circRPS28 (hsa_circ_0049055) is a novel contributor for papillary thyroid carcinoma by regulating cell growth and motility via functioning as ceRNA for miR-345-5p to regulate frizzled family receptor 8 (FZD8)

Yu Mao, Yajie Huo, Jing Li, Yanli Zhao, Yuan Wang, Ling Sun and Zhiqiang Kang

Department of Endocrinology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, 450007, China

Abstract. Circular RNA 40S ribosomal protein S28 (circRPS28; hsa_circ_0049055) is upregulated in papillary thyroid carcinoma (PTC) patients. However, its role remained uncovered in the progression of PTC. Above all, expression of circRPS28 was determined in PTC samples by real-time quantitative PCR and circRPS28 was highly expressed in tumor tissues and cells. Besides, circRPS28 was predominantly distributed in the cytoplasm. Functional experiments were launched using colony formation assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 5-ethynyl-2-deoxyuridine (EdU) assay, transwell assays, scratch wound assay, and flow cytometry. As a result, blocking circRPS28 restrained PTC cell viability, EdU positive cell rate, colony formation number, wounding healing rate, and numbers of migration and invasion cells, accompanied with apoptosis rate promotion. These effects paralleled with low B-cell lymphoma (Bcl)-2 level and high Bcl-2-associated X protein (Bax), matrix metalloproteinase-2 (MMP2), and MMP9 levels, as analyzed by western blotting. Overexpressing microRNA (miR)-345-5p exerted similar roles to circRPS28 silencing. Notably, dual-luciferase reporter assay and RNA immunoprecipitation confirmed the target relationship between circRPS28 and miR-345-5p, miR-345-5p and frizzled family receptor 8 (FZD8). Downregulating miR-345-5p abrogated effects of circRPS28 blockage in PTC cells, and restoring FZD8 counteracted miR-345-5p roles, either. Furthermore, xenograft tumor model was established in mice, and exhausting circRPS28 delayed the growth of PTC cells in vivo by regulating miR-345-5p and FZD8. In conclusion, we demonstrated that blocking circRPS28 and/or promoting miR-345-5p suppressed PTC cell growth and motility via regulating FZD8. This study might suggest a novel circRPS28/miR-345-5p/FZD8 competing endogenous RNA pathway in PTC.

Key words: circRPS28, miR-345-5p, Frizzled family receptor 8 (FZD8), Papillary thyroid carcinoma

PAPILLARY THYROID CARCINOMA (PTC) is the most common subtype of thyroid cancer [1]. It is defined as a malignant epithelial tumor with evidence of follicular differentiation and a series of specific nuclear features [2]. BRAFV600E is the most common mutation in PTC [3], and epigenetic alteration is also molecular pathogenesis and mechanism of PTC [4].

Transcriptomic signature associated with carcinogenesis and aggressiveness of PTC has been uncovered, including differentially expressed genes, microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) [5]. Dysregulated circRNA expression is considered to be correlated with clinicopathological characteristics of PTC patients [6, 7]. circRNAs are derived from the same host genes and comprise at least 10% of transcripts accumulating from their loci [8]. Moreover, circRNAs are more stable and abundant than associated linear miRNAs in vivo [9]. Transcriptome-wide landscapes of circRNAs have been reported in PTC tissues [10, 11]. Serum circRNAs might deliver diagnostic utility in PTC patients [12]. 40S ribosomal protein S28 (RPS28) could participate in ribosome biogenesis and bind near the mRNA exit tunnel [13], eventually modulating immunosurveillance and tumor progression [14, 15]. Hsa_circ_0049055 is one of circRNAs spliced from RPS28 and it can be nicknamed as circRPS28. A circRNA microarray assay reveals that circRPS28 is highly increased in PTC tissues than normal tissues [16]. However, it remained uncovered that whether circRPS28 played a role in PTC progression. MicroRNA (miR)-345-5p is versatile in human cancers [17, 18], and it has been proposed as a tumor suppressor in PTC [19]. Frizzled family receptor 8 (FZD8) is Wnt
receptor in canonical Wnt signaling pathway that is therapeutic target to antagonize human cancers [20]. Nevertheless, FZD8 role in thyroid cancer was still vague.

