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Circular RNA circ_0067934 exhaustion expedites cell apoptosis and represses cell proliferation, migration and invasion in thyroid cancer *via* sponging miR-1304 and regulating CXCR1 expression

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Abstract. – OBJECTIVE: Distant metastasis or local recurrence is the leading cause of death in some patients with thyroid cancer (TC), a malignant tumor of the endocrine system. Circular RNA circ_0067934 (circ_0067934) has been reported to be connected with the tumorigenesis of multiple tumors. However, there are few reports on the role and regulatory mechanisms of circ_0067934 in TC.

MATERIALS AND METHODS: The expression levels of circ_0067934, protein kinase C iota (PRKCI), microRNA-1304 (miR-1304), and C-X-C chemokine receptor types 1 (CXCR1) were detected with quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The proliferation, apoptosis, migration, and invasion of TC cells were evaluated by Cell Counting Kit-8 (CCK8) assay, flow cytometry assay, and transwell assay, respectively. The relationship between circ_0067934 or CXCR1 and miR-1304 was confirmed with Dual-Luciferase reporter assay or RNA immunoprecipitation (RIP) assay. Protein level of CXCR1 was analyzed *via* Western blot analysis. Xenograft assay was executed to verify the role of circ_0067934 *in vivo*.

RESULTS: Circ_0067934 and CXCR1 were enhanced and miR-1304 decreased in TC tissues and cells. Circ_0067934 downregulation triggered apoptosis and curbed proliferation, migration, and invasion of TC cells *in vitro*, as well as repressed tumor growth *in vivo*. Notably, circ_0067934 regulated CXCR1 expression *via* sponging miR-1304 in TC cells. Both miR-1304 silencing and CXCR1 elevation reversed the facilitation of apoptosis and the retardation of proliferation, migration, and invasion induced by circ_0067934 reduction in TC cells.

CONCLUSIONS: Circ_0067934 downregulation expedited apoptosis and retarded proliferation, migration, and invasion of TC cells through miR-1304/CXCR1 axis.

Key Words:
Thyroid cancer, Circ_0067934, MiR-1304, CXCR1.

Introduction

Thyroid cancer (TC) is a common malignant tumor of the endocrine system, and its morbidity and mortality are on the rise[1]. According to statistics, from 2006 to 2015, the average annual growth rate of TC in men and women in the United States was 3.2% and 3.1%, respectively[2]. It is estimated that there will be 52,070 new cases of TC in the United States in 2019, including 14,260 males and 37,810 females[2]. At present, most patients with TC have a good prognosis based on surgical resection and chemotherapy or radiotherapy, but some patients still die from distant metastasis or local recurrence within 10 years[3,4]. Therefore, it is imperative to find new molecular targets in tumorigenesis and progression of TC to provide a potential theory for the management of TC cases.

Circular RNAs (circRNAs) are a kind of covalent cyclic endogenous RNAs formed by the covalent attachment of a 3' splice donor of pre-mRNA to the 5' splice acceptor in the reverse order[5]. Increased researches revealed that circRNAs were connected with a variety of physiological and pathological processes, such as sponging microRNAs (miRNAs), regulating gene expression and protein-RNA interactions, and mediating alternative splicing[6]. It has been reported that circRNAs are promising cancer biomarkers and therapeutic targets or drugs[7]. Circular RNA circ_0067934

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(circ_0067934) is derived from the human protein kinase C iota (PRKCI) gene locus in the 3q26.2 region of the chromosome[8]. Circ_0067934 has been reported to be related to the tumorigenesis of multiple cancers, such as hepatocellular cancer[9], esophageal squamous cell cancer[8], and cervical cancer[10]. Also, previous literature revealed that enhanced circ_0067934 expression was associated with poor prognosis in TC[11]. Nevertheless, the biological role of circ_0067934 in TC and its related molecular mechanisms have rarely been reported.

MiRNAs (approximately 21 nucleotides) are a class of post-transcriptional regulators that regulate gene expression and play fundamental roles in cell metabolism and differentiation, organ development, tumorigenesis, and viral infection[12]. Studies have showed that miRNAs exert roles in a range of cancers by acting as tumor suppressors or oncogenes[13]. In addition, it was reported that the dysregulation of microRNA-1304 (miR-1304) was connected with the occurrence and progression of papillary thyroid cancer[14] and non-small cell lung cancer[15]. However, the role and potential molecular mechanisms of miR-1304 in TC need to be further elucidated.

Chemokines and their cognate receptors play vital roles in the immune system *via* mediating the activation and trafficking of immune cells[16]. C-X-C chemokine receptor types 1 (CXCR1) is a member of the chemokine family that has been revealed to be developed with a variety of cancers[17]. CXCR1 was reported to be associated with cisplatin resistance in osteosarcoma[18]. Also, CXCR1 could increase the chemoresistance of colorectal cancer to 5-fluorouracil[19]. Moreover, CXCR1 could promote the progression of gastric cancer[20]. Additionally, lymph node metastasis of papillary TC was correlated with elevated expression of CXCR1[21]. Nevertheless, it is unclear whether CXCR1 is regulated by circ_0067934 and miR-1304 in TC.

Herein, the expression pattern of circ_0067934 in TC tissues and cells was probed. Moreover, the role of circ_0067934 on TC cells *in vitro* and *in vivo* was also explored. Moreover, the mechanism of the circ_0067934/miR-1304/CXCR1 axis in TC cells was investigated, providing possible strategies for the treatment of TC.

Materials and Methods

TC Specimen Collection

The protocol of this research was approved by the Ethics Committee of the Anhui Provincial Hospital. 50 paired TC tissues and neighboring normal tissues were obtained from patients who underwent surgery at the Anhui Provincial Hospital. Patients with TC were diagnosed according to the American Joint Committee on Cancer (AJCC) diagnostic criteria and did not receive any treatment before surgical removal of the tumor. All participants in the study signed informed consents before surgery.

Cell Culture

TC cell lines (SW579, TPC-1, FTC-133, and IHH4) and normal human thyroid cell line Nthy-ori3-1 were purchased from BeNa Culture Collection (Suzhou, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with fetal bovine serum (FBS, 10%; HyClone, South-Logan, UT, USA), streptomycin (100 μg/mL; Life Technologies, Grand Island, NY, USA) and penicillin (100 U/mL; Life Technologies, Grand Island, NY, USA) were applied to culture the cells used in this study. An incubator with 5% CO_2 at 37°C was utilized to keep the growth of the above cell lines.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA of cells and TC tissues, as well as neighboring normal tissues, were extracted through TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2 μg) was maintained at 37°C for 15 min with or without RNase R (3 U/μg, Epicentre Technologies, Madison, WI, USA) to digest total RNA for the assessment of the stability of circ_0067934. Moloney Murine Leukemia Virus (M-MLV) First Strand Kit from Life Technologies was executed to synthesize the first-strand complementary DNA (cDNA) of circ_0067934, PRKCI, and CXCR1. The PARIS kit (Life Technologies, Grand Island, NY, USA) was applied to separate the nuclear and cytoplasmic fractions of TC cells to analyze the subcellular localization of circ_0067934. MiRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA for miR-1304. The expression of circ_0067934, PRKCI, miR-1304, and CXCR1 was evaluated through SYBR green PCR master mixes (Thermo Fisher Scientific, Waltham, MA, USA). The primers used were displayed as below: circ_0067934: 5'-TAGCAGTTCCCCAATCCTTG-3' (F) and 5'-CACAAATTCCCATCATTCCC-3' (R); CXCR1: 5'-CTGACCCAGAAGCGTCACTTG-3' (F) and 5'-CCAGGACCTCATAGCAAACTG-3' (R);

PRKCI: 5'-TACGCGCAAGGAACCTCAAG-3' (F) and 5'-CTCGCAACTTGGTCACGTCT-3' (R); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-GACTCCACTCACGGCAAATTCA-3' (F) and 5'-TCGCTCCTGGAAGATGGTGAT-3' (R) as well as U6 small nuclear RNA (snRNA): 5'-GCTCGCTTCGGCAGCACA-3' (F) and 5'-GAGGTATTCGCACCAGAGGA-3' (R). Also, the primer of the miR-1304 (cat.no.MS00031395) was obtained from Qiagen (Hilden, Germany). GAPDH or U6 snRNA was viewed as an internal control for circ_0067934, PRKCI, CXCR1, and miR-1304, and the expression of circ_0067934, PRKCI, miR-1304, and CXCR1 was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell Transfection

Small interference RNA (siRNA) targeting circ_0067934 (si-circ_0067934#1 and si-circ_0067934#2) and negative control (si-NC) were obtained from GenePharma (Shanghai, China). MiRNA mimics targeting miR-1304 (miR-1304) and scrambled mimics control (miR-NC), as well as a miR-1304 inhibitor (anti-miR-1304) and negative inhibitor control (anti-NC) were obtained from GenePharma. The sequence of CXCR1 was cloned into pLVX-IRES-Puro (Lv-NC; Promega, Madison, WI, USA) to construct the overexpression vector of CXCR1 (Lv-CXCR1). Also, the oligonucleotides or plasmids were transiently transfected into TC cells by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The sequences were displayed as the following: si-circ_0067934#1 (5'-UGUUGAUUGGGAUAUGUUAUU-3'), si-circ_0067934#2 (5'-CCGAAAUGUUGAUUGGGAUTT-3') and si-NC (5'-GCGCGATAGCGCGAATATA-3').

