

Circ-0012417/ miR-29c-5p Axis Stimulates Thyroid Cancer Growth

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Research

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Abstract

Background: There is an increasing tendency of the incidence rate of thyroid cancer. Despite the advance of widespread early diagnose and good prognosis of papillary thyroid cancer (PTC), some patients still suffered from the invasiveness and metastasis of thyroid cancer. More and more evidences demonstrated that circular RNAs (circRNAs) play important roles in tumorigenesis, tumor progression and metastasis. This study aims to identify oncogenic circRNAs and explore its underlying molecular mechanisms in thyroid cancers.

Methods: With circRNA chips in GEO database, we found that circ-0012417 was over-expressed in thyroid cancer. After silencing circ-0012417 or ectopic over-expressed circ-0012417, CCK-8 was used for detecting cell proliferation and transwell is used for examining metastasis and invasion abilities. Western Blot was used to detect changes of downstream proteins. Dual luciferase reporter assay was used to verify whether miR-29c-5p is the target of circ-0012417. Tumor xenografts in nude mice were used for further *in vivo* verifications.

Results: Circ-0012417 is overexpressed in thyroid cancer, and silencing circ-0012417 could inhibit cancer cell proliferation, metastasis and invasion. MiR-29c-5p may be the target of circ-0012417 and inhibiting miR-29c-5p could reverse this phenomenon. And further analysis of TCGA and GTEx databases showed that EGFL7 may be the target of miR-126 and a promising thyroid cancer prognosis indicator.

Conclusion: Circ-0012417 promotes thyroid cancer progression through regulating miR-29c-5p /EGFL7 pathway. Our finding provided novel therapeutic targets for thyroid cancer treatment.

Background

Thyroid cancer is one of most common endocrine tumors and its incidence rate is increasing. Thyroid cancer is classified in 4 types: follicular thyroid cancer (FTC), papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC) and medullary thyroid cancer (MTC). While FTC and PTC accounts for around 90% thyroid cancer in clinical. Early diagnose, radical surgery and TSH inhibition therapy and I¹³¹ is recommended for patients with tumor invasion and metastasis. Because of early invasion and metastasis, relapse and I¹³¹ resistance, a subset of ATC patients suffered with only 6 months overall survival (OS). Besides, narrow therapy strategies limited benefits for patients with significant invasion and metastasis.

Circular RNAs (circRNAs), as the name suggests, are a novel group of covalently closed loop RNAs without 5' hat or polyadenylation at their 3' ends[1]. CircRNAs are mainly constituted by back-spliced exons[2]. MiRNAs are a group of short non-coding RNAs (16–29 nt) with post-transcriptional regulation functions. CircRNAs can function as “miRNA-sponges” to protect the target mRNAs from miRNA-induced cleavage. In fact, circRNA-miRNA-mRNA network regulation had been found in several type of cancer[3]. For example, circ-ITCH could sponge miR-17/miR-224 to inhibit bladder cancer progression[4]. Concretely, these circRNA-miRNA networks inhibit the expression of target genes and regulate tumorigenesis, tumor

progression, invasion and metastasis. What's more, some circ-RNAs could also be reliable prognostic indicators. In advanced colon cancer, a four-circRNA-based classifier could be a good recurrence prognostic stratification[5].

To uncover the roles of circRNAs in thyroid cancer, we analyzed circRNA chips in GEO database and found that circ-0012417 was overexpressed in thyroid cancer. Thus, this study focused on demonstrating the functions and its molecular mechanisms of circ-0012417 in thyroid cancers.

Material And Methods

Patient tissue samples and cell lines

This study was approved by the ethics committee of the First affiliated hospital of Jinan University. All patients provided a written informed consent. Fresh-frozen cancer tissues and adjacent normal tissues from 30 patients with thyroid cancer were used for this study. Fresh-frozen tissues from 50 patients with benign thyroid nodules were used as control.