In this study, we aimed to annotate the role and mechanism of circRPS28 in regulating excessive cell growth and motility of PTC, and to investigate whether there was a competing endogenous RNA (ceRNA) axis involving circRPS28, miR-345-5p and FZD8.

Materials and Methods

PTC patients
Fifty PTC patients were recruited at the Zhengzhou Central Hospital Affiliated to Zhengzhou University, and the diagnosis of PTC was pathologically confirmed either intra- or post-operatively. These patients were selected according to the National Comprehensive Cancer Network (NCCN) guidelines (2014) for Thyroid Carcinoma [21], and none of the patients received preoperative treatment. Following the obtaining of written informed consents from all patients, 50 pairs of PTC tissues and corresponding noncancerous thyroid tissues were surgically resected and snap-frozen in liquid nitrogen. The normal thyroid tissues were located >2 cm away from the tumor margins, and all tissues received histopathological examination. This research was approved by the Ethics Committee of the Zhengzhou Central Hospital Affiliated to Zhengzhou University and complied with the Declaration of Helsinki.

Cells and cell culture
Human PTC cell lines including TPC-1 (#SCC147; Millipore, Billerica, MA, USA) and IHH-4 (#JCRB1079; JCRB, Osaka, Japan), as well as thyroid follicular epithelial cell line Nthy-ori3-1 (#90011609; ECACC, Salisbury, UK) were purchased. TPC cells were cultured in 1 to 1 mixture of DMEM (#M22650; R&D systems, Minneapolis, MN, USA) and RPMI-1640 medium (#M31050; R&D systems) with 10% heat-inactivated fetal bovine serum (FBS; S12550H; R&D systems), and Nthy-ori3-1 cells were in human thyroid follicular epithelial cells culture medium (#CM-H023; Procell, Wuhan, China) supplemented with 10% FBS. All cells were kept in a humidified atmosphere with 5% CO₂ at 37°C.

RNA isolation and real-time quantitative PCR (RT-qPCR)
Total RNA was isolated in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the operating instructions; cytoplasmic RNA and nuclear RNA were separated via Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada). RNA samples were used to reversely transcribe into cDNA using SuperScript First-strand Synthesis System (Invitrogen), and cDNA was amplified using SYBR Green qPCR Master Mix (Invitrogen) and corresponding primer pairs. The primer sequence for circRPS28, miR-345-5p and FZD8 was listed in Table 1, as well as the internal controls including glyceraldehyde-phosphate dehydrogenase (GAPDH) and U6. Gene expression was determined via $2^{-ΔΔCt}$ method [22].

Actinomycin D (ActD) and Ribonuclease R (RNase R) treatment
ActD (2 μg/mL; Amyjet, Wuhan, China) was added in culture medium of TPC-1 and IHH-4 cells, and total RNA was isolated after ActD treatment for 0, 6, 12, 18 and 24 h. RNase R could only degrade linear RNA without affecting circRNA, and the extracted total RNA from TPC-1 and IHH-4 cells was treated with 3 U/μg RNase R (Solarbio, Beijing, China) for 20 min. RT-qPCR was following performed to determine the expression of circRPS28 and the linear RPS28. And, the primers for circRPS28 and RPS28 were listed in Table 1.