Cell Counting Kit-8 (CCK8) Assay

CCK-8 from Dojindo (Dojindo Laboratories, Kumamoto, Japan) was utilized to assess the proliferation capacity of transfected TC cells. In short, transfected TC cells (5×10^3) were seeded in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) to cultivate for 24 h, 48 h, and 72 h. Then, each well was supplemented with CCK-8 (10 μL) at the specified time. 2 h later, the Microplate Absorbance Reader (Thermo Fisher Scientific, Waltham, MA, USA) was employed for the evaluation of the color reaction at 450 nm.

Flow Cytometry Assay

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes NJ, USA) was applied to determine the apoptosis of transfected TC cells. In brief, transfected TC cells were cultured for 48 h. After washing, transfected TC cells (1×10^5) were resuspended in the binding buffer. Afterward, Annexin V-FITC (10 μL) and PI (5 μL) were supplemented to binding buffer with transfected TC cells and incubated for 15 min in the dark. Lastly, the FACScan flow cytometry (BD Biosciences, Franklin Lakes NJ, USA) was performed to analyze the apoptosis rate of transfected TC cells.

Transwell Assay

The transwell assay was executed for analysis of the migration and invasion of transfected TC cells. Briefly, RPMI-1640 medium supplemented with FBS (10%) was added to the lower of the transwell chamber (8 μm, BD Biosciences, Franklin Lakes NJ, USA) as a chemoattractant. Contemporaneously, RPMI-1640 medium containing transfected TC cells (2×10^5) was replenished to the top chamber. After culturing for 48 h, the cells on the upper surface of the membrane were eliminated with a cotton swab. Following this, the methanol (100%) was used to fix the cells on the lower surface of the membrane. Moreover, crystal violet (0.25%, Sigma-Aldrich, St. Louis, MO, USA) was utilized to stain the migrated cells. In addition, the transwell chamber coated with Matrigel matrix (BD Biosciences, Franklin Lakes NJ, USA) was used for the execution of the invasion assay. Finally, the migrated or invaded cells were counted through a light microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Assay

The circular RNA Interactome database was employed for the prediction of the binding sites between circ_0067934 and miR-1304. Afterward, the pGL3-control vector (Promega, Madison, WI, USA) with the sequences of wild type circ_0067934 (WT-circ_0067934) (with predicted miR-1304 binding sites) and mutant circ_0067934 (MUT-circ_0067934) were established to check on the binding sites between circ_0067934 and miR-1304. Also, the TargetScan database was carried out to predict the binding sites between miR-1304 and CXCR1. Similarly, the sequences of wild type CXCR1 3'Untranslated Regions (UTR) (with predicted miR-1304 binding sites) and mutant CXCR1 3'UTR were synthesized and inserted into the pGL3-control vector for the construction of the Luciferase reporter vectors of CXCR1 3'UTR-WT and CXCR1 3'UTR-MUT.

After that, TC cells were cotransfected miR-NC or miR-1304 and Luciferase reporter vector to execute the Dual-Luciferase reporter assay. Eventually, the Luciferase activity of the Luciferase reporter vector was assessed by the Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

The Magna RIP kit (Millipore, Bedford, MA, USA) was utilized to analyze the interaction between circ_0067934 and miR-1304. In short, RIP lysis buffer supplemented with a protease-inhibitor cocktail (Hoffman-La Roche, Basel, Switzerland) and RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) were used to lyse the TC cells. Then, the RIP buffer harboring protein A/G magnetic beads conjugated IgG or Ago2 antibody (Millipore, Billerica, MA, USA) was supplemented to the lysates of TC cells and incubated at 4°C overnight. The enrichment of circ_0067934 and miR-1304 in precipitates was assessed with qRT-PCR.

Western Blot Analysis

The radio-immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the protein of paired TC tissues and neighboring normal tissues, as well as cells. Following this, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10%, SDS-PAGE) was employed to separate the extracted total protein. Next, the separated protein was transferred onto polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes through wet electrophoretic transfer method. After immersion in Tris-Buffered Saline Tween (TBST) buffer with 5% skim milk for 1 h, the PVDF membranes were incubated with primary antibodies: rabbit anti-CXCR1 (1:500, ab14936, Abcam, Cambridge, MA, USA) and rabbit anti-GAPDH (1:2500, ab9485, Abcam, Cambridge, MA, USA). GAPDH was regarded as a loading control. Then, the membranes were incubated with goat anti-rabbit IgG (1:2000, ab205718, Abcam, Cambridge, MA, USA). Finally, the Immobilon TM Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was utilized to visualize the protein bands.

Xenograft Assay

The animal experiments were approved by the Ethics Committee of Anhui Provincial Hospital. 10 BALB/c nude mice (4-6 weeks old) from Shanghai Experimental Animal Center (Shanghai, China) were used to execute xenograft assay.

Briefly, SW579 cells were transfected with lentivirus-mediated small hairpin RNA targeting circ_0067934 (sh-circ_0067934) and negative control (sh-NC). Then, sh-NC-transfected SW579 cells or stable lentiviral-mediated circ_0067934-reduced SW579 cells were subcutaneously injected into the dorsal side of nude mice (every 5 mice in a group) to establish the mouse xenograft models. The tumor volume was measured every 7 days with a digital caliper and calculated with the equation: Volume = (length × width^2) / 2. After 35 days, the mice were euthanized under anesthesia to separate the tumor tissues for subsequent experiments (evaluation of tumor weight and expression levels of circ_0067934, miR-1304, and CXCR1).

Statistical Analysis

The data of this study were derived from at least 3 independent experiments and were shown as mean ± standard deviation. Statistical Product and Service Solution SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) were executed for statistical analysis. The differences between two or among more groups were analyzed through Student's *t*-test or One-way variance analysis (ANOVA), and ANOVA was followed by Tukey's post-hoc test. Differences with $p < 0.05$ were statistically significant. Kaplan-Meier analysis was conducted to evaluate the survival rate of TC patients with high or low expression of circ_0067934. The relationship between miR-1304 and circ_0067934 or CXCR1 expression in TC tissues was assessed with Pearson's correlation analysis method.

Results

Expression Pattern and Characterization of Circ_0067934 in TC Tissues and Cells

To probe into the expression pattern of circ_0067934 in TC, qRT-PCR was executed for the detection of the level of circ_0067934 in 50 paired TC tissues and neighboring normal tissues. The results exhibited that circ_0067934 was aberrantly reinforced in TC tissues in comparison to that in the neighboring normal tissues (Figure 1A). Moreover, the relationship between circ_0067934 expression and TC severity (based on AJCC grade) was analyzed using qRT-PCR.

We found that augmented circ_0067934 expression was connected with the high AJCC grade (stage III and IV) and lymph node metastasis (Figure 1B and 1C). Besides, the survival rate of TC patients with high expression of circ_0067934 and low expression of circ_0067934 was analyzed by the Kaplan-Meier analysis. The results displayed that the prognosis of TC patients with higher circ_0067934 expression level was worse (p = 0.0268) (Figure 1D). Furthermore, the expression level of circ_0067934 in TC cell lines (SW579, TPC-1, FTC-133, and IHH4) was further detected. The results of qRT-PCR implied that circ_0067934 expression level was significantly elevated in 4 TC cell lines compared with the Nthy-ori3-1 cells. Also, the expression of circ_0067934 was relatively high in SW579 and TPC-1 cells compared to other TC cell lines (Figure 1E). In addition, the expression of PRKCI mRNA and circ_0067934 in RNase R-treated SW579 and TPC-1 cells was assessed with qRT-PCR. As presented in Figure 1F and 1G, the circ_0067934 was resistant to RNase R, whereas the liner PRKCI was dramatically reduced after RNase R treatment. Additionally, the cytoplasmic and nuclear fractions of SW579 and TPC-1 cells, as well as qRT-PCR, were utilized to analyze the subcellular localization of circ_0067934. The results exhibited that circ_0067934 was significantly enriched in the cytoplasm fraction of both SW579 and TPC-1 cells, indicating that circ_0067934 was mainly distributed in the cytoplasm (Figure 1H and 1I). Collectively, these data suggested that circ_0067934 was principally localized in the cytoplasm, and the high expression of circ_0067934 was connected with AJCC grade, lymph node metastasis, and survival rate in patients with TC.

Circ_0067934 Depletion Boosted Apoptosis and Constrained Proliferation, Migration, and Invasion of TC Cells In Vitro

To explore the biological role of circ_0067934, we assessed the expression level of PRKCI mRNA and circ_0067934 in SW579 and TPC-1 cells transfected with si-NC, si-circ_0067934#1 or si-circ_0067934#2. The results of qRT-PCR implied that circ_0067934 was apparently reduced in SW579 and TPC-1 cells transfected with si-circ_0067934#1 and si-circ_0067934#2 compared to the si-NC group, while there was no prominent change in PRKCI mRNA (Figure 2A and 2B). It was indicated that si-circ_0067934#1 and si-circ_0067934#2 could be utilized for subsequent loss-of-function experiments. Results of CCK-8 assay revealed that the proliferation capacity of SW579 and TPC-1 cells transfected with si-circ_0067934#1 or si-circ_0067934#2 was conspicuously impeded compared with the si-NC group (Figure 2C and 2D). Moreover, flow cytometry assay demonstrated that the apoptosis of SW579 and TPC-1 cells transfected with si-circ_0067934#1 or si-circ_0067934#2 was strikingly higher than that of the si-NC group (Figure 2E). In addition, transwell assay displayed that the migration and invasion capacities of SW579 and TPC-1 cells transfected with si-circ_0067934#1 or si-circ_0067934#2 were dramatically blocked *vs.* the control group (Figure 2F and 2G). From the above results, the downregulation of circ_0067934 could trigger apoptosis and repress proliferation, migration, and invasion of TC cells and the si-circ_0067934#1 was selected for subsequent exploration.