Thyroid cancer cell lines (K1, BHT-101, KHM-5M, B-CPAP) were all purchased from the Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were preserved as per the manufacturer's instructions. Briefly, the cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM penicillin and streptomycin (Gibco BRL, NY, USA). All cell lines were cultured in humidified air supplemented with 5% CO₂ at 37 °C.

CircRNAs expression profile analysis

The gene expression profiles (which one) of TC were reviewed from the Gene Expression Omnibus database (GEO, <http://www.ncbi.nlm.nih.gov/geo>). The online analysis tool GEO2R (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) was utilized to assess the differentially expressed genes (DEGs) in this study. The adjusted P values were then calculated using the Benjamini and Hochberg false discovery rate method to correct for the occurrence of false positive results.

RNase R resistance analysis of circRNAs

Hsa-circ-0012417 from BHT-101 and B-CPAP was treated with RNase ((4 U/mg, Epicenter) and incubated for 30 minutes at 37 °C. The treated RNAs were then reverse transcribed using specific primers. Next, quantitative real-time PCR (qRT-PCR) was used to determine the expression of has-circ-0012417 in the TC cell lines.

RNA extraction and quantitative real-time PCR (RT-PCR) assay

Total RNA was extracted from the thyroid cancer tissues/cell lines and adjacent normal tissues/cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After assuring RNA quantification and quality using NanoDro2000c (Thermo Scientific, Waltham, USA), 2 µg of total RNA were reverse transcribed to cDNA

using the Bestar™ qPCR RT kit (#2220, DBI Bioscience, China). Quantitative polymerase chain reaction (RT-PCR) was then done using the Bestar™ qPCR MasterMix (#2043, DBI Bioscience, China).

Lentivirus constructs and cell transfection

For *in vivo* studies, circRNA0012417- knockdown cells (si-circRNA0012417) and negative control cells (NC) were utilized. Lentivirus particles were bought from Hanbio Biotechnology (Shanghai, China). 48 hours after transfection, stable-transfection cells were established by puromycin selection (2 µg/ml) applied for 10 days. LV003-has-circ-0012417 overexpression and negative cells (LV003 vector control) were established in the same way.

The miR-29c, and the miR-miR-29c inhibitor were acquired from GenePharma (Shanghai, China). B-CPAP and BHT101 cells were seeded in 6-well plates and then transfected the next day using Lipofectamine3000 (Invitrogen, CA, USA) as per the manufacturer's instructions. Cells were harvested at 48 hours post transfection followed by confirmation of transfection efficiency using qRT-PCR.

Cell proliferation assay

The cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo, Osaka, Japan). B-CPAP and BHT101 cells in different groups were seeded into 96-well plates with serum-free DMEM medium. Each group was duplicated with three independent wells. Cell proliferation was observed at 24, 48 and 72 hours. Prior to observation, each well was treated with 10 µl CCK-8 reagent and then cells were incubated at 37°C for another 3 hours before measuring absorbance at 450 nm utilizing a spectrophotometer. All experiments were done in triplicate.

Transwell migration assay and invasion assay

B-CPAP and BHT101 cells (5×10^4) in different groups were resuspended in 200 µl serum-free medium and seeded into the upper chamber of 24-well plates (Corning, New York, NY, USA) with (invasion) or without (migration) Matrigel (BD Biosciences, New York, NY, USA), while 600 µl medium containing 20% FBS were supplied at the lower chamber as chemoattractant. After incubation at 5% CO₂ /37 °C for 20 hours, cells were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet for 30 min. The number of cells that migrated or invaded was determined in five randomly selected fields using an inverted microscope.

Cell cycle assay

Cells (2×10^6) were collected and fixed with 70% ethanol at 4 °C overnight, and 100 µl RNase A (Keygen Biotech, Nanjing, China) were then added, followed by incubation at 37 °C for 30 min. Cells were then stained with 400 µl propidium iodide (Keygen Biotech) for 30 min. A FACS Calibur flow cytometer (Becton Dickinson) was applied to evaluate cells at 488 nm, and ModFit LT software (Verity Software House) for analysis. All assays were repeated at least three times.