Cell transfection
CircRPS28 and miR-345-5p were transiently silenced by transfecting small interfering RNA (siRNA) targeting circRPS28 (si-circRPS28#1 and si-circRPS28#2) and inhibitor of miR-345-5p (anti-miR-345-5p), respectively. Besides, miR-345-5p mimic (miR-345-5p) and recombinant plasmid pcDNA3.1-FZD8 (FZD8) were severally used to transiently overexpress miR-345-5p and FZD8. The empty pcDNA3.1 (+) plasmid (#VT1001; YouBio, Changsha, China) was used as the internal control, as well as si-NC, miR-NC and anti-miR-NC. Stable transfection was also performed into TPC-1 cells with small hairpin RNA (shRNA) targeting circRPS28 (sh-circRPS28) and the negative control sh-NC which were separately cloned into pHBVL-U6-ZSGreen-puro lentivirus vector (HANBIO, Shanghai, China). Stable cells were selected with puromycin (2.0 μg/mL) for 15 days. TPC-1 and IHH-4 cells were seeded into 6-well plates 1 day before transfection and transfected with aforementioned nucleotides using Lipofectamine 2000 reagent (#11668-019; Invitrogen) following manufacturer’s instructions. The oligonucleotides were listed in Table 1. Subsequent experiments were carried out 36 h after transfection.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony formation assay
Transfected TPC-1 and IHH-4 cells were re-plated in 96-well plate at a density of 3,000 cells/well and cultured for 3 consecutive days for MTT assay. 10% MTT reagent
(5 mg/mL; Beyotime, Shanghai, China) was added to each well and incubated for 4 h at 37°C. The MTT-containing medium was changed to dimethylsulfoxide (150 μL; Beyotime) for 15 min-incubation. The optical density (OD) values were detected at 570 nm on a Sunrise microplate reader (TECAN, Männedorf, Switzerland). Five identical replicates were done in each transfection group.

For colony formation assay, 200 transfected TPC-1 and IHH-4 cells were re-plated in 12-well plate for another 15 consecutive days. Cell colonies were formed and stained with 0.5% crystal violet (Beyotime) for 30 min following cell fixation with 4% paraformaldehyde (Beyotime) for 30 min. Then, number of colonies was counted.

5-ethynyl-2-deoxyuridine (EdU) assay

EdU Assay Kit (Abcam, Cambridge, UK) was obtained, and 2× EdU solution was added in culture medium for another 4 h in the dark; then, these cells were fixed in 1× Working Fixative Solution for 15 min, washed with the Wash Buffer twice; cells were permeabilized after 1× Permeabilization Buffer incubation for 20 min. Eventually, cells were processed in the EdU Reaction Mix for 30 min without sunlight, followed by DAPI working solution. The stained cells were imaged under a fluorescence microscope (Olympus IX73; Olympus, Tokyo, Japan) equipped with filter for Ex/Em = 491/520 nm, and the percentage of EdU-positive cells in DAPI-positive cells was determined in five randomly selected fields (100×).

Scratch wound assay and transwell assay

After transfection for 36 h, TPC-1 and IHH-4 cells were starved for 6 h and then a scratch was created in the bottom of each well using a 200 μL-pipette tip. The remaining cells were cultured in serum-free medium for another 24 h. The wounds were captured at 0 and 24 h under an inverted microscope (Olympus, Tokyo, Japan), and analyzed on Image J v1.46 software (National Institute of Health, Bethesda, MD, USA). Eventually, the wound healing rate was calculated using 100% × (Width₀h - Width₂₄h)/Width₀h.