Circ_0067934 Served as a Sponge of MiR-1304 in TC Cells In Vitro

In order to investigative how circ_0067934 works, the circular RNA Interactome database (https://circinteractome.nia.nih.gov) was utilized for the prediction of the potential target of circ_0067934. As presented in Figure 3A, circ_0067934 held potential binding sites for miR-1304. Then, we constructed the Luciferase reporter vectors (WT-circ_0067934 and MUT-circ_0067934) to validate the predicted binding sites. Results of Dual-Luciferase reporter assay implied that the activity of WT-circ_0067934 in both SW579 and TPC-1 cells transfected with miR-1304 was strikingly lower than that in the control group, but there was no evident difference in the activity of MUT-circ_0067934 (Figure 3B and 3C). Subsequently, we conducted the RIP assay to verify the relationship between circ_0067934 and miR-1304 further. The results exhibited that circ_0067934 and miR-1304 were specially gathered in Ago2-containing beads relative to those harboring control IgG antibody (Figure 3D and 3E). Furthermore, the expression of miR-1304 in TC cells was detected using qRT-PCR. Compared with the Nthy-ori3-1 cells, the expression of miR-1304 was markedly reduced in SW579 and TPC-1 cells (Figure 3F). Moreover, the expression of miR-1304 was evidently reinforced in SW579 and TPC-1 cells transfected with circ_0067934#1 (Figure 3G). Also, we discovered that miR-1304 was apparently downregulated in 50 TC tissues

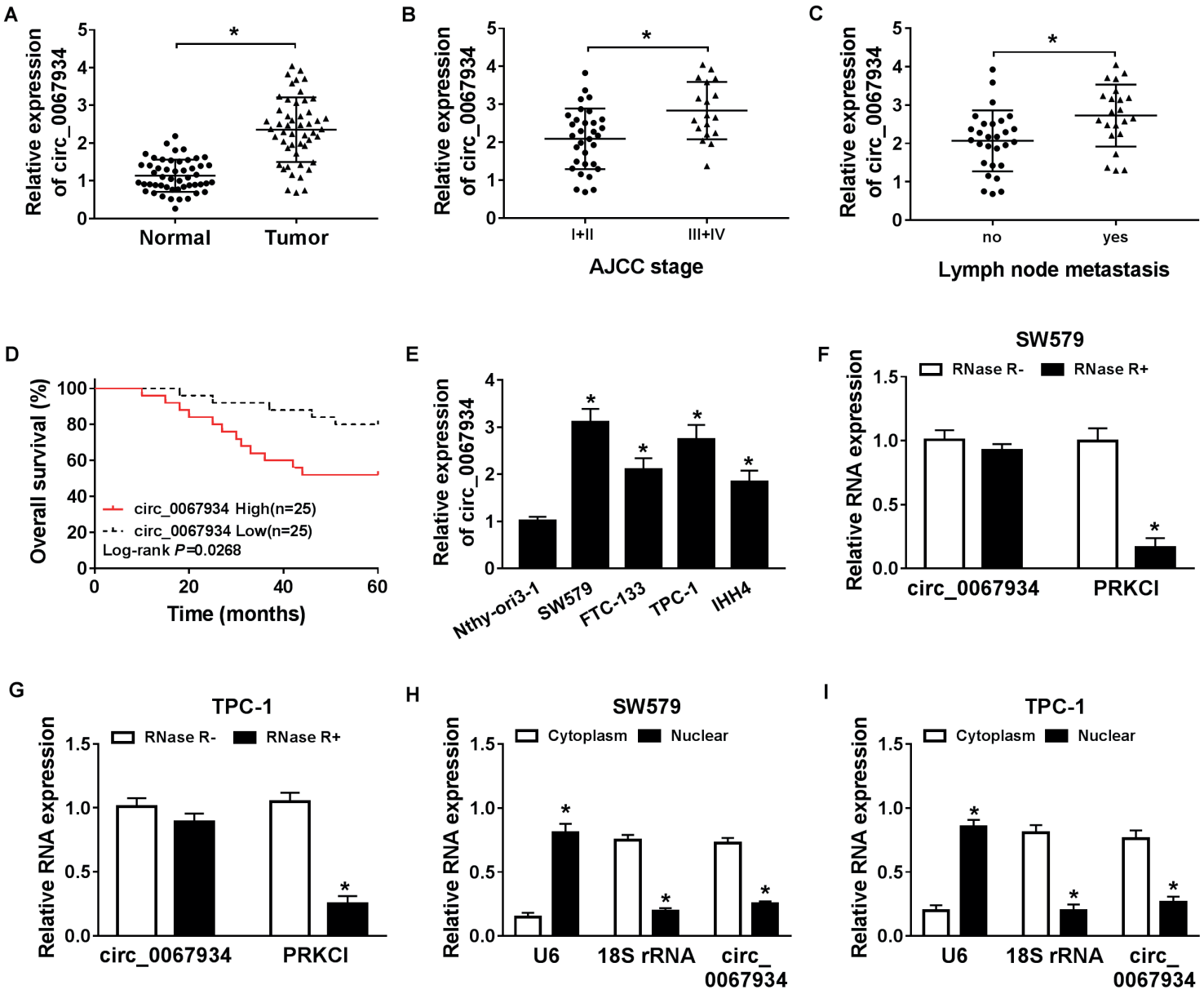

Figure 1. Expression pattern and characterization of circ_0067934 in TC. **A,** QRT-PCR was employed for the evaluation of the level of circ_0067934 in 50 paired TC tissues and neighboring normal tissues. **B,** The expression of circ_0067934 in TC tissues based on the AJCC grade was detected with qRT-PCR. **C,** The expression of circ_0067934 in TC tissues with or without lymph node metastasis was analyzed using qRT-PCR. **D,** Kaplan-Meier analysis was used to assess the survival rate of TC patients with high or low expression of circ_0067934 (divided base on median level). **E,** QRT-PCR was conducted to detect the expression of circ_0067934 in TC cell lines (SW579, TPC-1, FTC-133, and IHH4) and Nthy-ori3-1 cells. **F,** and **G,** QRT-PCR analysis of PRKCI mRNA and circ_0067934 levels in SW579 and TPC-1 cells with or without RNase R treatment. **H,** and **I,** The abundance of circ_0067934 in the cytoplasm or nucleus of SW579 and TPC-1 cells was analyzed using qRT-PCR. *$p < 0.05$.

in contrast to neighboring normal tissues (Figure 3H). Pearson correction analysis revealed that the relationship between circ_0067934 and miR-1304 expression was negatively correlated in TC tissues (Figure 3I). In sum, these results indicated that circ_0067934 could negatively regulate miR-1304 expression in TC cells.

MiR-1304 Inhibition Overturned Circ_0067934-Reduction-Mediated Proliferation, Apoptosis, Migration, and Invasion of TC Cells

Considering the above results, we further probed into whether circ_0067934 exerted its role *via* regulating miR-1304 expression. In the first place, the expression of miR-1304 in SW579 and TPC-1 cells transfected with anti-NC or anti-miR-1304 was detected through qRT-PCR. Compared to the control group, the expression of miR-1304 in SW579 and TPC-1 cells transfected with anti-miR-1304 was drastically restrained, indicating that anti-miR-1304 could be used in subsequent studies (Figure 4A). Afterward, the effects of miR-1304 inhibition on proliferation, apoptosis, migration, and invasion of circ_0067934-reduced SW579 and TPC-1 cells were further explored. CCK-8 assay presented that the repression of proliferation of SW579 and TPC-1 cells caused by circ_0067934 downregulation was reversed by miR-1304 deletion (Figure 4B and 4C). Furthermore, flow cytometry assay uncovered that downregulated miR-1304 expression recovered the facilitation of apoptosis of SW579 and TPC-1 cells following circ_0067934 restraint (Figure 4D). Also, transwell assay showed that the repressive effects of circ_0067934 knockdown on migration and invasion of SW579 and TPC-1 cells were abolished by miR-1304 suppression (Figure 4E and 4F). Taken together, these data indicated that circ_0067934 mediated proliferation, apoptosis, migration, and invasion of TC cells by regulating miR-1304 expression.