Luciferase reporter assay

A mutant circRNA12417 without miR-29c binding sites were obtained by overlap extension PCR with mutant primers. Briefly, BHT-101 and B-CPAP cells were co-transfected with psiCHECK-circRNA12417-WT or psiCHECK-circRNA12417-Mut and miR-29c mimics or miR-NC using Lipofectamine3000 (Invitrogen). Luciferase activity was measured at 48 hours after transfection by a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) as per the manufacturer's instructions.

Western blotting

Total protein was extracted from cells using RIPA lysis buffer (Keygen Biotech). The lysate protein was separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in TBST for 2 h, proteins were then incubated with primary and secondary antibodies. Signal detection was visualized by using enhanced chemiluminescence. Finally, relative concentration was calculated using Quantity One software (Bio-Rad). Primary antibodies included those against CBX8 (dilution 1:1000, 14696) from Cell Signaling Technology, (CA, USA), and Cyclin D1 (dilution 1:200, 60186-1-Ig), Bax (dilution 1:2000, 50599-2-Ig), and GAPDH (dilution 1:5000, 10494-1-AP) from Proteintech (Chicago, USA).

Tumor formation in nude mice

The validated thyroid cancer cell line hsa-circ-0012417 (B-CPAP Circ) and its negative control strain (B-CPAP NC) were expanded and cultured. 1×10^7 cells was injected subcutaneously into 10 BALB/c-nu/nu female nude mice weighing between 18–22 g (4–5 weeks old). 14 days after transfection, the length and width of the tumor were measured once a week for 5 weeks. After 5 weeks, the mice were sacrificed by cervical dislocation. The tumor length, width and weight were all measured and photographed.

Statistical Analysis

Statistical analysis was done using SPSS 18.0 software (SPSS, Chicago). T test was used to analyze the relationship between the indicators of each group. P value less than 0.05 was deemed as statistically significant.

Results

Hsa-circ-0012417 is overexpressed in thyroid cancer

According to circRNA chips of thyroid cancer in GEO database, we focused on 4 dysregulated RNAs: hsa-circ-0012417, hsa-circ-0000690, hsa-circ-0006357, hsa-circ-0007694. We then measured in 14 pairs of human thyroid cancer tissues and adjacent normal tissues, and we found that only has-circ-0012417 was significantly elevated in cancer tissues compared with respective normal tissues ($p < 0.05$) (Fig. 1A). We then tried amplify circ-0012417 using human complementary DNA (cDNA) and genomic DNA (gDNA) as templates, and we got the circ-0012417 fragment from cDNA templates, but we failed to obtain circ-0012417 from gDNA (Fig. 1B). This result indicating that the intact circ-0012417 fragment contains different splicing sites and it could be generated by back-splicing. Sanger sequencing was further

performed to verify the sequence information and the conjugation site information of hsa-circ-0012417 was showed (Fig. 1C). These results demonstrated that circ-0012417 was identified as a novel circRNA, which is overexpressed in thyroid cancers.

Silence of circ-0012417 inhibits proliferation in thyroid cancer cells

Since circ-0012417 was higher-expression in thyroid cancer tissues, we next test thyroid cancer functions in thyroid cancer cells. Firstly, we determined circ-0012417 expression in four different thyroid carcinoma cell lines: KHM-5M, K1, BHT101 and B-CPAP and found that hsa-circ-0012417 was relatively high expressed in KHM-5M, BHT101 and B-CPAP than in K1 (Fig. 2A). Then we constructed two si-circ0012417-1 and si-circ0012417-2 which could successfully inhibit circ-0012417 levels (Fig. 2B). Finally, we detected that the proliferation of B-CPAP and BHT101 was inhibited when transfected with si-circ0012417 (Fig. 2C) ($p < 0.01$ and $p < 0.05$, respectively).