The starved cells (5 × 10⁵ cells) were re-suspended in

| Name                      | Sequence                                                                 |
|---------------------------|--------------------------------------------------------------------------|
| si-circRPS28#1            | 5'-UAACUUGAAACACAAACGCUdTdT-3'                                           |
| si-circRPS28#2            | 5'-UAACUUGAAACACAAACGCUdTdT-3'                                           |
| sh-circRPS28              | Sense 5'-CGUUGUGUUGUUGUAAAGCUUACU-3'                                    |
|                            | Antisense 5'-UUACUUGAAGAACCAACAGGCU-3'                                  |
| miR-345-5p mimic          | 5'-GCUGACUCUUAGCCAGGAGCUC-3'                                            |
| anti-miR-345-5p           | 5'-GAGCCCGUGACUAGGAGUCACG-3'                                            |
| si-NC                     | 5'-UCUUCGAACGUGACAGC-3'                                                 |
| sh-NC                     | Sense 5'-GGAGUAGGGAGCACAACAUAGGAA-3'                                    |
|                            | Antisense 5'-UUCUACUAAGGUGCUCACUAAGC-3'                                 |
| miR-NC mimic              | 5'-ACGUGAGCACGCAGGAGAATT-3'                                             |
| anti-miR-NC               | 5'-CAGUAACUUUGUGAGUGAACA-3'                                             |
| circRPS28 (109 nt)        | Forward primer 5'-CGATGGGAATGGTCTGTCAC-3'                               |
|                            | Reverse primer 5'-GGTCTGGCGAGAGGTTACT-3'                                |
| RPS28 (145 nt)            | Forward primer 5'-ACAGGAGCGTCTACGTTAGC-3'                               |
|                            | Reverse primer 5'-TCTTGAAGATGCTCAGG-3'                                  |
| miR-345p (75 nt)          | Forward primer 5'-GCTGACTCTCTAGTCCA-3'                                  |
|                            | Reverse primer 5'-GGTCTGGCGAGAGGTTACT-3'                                |
| FZD8 (85 nt)              | Forward primer 5'-GTGTAAGGGGACCATCGGCACT-3'                             |
|                            | Reverse primer 5'-AGAACTTGAAGATCGGCGAG-3'                               |
| GAPDH (104 nt)            | Forward primer 5'-GACAGTCGCGAGCAGTCTTC-3'                               |
|                            | Reverse primer 5'-GCGGCAATACGCACACAC-3'                                 |
| U6 (80 nt)                | Forward primer 5'-TCGCAGAGCAGCAGTCTTC-3'                                |
|                            | Reverse primer 5'-TTGCAGTCTTGCAGTCAGTCTTC-3'                            |

Table 1  The sequences of oligos and primers

The role of circRPS28 in PTC
200 μL serum-free medium, followed by the cell inoculation into the upper place of Transwell chamber (Corning, Carlsbad, CA, USA) for cell migration assay. For invasion assay, the chambers were pre-coated with 50 μL Matrigel (#354480; BD Biosciences) overnight at 4°C. 500 μL medium containing 20% FBS (R&D systems) was filled in the bottom place in 24-well plate. These chambers and plates were together incubated at 37°C for 48 h. Transferred cells were fixed with 4% paraformaldehyde (Beyotime) for 30 min and stained with 0.5% crystal violet (Beyotime) for 30 min. The migration number and invasion number were counted under an inverted microscope (Olympus) at 100×, and five randomly selected fields were used.

Flow cytometry (FCM)

Cell apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime) and flow cytometer (BD Biosciences, San Jose, CA, USA). According to the standard procedures, 5 × 10⁴ cells were double-stained with Annexin V-FITC and propidium iodide (PI), and apoptosis rate was determined by the percentage of right upper quadrant and right lower quadrant.

Western blotting

Total proteins were extracted from tissues and cells, and the following western blotting was performed as previously described [23]. The primary antibodies were against FZD8 (#ab155650; Abcam), matrix metalloproteinase-2 (MMP2; #ab86607; Abcam), MMP9 (#ab137867; Abcam), B-cell lymphoma (Bcl)-2 (Bcl-2- associated X protein (Bax; #ab32503; Abcam), as well as β-actin (#ab8227; Abcam). The secondary antibodies were against rabbit IgG H&L (ab205718; Abcam) and mouse IgG H&L (ab205719; Abcam).

Dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay

The mutant (MUT) of miR-345-5p-binding sites were designed in circRPS28 and 3’-untranslated region (3’UTR) of FZD8 (FZD8 3’UTR) according to the prediction on starbase database (http://starbase.sysu.edu.cn/index.php). Then, wild type (WT) and MUT sequence of circRPS28 and FZD8 3’UTR were cloned into pGL4 luciferase vector (Promega, Madison, WI, USA). Co-transfection of recombination pGL4 vectors and miR-345-5p or miR-NC was carried out in TPC-1 and IHH-4 cells for 48 h. The luciferase activity was detected by using Dual-Lucerase® Reporter (DLR™) System (Promega) and on Glomax20/20 luminometer fluorescence detector (Promega).

RIP was performed using Thermo Fisher RIP kit (Thermo Fisher, New York, NK, USA) based on the manufacturer’s protocol. Antibodies were targeting Argonaute 2 (Ago2; #ab186733, Abcam) and IgG (#ab182931, Abcam). The input, IgG-mediated precipitation and Ago-mediated precipitation were used to detect expression of circRPS28 and miR-345-5p with normalization to that in input.