CXCR1 Acted as a Target of MiR-1304 in TC Cells In Vitro

Based on the above results, we further investigated whether circ_0067934 could serve as a competing endogenous RNA of miR-1304 to regulate the expression of the target of miR-1304 in TC cells. Firstly, the TargetScan database (http://www.targetscan.org/vert_72/) was carried out to predict the target of miR-1304. As presented in Figure 5A, CXCR1 3' UTR contained putative miR-1304 binding sites. Afterward, we established the Luciferase reporter vectors of CXCR1 3'UTR-WT and CXCR1 3'UTR-MUT for the verification of the binding sites between CXCR1 and miR-1304. Subsequently, Dual-Luciferase reporter assay implied that miR-1304 upregulation strikingly reduced the Luciferase activity of CXCR1 3'UTR-WT compared with the control group, while the Luciferase activity of CXCR1 3'UTR-MUT was not affected (Figure 5B and 5C). Moreover, the mRNA and protein levels of CXCR1 in TC cells were detected with qRT-PCR or Western blot analysis. The results revealed that CXCR1 mRNA and protein levels were dramatically upregulated in SW579 and TPC-1 cells in comparison to the Nthy-ori3-1 cells (Figure 5D and 5E). Furthermore, the effects of miR-1304 on CXCR1 mRNA and protein expression were assessed. Results of qRT-PCR and Western blot analysis indicated that the expression levels of CXCR1 mRNA and protein were apparently reduced in miR-1304-elevated SW579 and TPC-1 cells, but CXCR1 mRNA and protein levels were distinctly augmented in miR-1304-inhibited SW579 and TPC-1 cells (Figure 5F-5H). Besides, qRT-PCR and Western blot analysis manifested that a distinct upregulation of CXCR1 mRNA and protein was existed in TC tissues compared to that in the neighboring normal tissues (Figure 5I and 5J). Pearson correction analysis exhibited a negative correlation between CXCR1 mRNA and miR-1304 expression in TC tissues (Figure 5K). Therefore, these results proved that miR-1304 negatively regulated CXCR1 expression in TC cells.

CXCR1 Augmentation Recovered Circ_0067934-Knockdown-Mediated Proliferation, Apoptosis, Migration and Invasion in TC Cells In Vitro

Given that miR-1304 could negatively regulate CXCR1 expression, we next explored the relationship between CXCR1 and circ_0067934 in TC cells. In the first, the mRNA and protein expression levels of CXCR1 in SW579 and TPC-1 cells transfected with Lv-NC or Lv-CXCR1 were analyzed with qRT-PCR or Western blot analysis. We observed that the mRNA and protein expression levels of CXCR1 were distinctly increased in SW579 and TPC-1 cells transfected with Lv-CXCR1 relative to the control group (Figure 6A and 6B). Subsequently, the si-NC, si-circ_0067934#1, si-circ_0067934#1+Lv-NC or si-circ_0067934#1+Lv-CXCR1 was individually transfected into SW579 and TPC-1 cells to

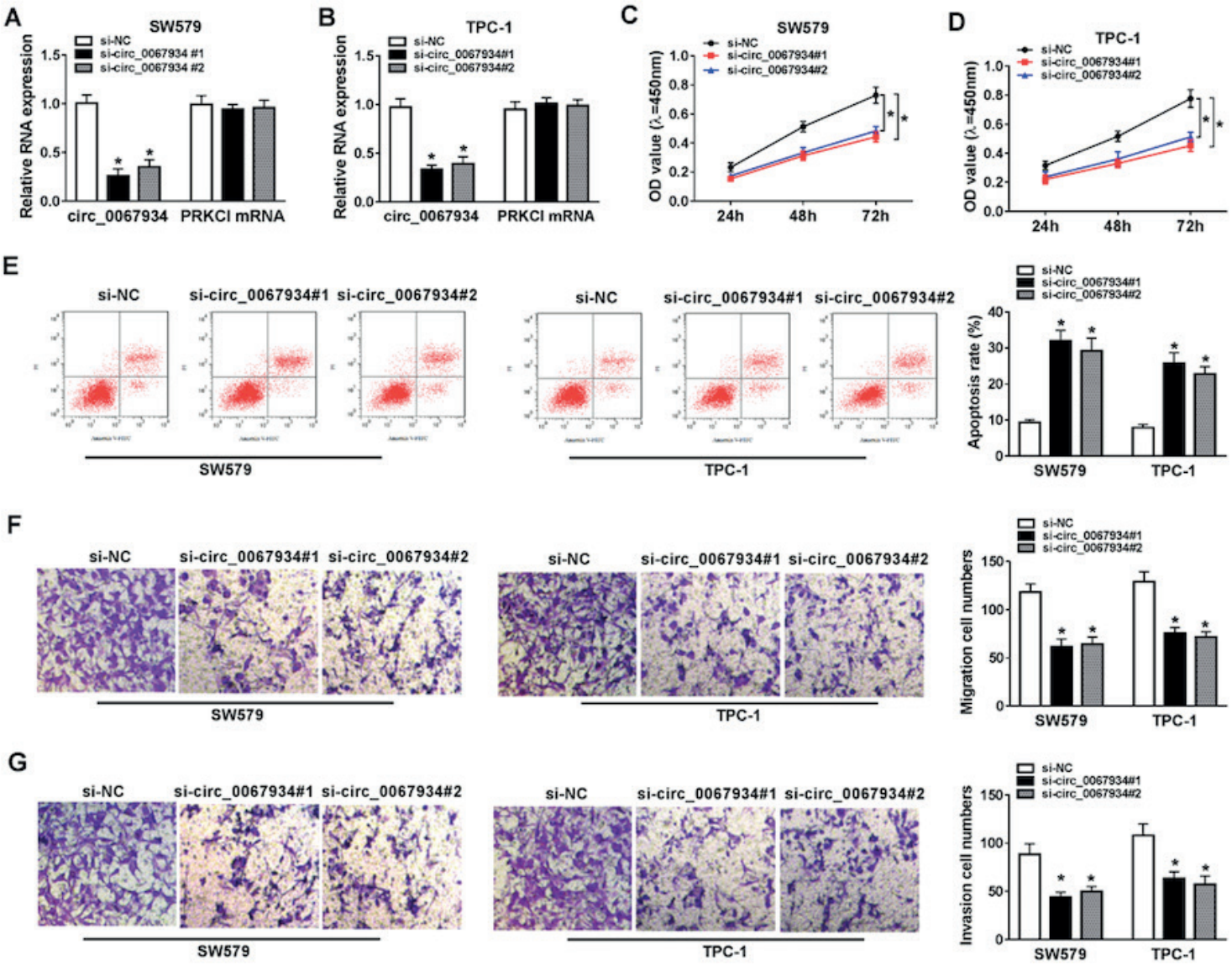

Figure 2. Effects of circ_0067934 downregulation on cell proliferation, apoptosis, migration, and invasion in TC cells. **A-G,** SW579 and TPC-1 cells were transfected with si-NC, si-circ_0067934#1 or si-circ_0067934#2. **A,** and **B,** QRT-PCR analysis was applied for the measurement of circ_0067934 expression level in SW579 and TPC-1 cells. **C,** and **D,** CKK8 assay was used for the determination of the proliferation of SW579 and TPC-1 cells. **E,** Flow cytometry assay was applied to assess the apoptosis of SW579 and TPC-1 cells. **F,** and **G (x300).** Transwell assay was performed for the evaluation of the migration and invasion of SW579 and TPC-1 cells. *$p < 0.05$.

