The expression of certain circRNAs has been reported in EC tissues and cells by a few previous studies (26,27). However, available information about the specificity and sensitivity of their expression as EC biomarkers, in addition to their functioning networks and mechanisms, is far from sufficient. In this study, we investigated the expression of circRNA-0008717 in two EC cell lines and supplied evidence that circRNA-0008717 sponges miR-203 to liberate Slug transcripts that are targeted by miR-203.

CircRNAs are highly stable and so could prove a useful diagnostic marker or therapeutic target for precision medicine (28,29). A previous study has demonstrated that circRNA-0008717 can promote osteosarcoma progression through sponging miR-203 (29). In our research, we found that the expression of circRNA-0008717 was upregulated and promoted the proliferation, migration, and invasion of EC cells. Consistent with earlier reports, our study supplies

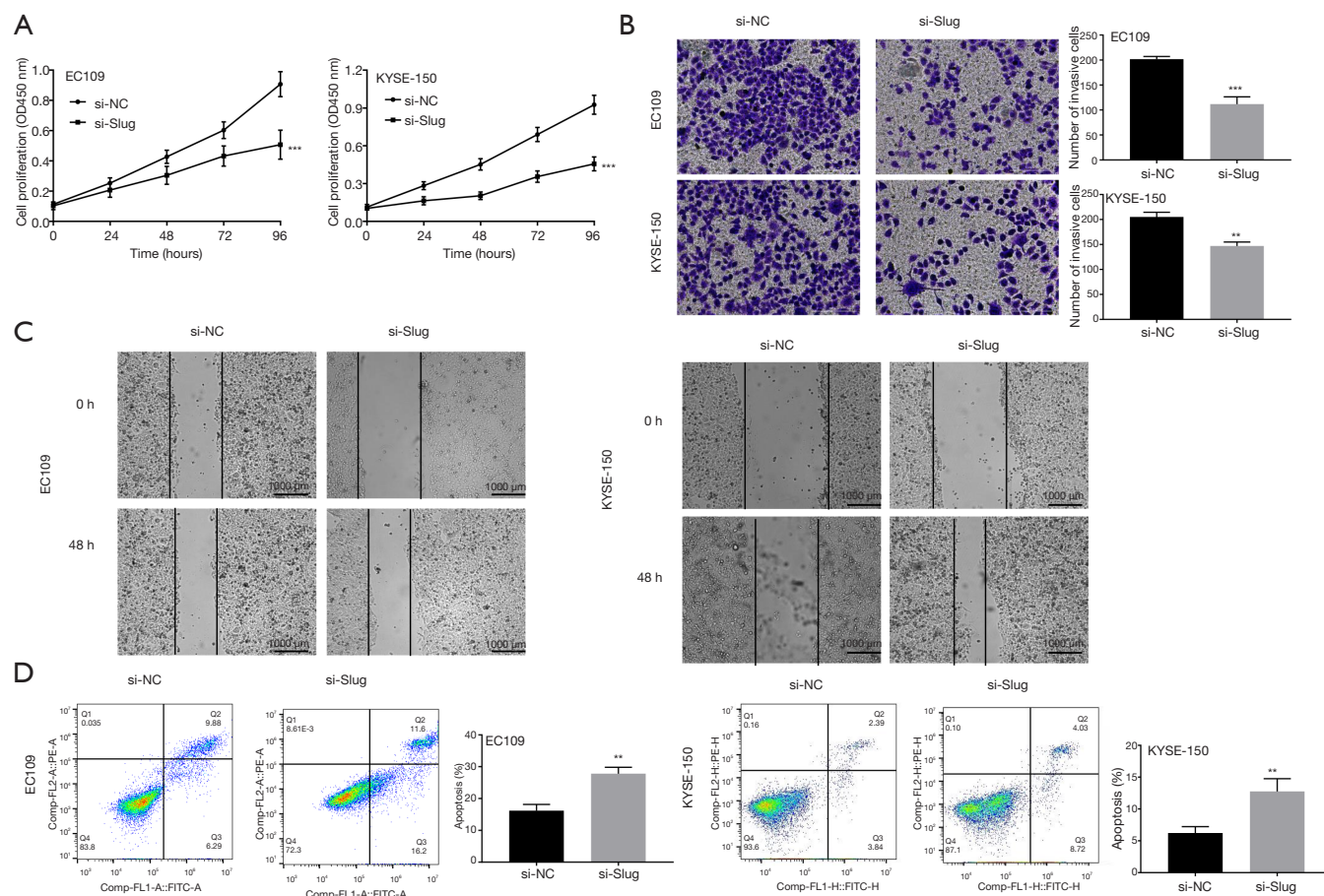


Figure 5 Slug siRNA inhibited the malignant evolution of esophageal cancer cells. (A) CCK-8 assay showed the proliferation of cells transfected with Slug siRNA (si-Slug). (B) Transwell assay showing the effects of the si-Slug on the invasion of EC109 and KYSE-150 cells (cells were dyed by 0.1% crystal violet, magnification $\times 40$; scale bar: 200 μm). (C) Scratch wound assay showing that the effects of the si-Slug on the migration of EC109 and KYSE-150 cells (magnification $\times 40$; scale bar: 1,000 μm). (D) Flow Cytometry assay showing the apoptosis of EC109 and KYSE-150 cells transfected with si-Slug. **, $P < 0.01$, and ***, $P < 0.001$ vs. si-NC group.