Xenograft experiment

A sum of 10 female nude BALB/c nude mice (5-week old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). The stably transfected TPC-1 cells were used to be inoculated into the subcutaneous location of the right flanks at a density of 5 × 10⁶ cells/mouse. Five mice were set for every group. Tumor volume was calculated once a week according to a formula: 0.5 × length × width². After 5-week later, the mice were sacrificed with 40 mg/kg sodium pentobarbital to excise tumor nodes. Tumors were weighted and tumor tissues were stored and lysed for total RNA or protein isolation. This study was approved by the Committee Ethics of Animal Center of Zhengzhou Central Hospital Affiliated to Zhengzhou University in accordance with the Guide for the Care and Use of Laboratory Animals from National Institutes of Health.

Statistical analysis

Data were presented as mean ± standard error of the mean, and processed by GraphPad Prism 6.0 software (La Jolla, CA, USA). Student’s t test and one-way analysis of variance compared the statistical difference between groups. P value was set at 0.05.

Results

CircRNA circRPS28 was highly and stably upregulated in PTC

Expression of circRPS28 in PTC tumor tissues was 3.5-fold of that in paired normal tissues (Fig. 1A), and its level was higher in human PTC cell lines (TPC-1 and IHH-4) than normal Nthy-ori3-1 cells (Fig. 1B). Besides, ActD incubation led to great decrease of RPS28 mRNA expression and little decline of circRPS28 expression in TPC-1 and IHH-4 cells (Fig. 1C and 1D); circRPS28 expression was unacted with RNase R treatment comparing to the linear counterpart (Fig. 1E and 1F). Moreover, circRPS28 expression was mainly detected in the cytoplasm of TPC-1 and IHH-4 cells (Fig. 1G and 1H). These results showed a structure stability of circRPS28 that was highly expressed in PTC patients and cell lines.
Blockage of circRPS28 functioned a suppressive role in PTC cell growth and motility

Special siRNAs transfection caused the silence of circRPS28 in both TPC-1 and IHH-4 cells (Fig. 1I). Due to si-circRPS28#1/#2 transfection, OD values of TPC-1 and IHH-4 cells were lower than si-NC transfection during 72 h according to MTT assay (Fig. 2A and 2B), and number of colonies and EdU positive cells were reduced (Fig. 2C and 2D). Exogenously blocking circRPS28 could highly induce apoptosis rate of TPC-1 and IHH-4 cells (Fig. 2E). Scratch wound assay manifested a loss of wound healing rate in si-circRPS28#1/#2-transfected TPC-1 and IHH-4 cells (Fig. 3A and 3B). Migration cell number and invasion cell number were simultaneously declined in TPC-1 and IHH-4 cells in the presence of si-circRPS28#1/#2 (Fig. 3C and 3D). Furthermore, allied with that was the decreased expression of MMP2, MMP9 and Bcl-2, and elevated Bax expression (Fig. S1A-S1D). These results indicated a suppressive role of circRPS28 blockage in cell growth and motility of PTC cells in vitro.

MiR-345-5p was identified as a target for circRPS28

The starbase and circbank databases predicted a panel of 6 miRNAs that were computational targets for circRPS28 (Fig. 4A); among these miRNAs, miR-125a-5p, miR-345-5p, miR-542-3p and miR-589-5p were significantly upregulated by circRPS28 deficiency via transfection (Fig. 4B and 4C). Next, the potential miR-345-5p-binding sites in circRPS28 WT were mutated (Fig. 4D). MiR-345-5p overexpression via transfection attenuated the luciferase activity of report vectors
expressing circRPS28 WT, and left alone that of MUT vectors (Fig. 4E-4G). What’s more, circRPS28 and miR-345-5p were concurrently enriched in Ago2 RIP from cell extract of TPC-1 and IHH-4 cells (Fig. 4H and 4I). What’s more, miR-345-5p was downregulated in human PTC tumor tissues and cell lines comparing with

**Fig. 2** Blockage of circRPS28 functioned a suppressive role in PTC cell growth. TPC-1 and IHH-4 cells were transfected with si-NC, si-circRPS28#1 or si-circRPS28#2. (A, B) MTT assay monitored OD value at 570 nm in 72 h. (C) Number of colonies was examined in colony formation assay. (D) EdU assay showed EdU positive cells (%). (E) FCM and Annexin V-FITC/PI staining method confirmed apoptosis rate (%). * p < 0.05.
normal tissues and cells (Fig. 4J and 4K). These data revealed that circRPS28 could directly target miR-345-5p in PTC cells.