Silence of circ-0012417 suppresses the metastasis of thyroid cancer cells

We then explore roles of has-circ-0012417 in biological behaviors of thyroid cancer. When transfected with si-circ0012417, the migration ability of B-CPAP and BHT101 cells were significantly decreased to 41.02% and 37.50% (Fig. 3A). Cell invasion decreased to 33.33% in the B-CPAP ($P < 0.01$), and to 56.91% in the BHT101 cell line respectively ($P < 0.01$) (Fig. 3B). Transfection of si-circ-0012417 also resulted in a significant decrease in the wound healing ability of B-CPAP and BHT101 cells (Fig. 3C). In conclusion, si-circ0012417 inhibit proliferation, migration, invasion and wound healing capacity of thyroid cancer cells.

Exogenous circ-0012417 stimulate he proliferation and metastasis of thyroid cancer cells

We then explore gain of functions experiments of circ-0012417 in B-CPAP and BHT101. Over-expressed circ-0012417 induced increased cell proliferation in B-CPAP and BHT101 (Fig. 4A&B). As expected, with exogenous circ-0012417, the migration and invasion of B-CPAP and BHT101 cells increased significantly ($P < 0.01$ and $P < 0.01$ in B-CPAP, $P < 0.001$ and $P < 0.001$ in BHT101, respectively) (Fig. 4C&D).

Furthermore, we wondered whether circ-0012417 regulated Wnt/ β -catenin signaling. Both western Blot and Real-time PCR results showed that β -catenin, c-myc and cyclin D1 were up-regulated by circ-0012417 (Fig. 5). Previous researches had proved that β -catenin activation regulate cell cycle and cell adhesion [6, 7], c-myc and cyclin-D1 could also promote cell cycle progression[8, 9]. With these results, circ0012417 may regulate cell cycle to stimulate cell proliferation by upregulating β -catenin, c-myc and cyclin D1 proteins.

hsa-circ-0012417 targets hsa-miR-29c-5p

To gain insights into the molecular mechanism by which the hsa-circ-0012417 suppresses cell proliferation, migration and invasion, and induces cell apoptosis, we screened the possible binding site of hsa-circ-0012417 to microRNAs via circBase software. MiR-29c-5 attracted our attentions. QPCR results also demonstrated that silencing hsa-circ-0012417 increased miR-29c-5p expression (Fig. 6A). Dual luciferase assay indicated that circ-0012417 could directly target miR-29c-5(Fig. 6B). MiR-29c-5 inhibitor could reverse cell proliferation inhibition of which is induced by silencing hsa-circ-0012417 (Fig. 6C). What's more, miR-29c-5 inhibitor could antagonize decreased cell migration and invasion induced by silencing hsa-circ-0012417(Fig. 6D-E). These results concluded that hsa-circ-0012417 acts as a molecular sponge for miR-29c-5p.

Silencing hsa-circ-0012417 inhibits tumor growth in vivo

Following these *in vitro* studies, we further build xenograft models with B-CPAP cell line. Consistent with previous results, compared with NC group, silencing hsa-circ-0012417 significantly inhibited tumor growth by measurement of tumor size once a week (Fig. 7A). At the end of the animal experiment, tumor weight in si-circ-0012417 group is obviously decreased than NC treatment (Fig. 7B&C). Following transfection with si-circ0012417, we observe a significant decrease in tumor volume and weight *in vivo*, supporting the hypothesis that hsa-circ-0012417 is a tumor growth factor.