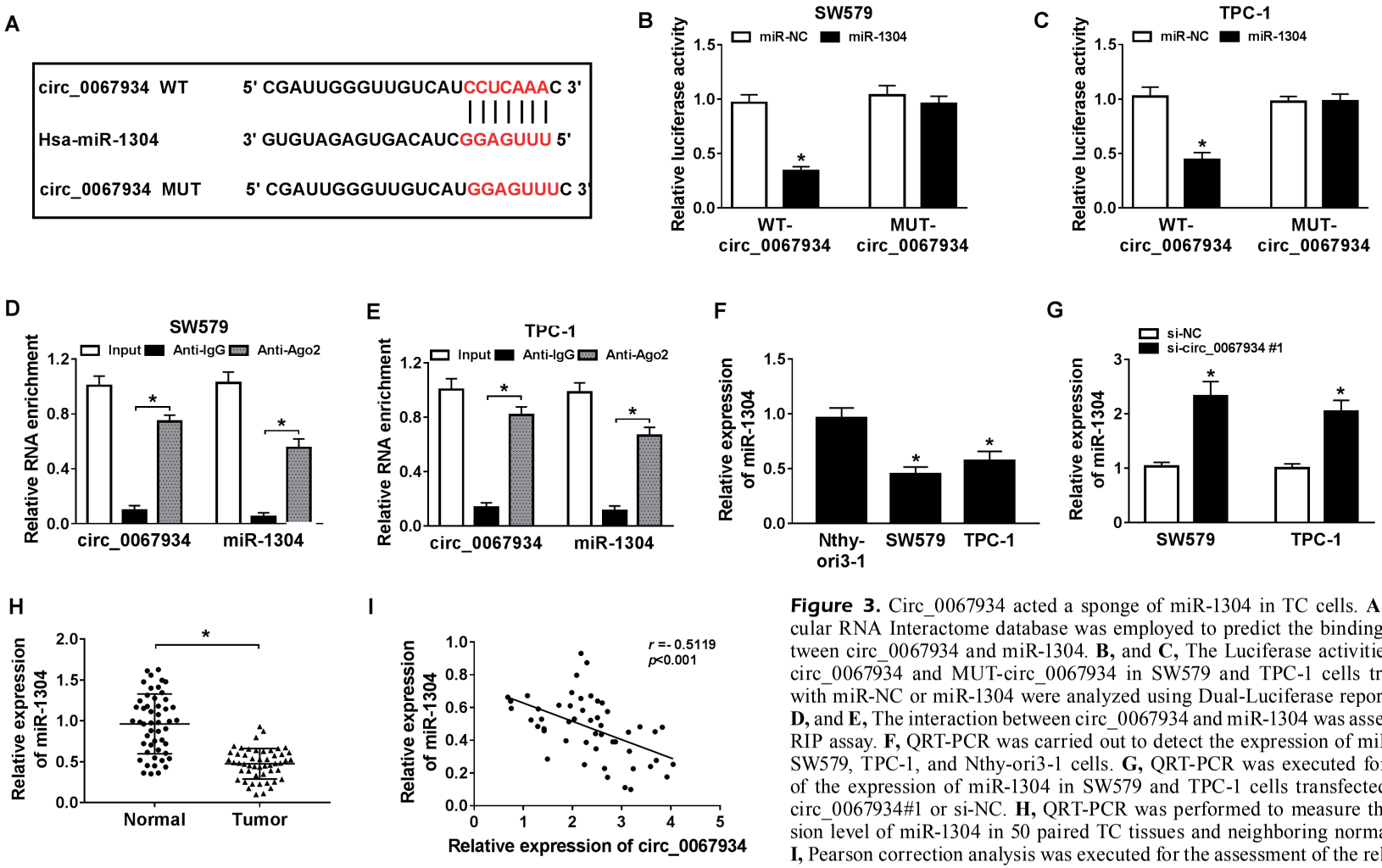

Figure 3. Circ_0067934 acted a sponge of miR-1304 in TC cells. **A,** The circular RNA Interactome database was employed to predict the binding sites between circ_0067934 and miR-1304. **B,** and **C,** The Luciferase activities of WT-circ_0067934 and MUT-circ_0067934 in SW579 and TPC-1 cells transfected with miR-NC or miR-1304 were analyzed using Dual-Luciferase reporter assay. **D,** and **E,** The interaction between circ_0067934 and miR-1304 was assessed with RIP assay. **F,** QRT-PCR was carried out to detect the expression of miR-1304 in SW579, TPC-1, and Nthy-ori3-1 cells. **G,** QRT-PCR was executed for analysis of the expression of miR-1304 in SW579 and TPC-1 cells transfected with si-circ_0067934#1 or si-NC. **H,** QRT-PCR was performed to measure the expression level of miR-1304 in 50 paired TC tissues and neighboring normal tissues. **I,** Pearson correction analysis was executed for the assessment of the relationship between circ_0067934 and miR-1304 expression in TC tissues. *$p < 0.05$.

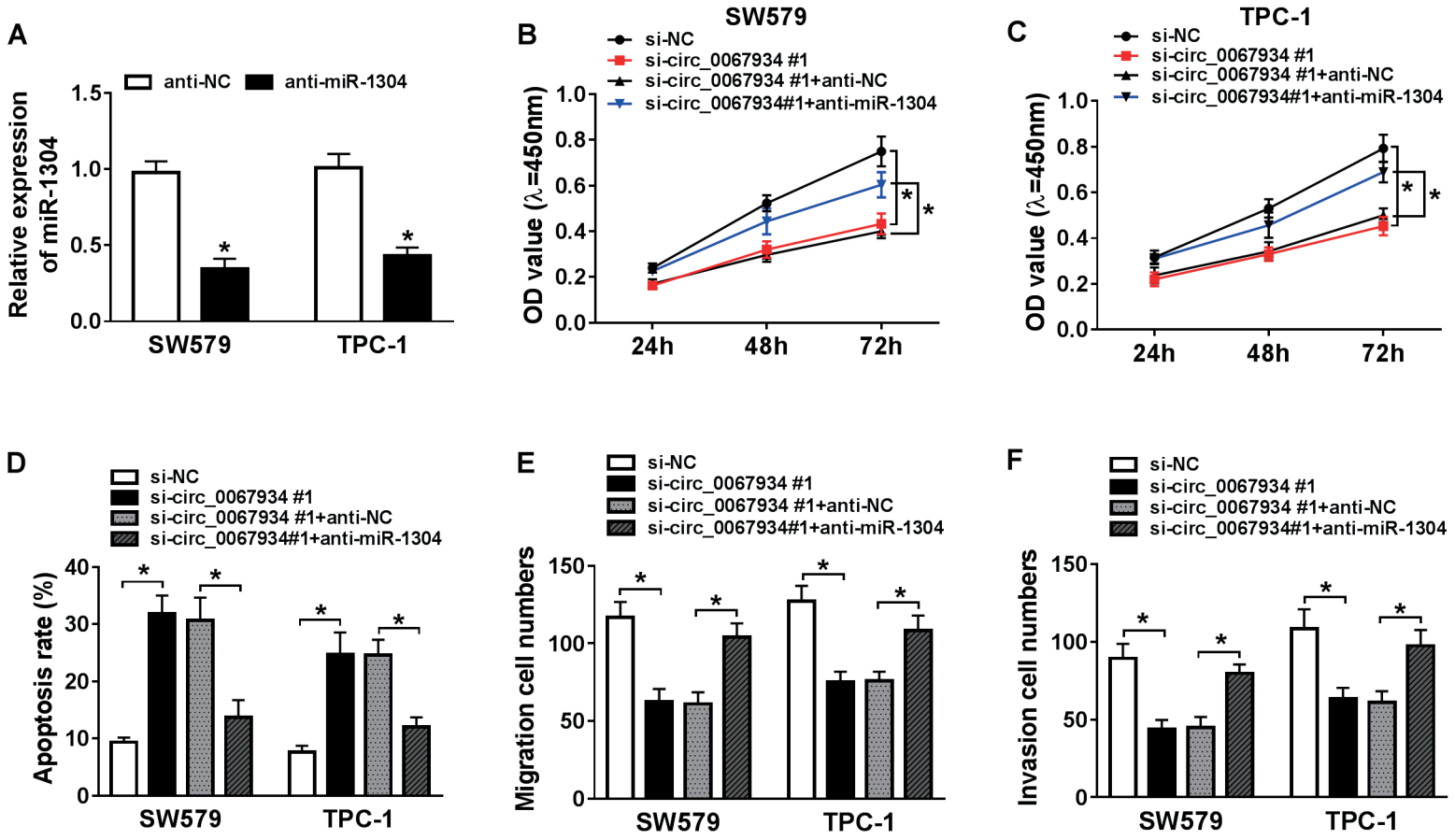

Figure 4. Circ_0067934 worked *via* regulating miR-1304 expression. **A,** QRT-PCR was employed to analyze miR-1304 expression in SW579 and TPC-1 cells transfected with anti-NC or anti-miR-1304. **B-F,** SW579 and TPC-1 cells were transfected with si-NC, si-circ_0067934#1, si-circ_0067934#1+anti-NC or si-circ_0067934#1+anti-miR-1304. **B,** and **C,** The proliferation of SW579 and TPC-1 cells was evaluated with CCK-8 assay. **D,** The apoptosis rate of SW579 and TPC-1 cells was analyzed using flow cytometry assay. **E,** and **F,** The migration and invasion of SW579 and TPC-1 cells were detected *via* transwell assay. *$p < 0.05$.