**Fig. 3**  Blockage of circRPS28 functioned a suppressive role in PTC cell motility. TPC-1 and IHH-4 cells were transfected with si-NC, si-circRPS28#1 or si-circRPS28#2. (A, B) Scratch wound assay determined wound healing rate (%). (C, D) Migration cell number and invasion cell number were tested in transwell assays. *p < 0.05.

**Downregulation of miR-345-5p abated the role of circRPS28 blockage in PTC cell growth and motility**

Transfecting anti-miR-345-5p could exogenously silence miR-345-5p in TPC-1 and IHH-4 cells (Fig. 5A),
Identification of miR-345-5p as a target for circRPS28. (A) Two prediction databases searched the potential miRNA-binding sites in circRPS28. (B, C) RT-qPCR detected relative miRNAs expression in TPC-1 and IHH-4 cells transfected with si-NC or si-circRPS28#2. (D) CircRPS28 MUT was synthesized by mutating miR-345-5p-binding sites in circRPS28 WT. (E) RT-qPCR detected relative miR-345-5p expression in TPC-1 and IHH-4 cells transfected with miR-345-5p or miR-NC. (F, G) Relative luciferase activity of report vector expressing circRPS28 WT and circRPS28 MUT in TPC-1 and IHH-4 cells co-transfected with miR-345-5p or miR-NC. (H, I) Relative RNA enrichment by Ago2 was determined by RIP assay and RT-qPCR with normalization to that by IgG. (J, K) RT-qPCR detected relative miR-345-5p expression in tumor tissues, normal tissues, PTC cells (TPC-1 and IHH-4), and thyroid follicular epithelial cells (Nthy-ori3-1). * p < 0.05.
and this transfection partially but significantly elevate OD values of circRPS28-blocked cells during 72 h (Fig. 5B and 5C), as well as numbers of colonies and EdU positive cells (Fig. 5D and 5E). Wound healing rate and numbers of migration cells and invasion cells in TPC-1 and IHH-4 cells were totally reduced with si-circRPS28#2 transfection, and these data were improved with the additional transfection of anti-miR-345-5p (Fig. 5F-5H). CircRPS28 knockdown via transfection mediated a promotion of apoptosis rate in TPC-1 and IHH-4 cells, which could be weakened by further blocking miR-345-5p (Fig. 5I). Molecularly, si-circRPS28#2 displayed effects on expression of MMP2, MMP9, Bcl-2, and Bax in TPC-1 and IHH-4 cells were overall counteracted with co-transfection with anti-miR-345-5p (Fig. S2A-S2D). These results demonstrated that miR-345-5p downregulation could abate the suppression of circRPS28 blockage on PTC cell growth and motility in vitro.

**FZD8 was a target gene for miR-345-5p**

According to starbase prediction, miR-345-5p-binding sites were mutated in FZD8 3’UTR WT (Fig. 6A), and miR-345-5p transfection could only decrease the luciferase activity of report vectors expressing FZD8 3’UTR WT (Fig. 6B and 6C). The expression of FZD8 both at mRNA level and protein level was upregulated in human PTC tumor tissues and cell lines (Fig. 6D-6G). Additionally, both miR-345-5p overexpression and circRPS28 knockdown via transfection resulted in FZD8 depression (Fig. 6H-6K), and si-circRPS28#2-mediated FZD8 depression could be rescued with additionally transfecting anti-miR-345-5p (Fig. 6J and 6K). These data
revealed that circRPS28 could modulate miR-345-5p-targeted FZD8 in PTC cells.