evidence that circRNA-0008717 could be a potential therapeutic target to improve the prognosis of EC.

Gene regulation via miRNAs has been implicated in processes that are involved in the development and prognosis of cancers (30). MiRNAs are involved in the post-transcriptional regulation of gene expression by interacting with mRNAs through corresponding miRNA response elements (MRE) (31). In recent years, the sponge role of circRNAs in regulating interactions between miRNAs and oncogenic genes has been realized. First, ciRS-7 was shown to act as a sponge to inhibit the miRNA (miR-7) activity known to have dysregulated expression in cancerous cells (32). Subsequently, it was reported that circRNA-0008717 upregulated Bmi-1 expression by sponging miR-203,

which resulted in the progression of osteosarcoma (29). In agreement with this finding, we have shown that circRNA-0008717 sponges miR-203 in EC cells. Previously, the aberrant expression of miR-203 has been linked to multiple malignancies, including EC (33). In line with earlier studies, we found that miR-203 was a key target of circRNA-0008717 and that miR-203 expression was down-regulated in EC.

The Slug transcription factor is a crucial regulator of multiple tumorigenic processes, including cell invasion, metastasis, cell survival, and proliferation (21). In gastric cancer, a reduction in Slug by miRNA-203 was shown to suppress tumor cell proliferation, migration, and invasion (17,34). Here, we also found Slug as a novel target for