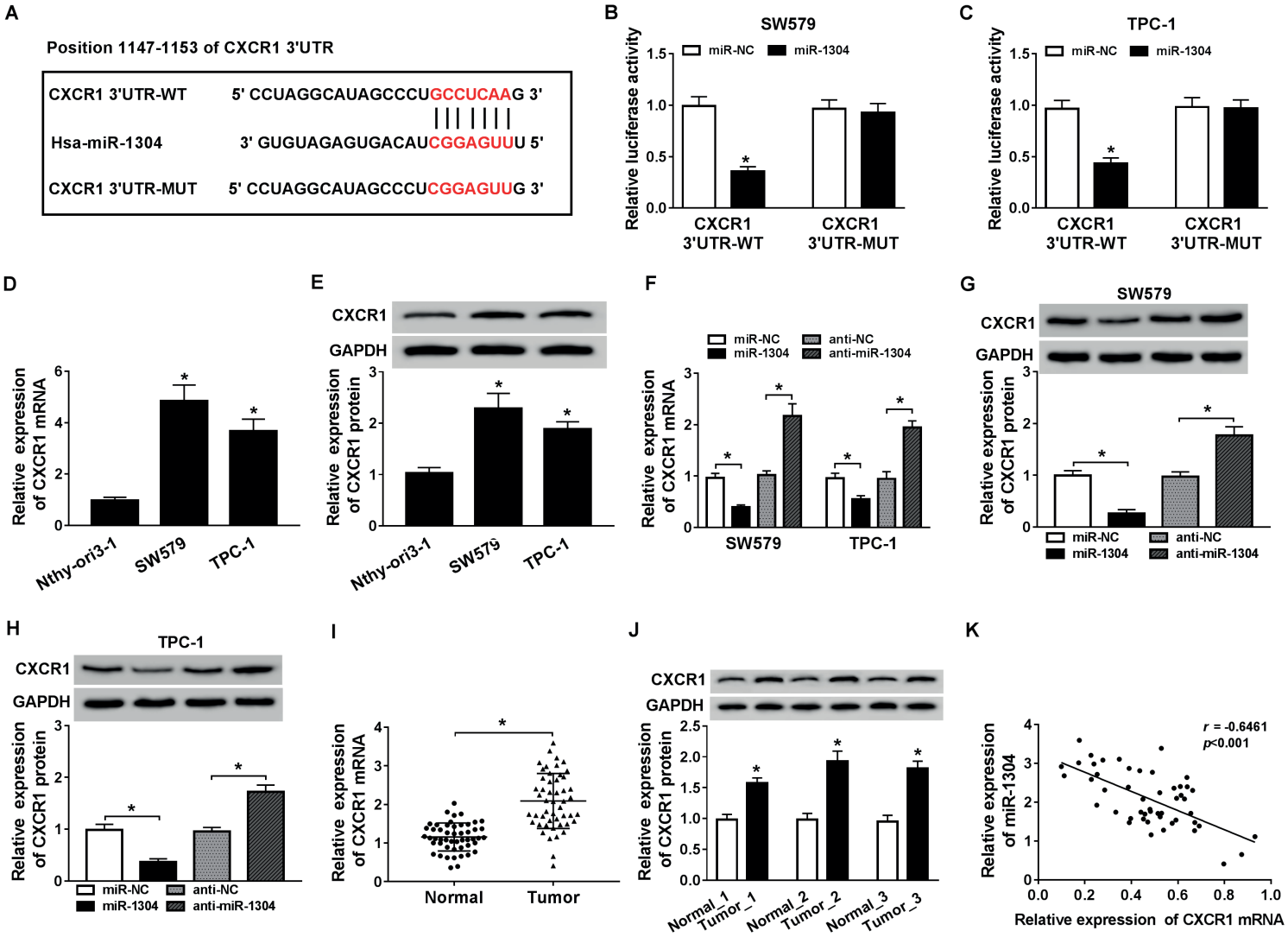

Figure 5. MiR-1304 targeted CXCR1 in TC cells. **A,** The potential binding sites of miR-1304 in the 3'UTR of CXCR1 were predicted with TargetScan database. **B,** and **C,** Dual-Luciferase reporter assay was executed for the detection of the Luciferase activity in SW579 and TPC-1 cells co-transfected CXCR1 3'UTR-WT or CXCR1 3'UTR-MUT and miR-NC or miR-1304. **D,** and **E,** The mRNA and protein levels of CXCR1 in Nthy-ori3-1, SW579, and TPC-1 cells were detected with qRT-PCR or Western blot analysis. **F-H,** QRT-PCR or Western blot analysis was conducted to analyze the mRNA and protein expression levels of CXCR1 in SW579 and TPC-1 cells transfected with miR-NC, miR-1304, anti-NC or anti-miR-1304. **I,** and **J,** QRT-PCR or Western blot analysis was carried out for the assessment of the mRNA and protein expression levels of CXCR1 in 50 paired TC tissues and neighboring normal tissues. **K,** The relationship between miR-1304 and CXCR1 expression in TC tissues was assessed with Pearson correction analysis. *$p < 0.05$.

probe into cell proliferation, apoptosis, migration, and invasion. CCK-8 assay revealed that reinforced CXCR1 expression could rescue the inhibition of proliferation of SW579 and TPC-1 cells caused by decreasing of circ_0067934 (Figure 6C and 6D). Flow cytometry assay indicated that CXCR1 elevation overturned the change of cell apoptosis induced by circ_0067934 downregulation (Figure 6E). Also, transwell assay implied that the enhancement of CXCR1 could abolish circ_0067934-exhaustion-mediated repression of migration and invasion of SW579 and TPC-1 cells (Figure 6F and 6G). Together, these results suggested that circ_0067934 mediated the proliferation, apoptosis, migration, and invasion of TC cells *via* CXCR1.

Circ_0067934 Regulated CXCR1 Expression Via Sponging MiR-1304 in TC Cells

Considering that circ_0067934 played its role in TC cells through miR-1304 and CXCR1, we further speculated that circ_0067934 could regulate CXCR1 expression by sponging miR-1304. To verify this speculation, the mRNA and protein expression levels of CXCR1 in SW579 and TPC-1 cells transfected with si-NC, si-circ_0067934#1, si-circ_0067934#1+anti-NC or si-circ_0067934#1+anti-miR-1304 were detected by qRT-PCR or Western blot analysis. The results showed that the mRNA and protein expression levels of CXCR1 were drastically reduced in circ_0067934-inhibited SW579 and TPC-1 cells, while this downregulation was abrogated by the repression of miR-1304 (Figure 7A-7D). Therefore, these data proved that circ_0067934 could regulate CXCR1 expression by binding to miR-1304 in TC cells.

Circ_0067934 Silencing Retarded Tumor Growth In Vivo

To further probe into the role of circ_0067934 in TC, we injected stable lentiviral-mediated circ_0067934-decreased SW579 cells or sh-NC-transfected SW579 cells into nude mice for the establishment of the mouse xenograft models. The size of tumor was measured in each group every 7 days. As displayed in Figure 8A and 8B, the size and weight of the tumor were remarkably reduced in the sh-circ_0067934 group compared with the sh-NC group. Moreover, the results of qRT-PCR proved that circ_0067934 was strikingly downregulated and miR-1304 was significantly enhanced in the tumor of mice injected with sh-circ_0067934 than the control group (Figure 8C). Also, qRT-PCR and Western blot analysis revealed that the expression of CXCR1 mRNA and protein were evidently inhibited in lentiviral-mediated sh-circ_0067934 group in comparison with the sh-NC group (Figure 8D and 8E). Above all, these results indicated that circ_0067934 silencing could suppress TC tumor growth *in vivo*.

Discussion

Great progress has been made in the diagnosis and treatment of TC, but tumor recurrence and metastasis, as well as drug resistance and severe toxicity during treatment, are inevitable[22,23]. Hence, it is indispensable to develop new molecular targets and therapeutic approaches for the improvement of the prognosis of TC.

Aurtors[24-26] revealed that circRNAs exerted a key regulatory role in tumor progression, including TC. Circ_0067934 was reported to be an oncogene in various cancers[10,27,28]. For instance, enhanced circ_0067934 expression was correlated with lymph node metastasis, advanced stage, and poor prognosis in cervical cancer patients, and circ_0067934 silencing impeded the colony formation, invasion, migration, proliferation, and epithelial-mesenchymal transition (EMT) of cervical cancer cells[10]. Xia et al[8] revealed that circ_0067934 was strikingly elevated in esophageal squamous cell cancer tissues and cells, and downregulated expression of circ_0067934 clogged the progression of cell cycle and repressed the migration and proliferation of esophageal squamous cell cancer cells. The previous study claimed that a conspicuous augmentation of circ_0067934 was discovered in TC tissues and cells and circ_0067934 inhibition suppressed the migration, proliferation, and invasion of TC cells[11]. Consistent with several data of the above studies, we discovered that circ_0067934 was upregulated in TC tissues and cells and upregulated circ_0067934 was correlated with AJCC grade, lymph node metastasis, and survival rate in patients with TC. Moreover, circ_0067934 knockdown induced apoptosis and blocked proliferation, migration, and invasion of TC cells *in vitro*, as well as repressed tumor growth *in vivo*. Therefore, the results in this research disclosed that circ_0067934 exerted a carcinogenic role in TC.

Many researches[9,10] have stated that circ_0067934 could exert its biological function as a sponge of miRNA. For example, Hu et al[10] reported that