**Upregulation of FZD8 abolished the inhibitory effect of miR-345-5p overexpression on PTC cell growth and motility**

Cell proliferation of TPC-1 and IHH-4 cells was suppressed by transfecting miR-345-5p, as evidenced by the lower OD values during 72 h and EdU positive cells rate (Fig. 7B, 7C and 7E). Numbers of the colonies, migratory cells and invasive cells in TPC-1 and IHH-4 cells were totally reduced by re-expression of miR-345-5p via transfection (Fig. 7D, 7G and 7H). Moreover, miR-345-5p upregulation inhibited wound healing rate of TPC-1 and IHH-4 cells (Fig. 7F), whereas induced higher apoptosis rate (Fig. 7I). Expression of MMP2, MMP9 and Bcl-2 was depressed, and Bax expression was augmented in miR-345-5p-transfected TPC-1 and IHH-4 cells.
IHH-4 cells than miR-NC-transfected above cells (Fig. S3A-S3D). These results demonstrated that miR-345-5p upregulation could restrain PTC cell growth and motility in vitro. Then, transfecting FZD8 vector could exogenously overexpress FZD8 protein expression (Fig. 7A), and this transfection could overall attenuated above effects of miR-345-5p overexpression in TPC-1 and IHH-4 cells (Fig. 7B-7I and Fig. S3A-S3D).

Silencing circRPS28 delayed tumor growth of PTC cells in vivo
Stably transfected TPC-1 cells could induce xenograft tumors in nude mice (Fig. 8A), and sh-circRPS28 transfection lowered tumor volume and weight than sh-NC, (Fig. 8B and 8C). Moreover, the expression of circRPS28 was diminished in the xenograft tumor tissues (Fig. 8D), accompanying with higher level of miR-345-5p and lower level of FZD8 (Fig. 8E and 8F). These data displayed a tumor-suppressive role of circRPS28 blockage in PTC cell growth in vivo by regulating miR-345-5p and FZD8.

Discussion
In this study, we discovered an upregulation of circRPS28 and FZD8 and a downregulation of miR-345-5p in PTC patients and cells, as well as a tumor-suppressive role of circRPS28 exhaustion and miR-345-5p re-expression in PTC cell growth and motility. Notably, circRPS28 could sponge miR-345-5p

Fig. 7 Upregulation of FZD8 abolished the inhibitory effect of miR-345-5p overexpression on PTC cell growth and motility. (A) Western blotting detected relative FZD8 protein expression in TPC-1 and IHH-4 cells transfected with FZD8 vector or control vector. (B-L) TPC-1 and IHH-4 cells transfected with miR-345-5p, miR-NC, or miR-345-5p together with FZD8 vector or control vector. (B, C) MTT assay monitored OD value at 570 nm in 72 h. (D) Number of colonies was examined in colony formation assay. (E) EdU assay showed EdU positive cells (%). (F) Scratch wound assay determined wound healing rate (%). (G, H) Migration cell number and invasion cell number were tested in transwell assays. (I) FCM and Annexin V-FITC/PI staining method confirmed apoptosis rate (%).
to fine-tune gene expression of FZD8. These findings disclosed a novel circRPS28/miR-345-5p/FZD8 ceRNA regulatory axis in regulating PTC progression, helping to better understand the molecular mechanism of PTC. Therefore, this study might provide a potential therapeutic target to boycott PTC.

CircRPS28 expression was declared to be higher in this cohort of human PTC tissues than paired normal tissues, and this was in support of previous circRNA microarray data [16]. Moreover, Ye et al. [16] noticed that circRPS28 was one of the top 5 upregulated circRNAs in PTC patients’ tumors. However, this circRNA seemed heretofore little investigated, and there was probably no more research focusing on its dysregulation in other human cancers. This was the first description that circRPS28 was predominantly located in the cytoplasm with resistance to RNase R and ActD; thia finding suggested that circRPS28 might be a stably upregulated circRNA in PTC via functioning as miRNAs sponge. Next, we not only showed the anti-tumor role of blocking circRPS28 in cell proliferation, apoptosis, tumor growth, colony formation, wound healing, migration and invasion in PTC cells, but also deciphered the target relationship between circRPS28 and miR-345-5p. These outcomes illuminated that silencing circRPS28 might be a potential therapeutic approach to eliminate PTC.