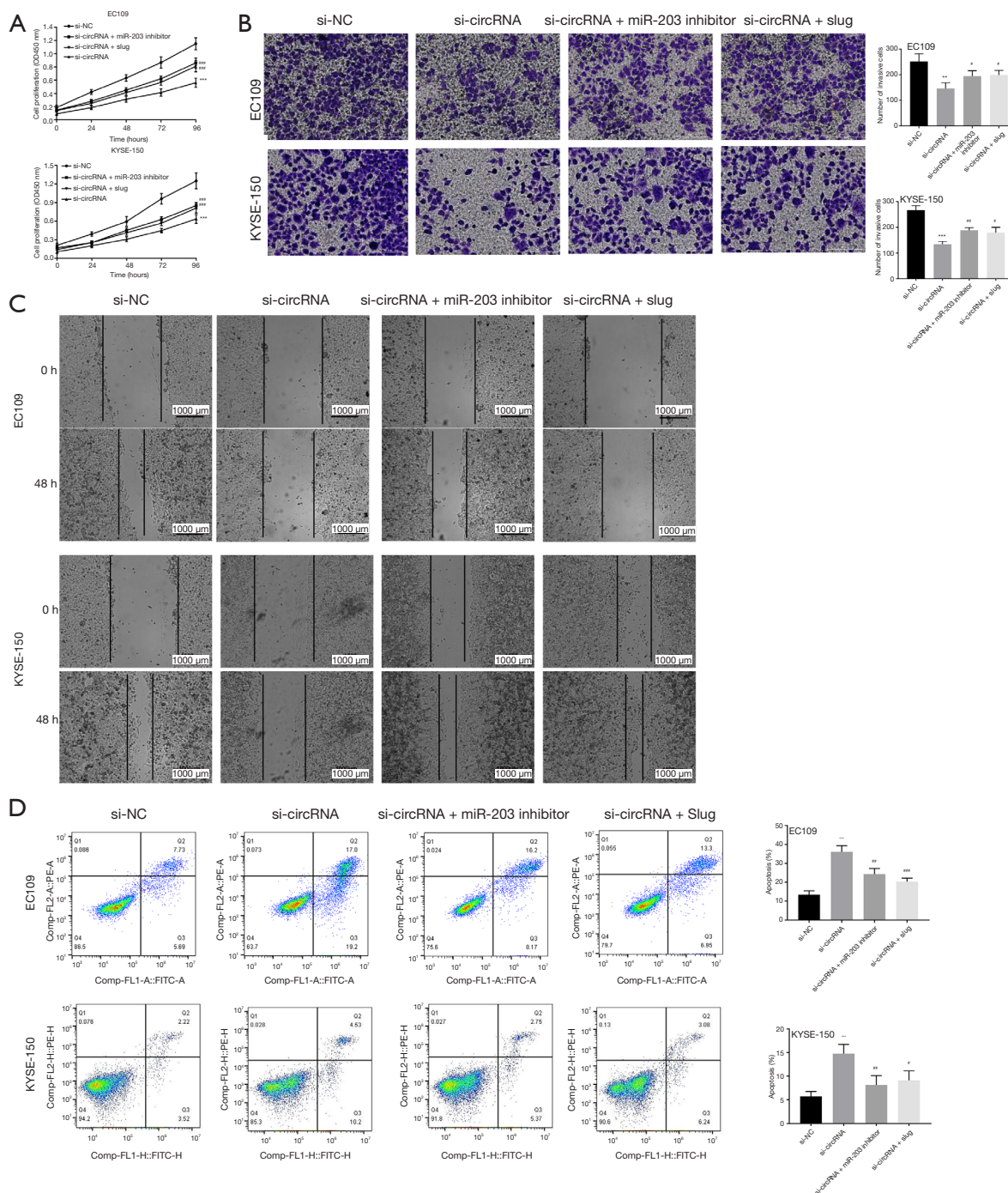


Figure 6 CircRNA-0008717 promoted EC progression by sponging the miR-203 and upregulating the expression of Slug. (A) CCK-8 assay showed the proliferation of cells co-transfected with a miR-203 inhibitor and circRNA-0008717 siRNA or co-transfection of pcDNA Slug and circRNA-0008717 siRNA. (B) Transwell assay showing the effects of the miR-203 inhibitor, pcDNA Slug, and circRNA-0008717 siRNA on the invasion of EC109 and KYSE-150 cells (cells were dyed by 0.1% crystal violet, magnification $\times 40$; scale bar: 200 μm). (C) Scratch wound assay showing that the effects of the miR-203 inhibitor, pcDNA Slug, and circRNA-0008717 siRNA on the migration of EC109 and KYSE-150 cells (magnification $\times 40$; scale bar: 1,000 μm). (D) Flow Cytometry assay showing the apoptosis of EC109 and KYSE-150 cells transfected with si-circRNA-0008717, the miR-203 inhibitor, pcDNA Slug. **, $P < 0.01$, and ***, $P < 0.001$ vs. si-NC group; #, $P < 0.05$, ##, $P < 0.01$, and ###, $P < 0.001$ vs. si-circRNA group.

miR-203 in EC. Furthermore, we found that inhibition of miR-203 increased the expression of Slug and vimentin expression and decreased the expression of E-cadherin. According to previous studies, we know that Slug can increase the invasion of cancer cells in various malignancies by repressing E-cadherin expression (20).

Furthermore, miR-203 inhibits cell motility and invasiveness through Slug/E-cadherin signals (17). In this study, we have shown that circRNA-0008717 can regulate Slug expression via miR-203. Furthermore, transfection with a miR-203 inhibitor reverses the effect of knocking down circRNA-0008717 expression in EC cells. Thus, we have confirmed that circRNA-0008717 can promote the proliferation, migration, and invasion of EC cells by sponging the miR-203.

Our results showed that circRNA-0008717 expression was upregulated in EC cells. Moreover, we have supplied evidence that circRNA-0008717 acts as a sponge that inhibits miR-203, thereby increasing Slug expression, which promotes the proliferation, migration, and invasion of EC cells. This research has proved a novel circRNA regulatory mechanism linked to the progression of EC and has provided a potential therapeutic target that could improve the prognosis of EC patients.

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Footnote

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