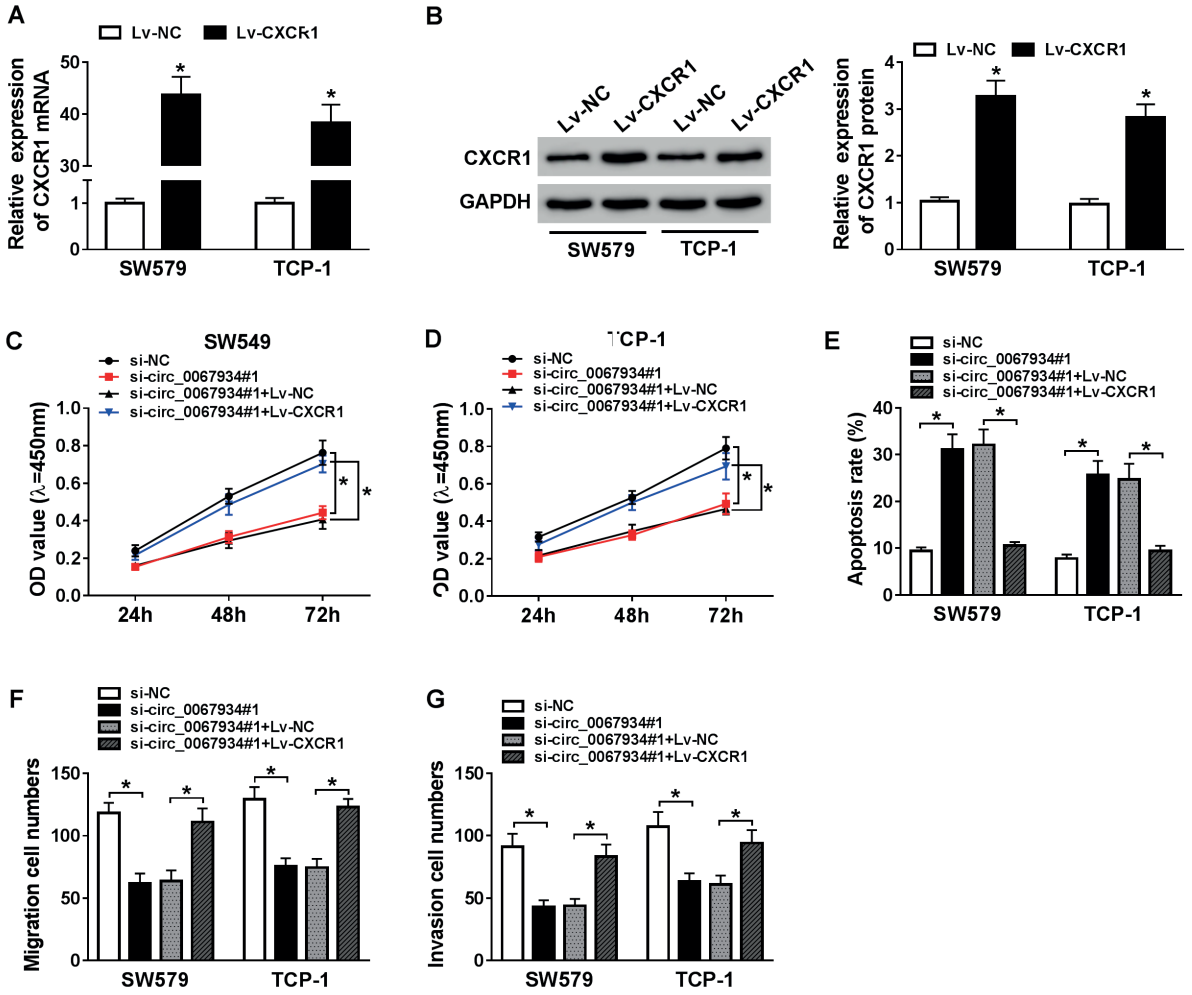

Figure 6. CXCR1 reversed the effects of circ_0067934 knockdown on TC cells. **A,** and **B,** The CXCR1 mRNA and protein expression of in SW579 and TPC-1 cells transfected with Lv-NC or Lv-CXCR1 were analyzed with qRT-PCR or Western blot analysis. **C-G,** SW579 and TPC-1 cells were transfected with si-NC, si-circ_0067934#1, si-circ_0067934#1+Lv-NC or si-circ_0067934#1+Lv-CXCR1. **C,** and **D,** The proliferation of SW579 and TPC-1 cells was evaluated with CCK8 assay. **E,** The apoptosis rate of SW579 and TPC-1 cells was assessed using flow cytometry assay. **F,** and **G,** Transwell assay was performed to determine the migration and invasion of SW579 and TPC-1 cells. *$p < 0.05$.

circ_0067934 could directly interact with miR-545 in cervical cancer cells. Hence, we predicted that circ_0067934 might be a sponge of miR-1304 using a circular RNA Interactome database. Li et al[15] revealed that miR-1304 was specially down-regulated in non-small cell lung cancer tissues and cells and miR-1304 enhancement facilitated cell apoptosis and restrained cell colony formation and viability in non-small cell lung cancer cells. Pan et al[14] claimed that the reduction of miR-1304 caused by circRNA circ_0025003 upregulation could repress cell apoptosis and expedite cell proliferation, invasion, and migration in papillary TC cells. In this study, we also proved that miR-1304 was apparently decreased in TC tissues and cells. Moreover, miR-1304 was negatively regulated by circ_0067934 and silenced expression of miR-1304 could reverse the effects of circ_0067934 inhibition-mediated cell proliferation, apoptosis, migration, and invasion in TC cells. Some our results were consistent with the above studies, indicating that miR-1304 played an anti-tumor role in TC.

Afterward, we further demonstrated that CXCR1 was a target of miR-1304 by Dual-Luciferase reporter assay. Moreover, we discovered that CXCR1 was overexpressed and inversely correlat-

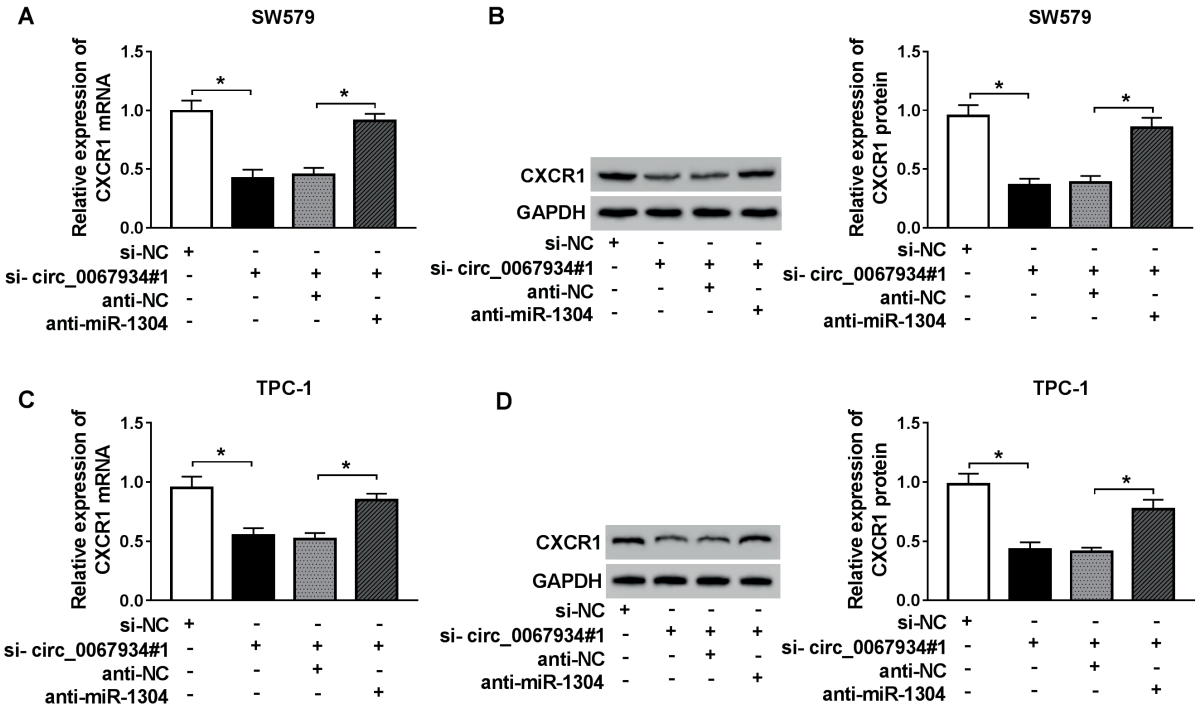

Figure 7. Circ_0067934 bounds to miR-1304 to regulate CXCR1 expression in TC cells. **A-D,** The mRNA and protein levels of CXCR1 in SW579 (**A** and **B**) and TPC-1 (**C** and **D**) cells transfected with si-NC, si-circ_0067934#1, si-circ_0067934#1+anti-NC or si-circ_0067934#1+anti-miR-1304 were detected by qRT-PCR or Western blot analysis. *$p < 0.05$.

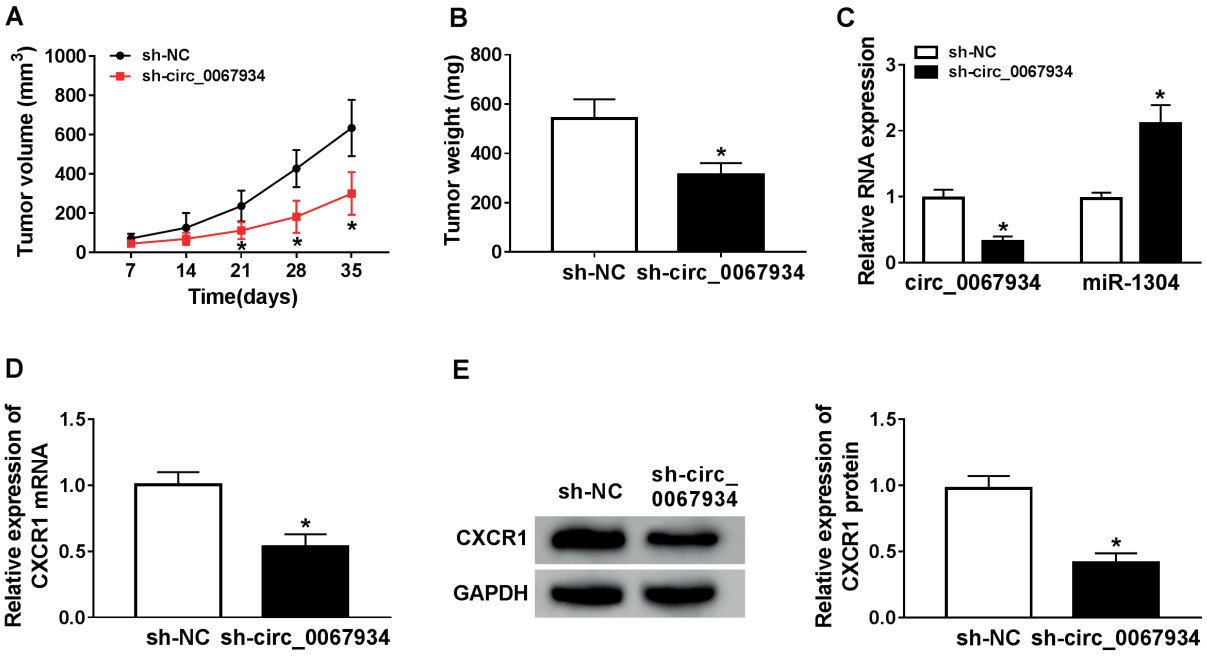

Figure 8. Inhibition of circ_0067934 constrained tumor growth *in vivo*. **A,** Tumor volume was measured every 7 days with a digital caliper for 35 days. **B,** Circ_0067934 silencing reduced tumor weight in nude mice. **C,** QRT-PCR was conducted to evaluate the expression of circ_0067934 and miR-1304 in xenograft tumors. **D,** and **E,** QRT-PCR and Western blot analysis were performed to assess the mRNA and protein expression of CXCR1 in xenograft tumors. *$p < 0.05$.