Here, we monitored a regulatory effect of circRPS28 on miR-125a-5p, miR-345-5p, miR-542-3p and miR-589-5p in PTC cells. MiR-125a-5p and miR-345-5p could suppress cell malignancy and glucose metabolism of PTC cells [19, 24]; miR-589-5p could target thyroid receptor interacting protein 6 to play as a tumor suppressor in endometrial carcinoma [25]. Yet, expression and role of miR-589-5p and miR-542-3p remained unstudied in PTC tumorigenesis. Since miR-345-5p was the most elevated miRNA in circRPS28-depleted PTC cells, we carried out dual-luciferase reporter system and Ago2 RIP assay to further confirmed the predicted circRPS28/miR-345-5p interaction. Expression of miR-345-5p was low in PTC tumor tissues than neighboring normal tissues, which was in favor with former data [19]. Functionally, overexpressing miR-345-5p was responsible for suppressing PTC cell progression by regulating cell proliferation, apoptosis, colony formation, wound healing, migration, and invasion. Similarly, Zhao et al. [19] claimed the anti-tumor role of miR-345-5p in PTC cell viability, colony formation and apoptosis in both loss-of-functional and gain-of-functional experiments. Advanced results of miR-345-5p role had been achieved in this study, and miR-345-5p exerted not only anti-growth role in PTC cells but also anti-migration/invasion role. Comparing to the existing miR-345-5p/SETD7 axis [19],

**Fig. 8** Silencing circRPS28 delayed tumor growth of PTC cells in vivo. TPC-1 cells were stably transfected with sh-circRPS28 or sh-NC, and then inoculated in nude mice. (A) The most representative tumor in sh-NC group (n = 5) and sh-circRPS28 (n = 5). (B) Tumor volume was monitored in 35 days. (C) Tumor weight was examined at the last day. (D-F) RT-qPCR detected relative RNA expression of circRPS28 and miR-345-5p, and western blotting detected relative protein expression of FZD8 in the xenograft mouse tumors tissues with normalization to β-actin. *p < 0.05.
FZD8 was here identified as a new target functional gene in PTC. Besides, miR-345-5p could exert anti-proliferation and anti-metastasis role in other human cancers, such as breast cancer [26], gastric cancer [27], and pancreatic cancer [17].

MiR-345-5p, sponged by circPRS28, was a direct regulator of functional gene FZD8 in development of PTC cells. FZD8 downregulation was hidden in the protective role of circPRS28 knockdown and miR-345-5p restoration against PTC cell growth and motility. Chen et al. [28] clarified the role of FZD8 in PTC cell proliferation, apoptosis and invasion by exogenously overexpressing and silencing FZD8, respectively. In this study, restoring FZD8 could restrain cell proliferation, colony formation, wound healing, migration, and invasion, but enhance apoptosis of PTC cells with miR-345-5p overexpression. These results collectively determined FZD8 as a PTC-related gene. The induction of FZD8 was known to activate Wnt pathway, and FZD8 was a potential therapeutic target for prostate cancer by integrating with Wnt-11 and serving as a target for Ets-related gene (ERG) and p53 [29-31]. In addition, p53 was one of the best-characterized genetic alterations, and its loss led to the development of poorly differentiated thyroid tumors through a complex network [32]; p53 could regulate the expression of miRNAs, lncRNAs and circRNAs, and thus participated in thyroid oncogenesis [33-35]. Whereas, the interaction between circPRS28/miR-345-5p/FZD8 and Wnt signaling pathway or p53 status was left to be further explored.

All in all, we demonstrated that blocking circPRS28 and/or restoring miR-345-5p could suppress growth and motility of PTC cells by inhibiting FZD8. Mechanically, circPRS28/miR-345-5p/FZD8 ceRNA pathway was considered as a novel mechanism underlying PTC pathogenesis and treatment.

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**Disclosure of Interest**

The authors declare that they have no conflicts of interest.

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