ed with miR-1304 expression in TC tissues. Furthermore, both the promotion of apoptosis and the repression of proliferation, migration, and invasion of TC cells caused by circ_0067934 knockdown were recovered by CXCR1 enhancement. Besides, Chen et al[29] claimed that the poor overall survival of metastatic renal cell cancer was associated with high expression of CXCR1. Li et al[30] proved that CXCR1 overexpression accelerated cell invasion and migration in gastric cancer cells. Moreover, upregulation of miR-215-3p targeting CXCR1 could increase colorectal cancer sensitivity to 5-fluorouracil[19]. Also, Fang et al[31] pointed out that the metastatic ability of papillary TC could be boosted by the interaction of CXCR1/2 and CXCL8. In agreement with the above reports, the results in this research stated that CXCR1 was an oncogene in TC. Additionally, we proved that circ_0067934 could regulate CXCR1 expression *via* binding to miR-1304 in TC cells by Western blot analysis. From all these data, we concluded that circ_0067934 silencing downregulated CXCR1 expression *via* sponging miR-1304, thus expediting apoptosis and repressing proliferation, migration, and invasion of TC cells.

Conclusions

Circular RNA circ_0067934 was augmented in TC tissues and cells. Notably, the downregulation of circ_0067934 contributed to apoptosis and impeded proliferation, migration, and invasion of TC cells *via* sponging miR-1304 and reducing CXCR1 expression. Hence, this research contributed to better comprehending the molecular mechanism of TC progression, providing a promising circRNA-targeted therapy for TC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Zaballos MA, Santisteban P. Key signaling pathways in thyroid cancer. J Endocrinol 2017; 235: R43-R61.
2) Siegel RL, Miller KD. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.
3) Fagin JA, Wells SA, Jr. Biologic and clinical perspectives on thyroid cancer. N Engl J Med 2016; 375: 1054-1067.
4) Regalbuto C, Frasca F, Pellegriti G, Malandrino P, Marturano I, Di Carlo I, Pezzino V. Update on thyroid cancer treatment. Future Oncol 2012; 8: 1331-1348.
5) Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S. CircRNA biogenesis competes with pre-mRNA splicing. Mol Cell 2014; 56: 55-66.
6) Meng X, Li X, Zhang P, Wang J, Zhou Y, Chen M. Circular RNA: an emerging key player in RNA world. Brief Bioinform 2017; 18: 547-557.
7) Kristensen LS, Hansen TB, Veno MT, Kjems J. Circular RNAs in cancer: opportunities and challenges in the field. Oncogene 2018; 37: 555-565.
8) Xia W, Qiu M, Chen R, Wang S, Leng X, Wang J, Xu Y, Hu J, Dong G, Xu PL, Yin R. Circular RNA has_circ_0067934 is upregulated in esophageal squamous cell carcinoma and promoted proliferation. Sci Rep 2016; 6: 35576.
9) Zhu Q, Lu G, Luo Z, Gui F, Wu J, Zhang D, Ni Y. CircRNA circ_0067934 promotes tumor growth and metastasis in hepatocellular carcinoma through regulation of miR-1324/FZD5/Wnt/beta-catenin axis. Biochem Biophys Res Commun 2018; 497: 626-632.
10) Hu C, Wang Y, Li A, Zhang J, Xue F, Zhu L. Overexpressed circ_0067934 acts as an oncogene to facilitate cervical cancer progression via the miR-545/EIF3C axis. J Cell Physiol 2019; 234: 9225-9232.
11) Wang H, Yan X, Zhang H, Zhan X. CircRNA circ_0067934 overexpression correlates with poor prognosis and promotes thyroid carcinoma progression. Med Sci Monit 2019; 25: 1342-1349.
12) Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11: 597-610.
13) Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.
14) Pan Y, Xu T, Liu Y, Li W, Zhang W. Upregulated circular RNA circ_0025033 promotes papillary thyroid cancer cell proliferation and invasion via sponging miR-1231 and miR-1304. Biochem Biophys Res Commun 2019; 510: 334-338.
15) Li CG, Pu MF, Li CZ, Gao M, Liu MX, Yu CZ, Yan H, Peng C, Zhao Y, Li Y, Ma ZL, Qi XM, Wang YZ, Miao LL, Ren J. MicroRNA-1304 suppresses human non-small cell lung cancer cell growth in vitro by targeting heme oxygenase-1. Acta Pharmacol Sin 2017; 38: 110-119.
16) Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases. Theranostics 2017; 7: 1543-1588.
17) Liu Q, Li A, Tian Y, Wu JD, Liu Y, Li T, Chen Y, Han X, Wu K. The CXCL8-CXCR1/2 pathways in cancer. Cytokine Growth Factor Rev 2016; 31: 61-71.
18) Han XG, Du L, Qiao H, Tu B, Wang YG, Qin A, Dai KR, Fan QM, Tang TT. CXCR1 knockdown improves the sensitivity of osteosarcoma to cisplatin. Cancer Lett 2015; 369: 405-415.

19) Li XW, Qiu SJ, Zhang X. Overexpression of miR-215-3p sensitizes colorectal cancer to 5-fluorouracil induced apoptosis through regulating CXCR1. Eur Rev Med Pharmacol Sci 2018; 22: 7240-7250.

20) Wang J, Hu W, Wu X, Wang K, Yu J, Luo B, Luo G, Wang W, Wang H, Li J, Wen J. CXCR1 promotes malignant behavior of gastric cancer cells *in vitro* and in vivo in AKT and ERK1/2 phosphorylation. Int J Oncol 2016; 48: 2184-2196.

21) Tang C, Yang L, Wang N, Li L, Xu M, Chen GG, Liu ZM. High expression of GPER1, EGFR and CXCR1 is associated with lymph node metastasis in papillary thyroid carcinoma. Int J Clin Exp Pathol 2014; 7: 3213-3223.

22) Wagle N, Grabiner BC, Van Allen EM, Amin-Mansour A, Taylor-Weiner A, Rosenberg M, Gray N, Barletta JA, Guo Y, Swanson SJ, Ruan DT, Hanna GJ, Haddad RI, Getz G, Kwiatkowski DJ, Carter SL, Sabatini DM, Janne PA, Garraway LA, Lorch JH. Response and acquired resistance to everolimus in anaplastic thyroid cancer. N Engl J Med 2014; 371: 1426-1433.

23) Hempel JM, Kloeckner R, Krick S, Pinto Dos Santos D, Schadmand-Fischer S, Boessert P, Bisdas S, Weber MM, Fottner C, Musholt TJ, Schreckenberger M, Miederer M. Impact of combined FDG-PET/CT and MRI on the detection of local recurrence and nodal metastases in thyroid cancer. Cancer Imaging 2016; 16: 37.

24) Wei H, Pan L, Tao D, Li R. Circular RNA circZFR contributes to papillary thyroid cancer cell proliferation and invasion by sponging miR-1261 and facilitating C8orf4 expression. Biochem Biophys Res Commun 2018; 503: 56-61.

25) Bi W, Huang J, Nie C, Liu B, He G, Han J, Pang R, Ding Z, Xu J, Zhang J. CircRNA circRNA_102171 promotes papillary thyroid cancer progression through modulating CTNNBIP1-dependent activation of β-catenin pathway. J Exp Clin Cancer Res 2018; 37: 275.

26) Li X, Tian Y, Hu Y, Yang Z, Zhang L, Luo J. CircNUP214 sponges miR-145 to promote the expression of ZEB2 in thyroid cancer cells. Biochem Biophys Res Commun 2018; 507: 168-172.

27) Xin J, Zhang XY, Sun DK, Tian LQ, Xu P. Up-regulated circular RNA hsa_circ_0067934 contributes to glioblastoma progression through activating PI3K-AKT pathway. Eur Rev Med Pharmacol Sci 2019; 23: 3447-3454.

28) Wang J, Li H. CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC. Eur Rev Med Pharmacol Sci 2018; 22: 3053-3060.

29) Chen X, Liu L, Wang J, Lin Z, Xiong Y, Qu Y, Wang Z, Yang Y, Guo J, Xu J. CXCR1 expression predicts benefit from tyrosine kinase inhibitors therapy in patients with metastatic renal cell carcinoma. Urol Oncol 2018; 36: 242.e215-242.e21.

30) Li Z, Wang Y, Dong S, Ge C, Xiao Y, Li R, Ma X, Xue Y, Zhang Q, Lv J, Tan Q, Zhu Z, Song X, Tan J. Association of CXCR1 and 2 expressions with gastric cancer metastasis in ex vivo and tumor cell invasion in vitro. Cytokine 2014; 69: 6-13.

31) Fang W, Ye L, Shen L, Cai J, Huang F, Wei Q, Fei X, Chen X, Guan H, Wang W, Li X, Ning G. Tumor-associated macrophages promote the metastatic potential of thyroid papillary cancer by releasing CXCL8. Carcinogenesis 2014; 35: 1780-1787.