Discussion

Thyroid cancer is originated from thyroid follicular cells or parafollicular cells and its morbidity is increasing worldwide in recent years[11]. The 5-year survival of patients with papillary thyroid microcarcinoma was around 99%[11]. But there're still a part of patients who suffered thyroid cancer metastasis and recurrence. CircRNAs are a group of endogenous noncoding RNAs (ncRNAs), and characterized by a closed covalently loop with back-spliced exons[12]. CircRNAs are dysregulated in many solid tumors like hepatocellular carcinoma[13, 14], gastric carcinoma[15, 16] and colorectal cancer[17, 18]. CircRNAs usually sponge miRNA to modulate energy metabolism, angiogenesis, invasion and metastasis in carcinoma. Recent researches had found that in thyroid cancer, circRNA_10217, circRNA_NEK6 and circ_0067934 could promote tumor growth[19–21], while circ-ITCH could suppress tumor progression[22]. In this manuscript, we first screened thyroid cancer related circRNA chips results from GEO database. Then hsa-circ-0012417 was upregulated in thyroid tumor via pPCR in 14 pairs of thyroid tumor tissues and para-carcinoma tissue (Fig. 1A). DNA gel and sanger sequencing supported that hsa-circ-0012417 is a circular RNA (Fig. 1B-D). Through gain of functions and loss of functions experiments, circ-001241 could stimulate cell division and promote cell proliferation, metastasis and invasion (Fig. 2–5). Researches had demonstrated that activation of WNT/ β -catenin pathway take parts in the occurrence and progression of thyroid carcinoma[23], and aberrant WNT/ β -catenin pathway expression is related with the survival of patients with thyroid carcinoma[24]. As expected, we found that silencing circ-0012417 could inhibit WNT/ β -catenin. Further we found that inhibiting circ-0012417 could stimulate miR-29c-5p expression, and inhibiting miR-29c-5p could rescue decreased migration and invasion induced by silencing hsa-circ-001241, while dual luciferase report assay demonstrated that circ-

0012417 may directly target miR-29c-5p (Fig. 6). And in vivo study showed that silencing hsa-circ-001241 could inhibit tumor growth significantly (Fig. 7). Finally, with TCGA and GEO database, we found that EGFL7 and LEMD1, the possible target of hsa-circ-001241, could be possible prognostic predictors in thyroid cancer.

CircRNAs are new class of non-coding RNAs and are mostly generated by back-splicing of exons of protein-coding genes. Like microRNAs and long non-coding RNAs, it is well-established that circRNAs exerts important physiological functions, especially in cancers[25]. Given that circRNAs are highly stable and their expression pattern highly depends on cell specificity, circRNAs could be considered as the novel biomarker for cancer diagnosis[26]. In present study, we identified that hsa-circ-0012417 is highly enriched in human thyroid cancer tissues and it was required for thyroid cancer cell proliferation and invasion. Although, the specificity and accuracy of applying hsa-circ-0012417 in thyroid cancer diagnosis needed to further evaluation, our finding suggested that hsa-circ-0012417 could be considered as a novel biomarker candidate for thyroid cancers.

Conclusions

In this study, we demonstrated that circ-0012417 could stimulate tumor growth, and circ-0012417/miR-29c-5p/EGFL7 and circ-0012417/miR-29c-5p/LEMD1 could the possible mechanisms. Circ-0012417, EGFL7 and LEMD1 may be valuable targets in thyroid cancer treatment.

Abbreviations

ATC

anaplastic thyroid cancer (ATC)

cDNA

complementary DNA

FTC

follicular thyroid cancer (FTC)

gDNA

genomic DNA

MTC

medullary thyroid cancer

PTC

papillary thyroid cancer

Declarations

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Author information

Fan Zhang, Jing Luo and Xingyuan Shi contributed equally to this work.

Contributions

Fan Zhang and Xingyuan Shi designed and conceived the project; Jing Luo, Jiming Liu and Ting Chen performed the experiments; Guo Chen contributed to writing the manuscript; Fan Zhang, Huizeng Lv, Jianlei Hao co-supervised this study. All authors have agreed and approved the content of the manuscript.

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Ethics declarations:

Ethics approval and consent to participate

This study was approved by the ethics committee of the First affiliated hospital of Jinan University. All patients provided a written informed consent. Fresh-frozen cancer tissues and adjacent normal tissues from 30 patients with thyroid cancer were used for this study. Fresh-frozen tissues from 50 patients with benign thyroid nodules were used as control.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

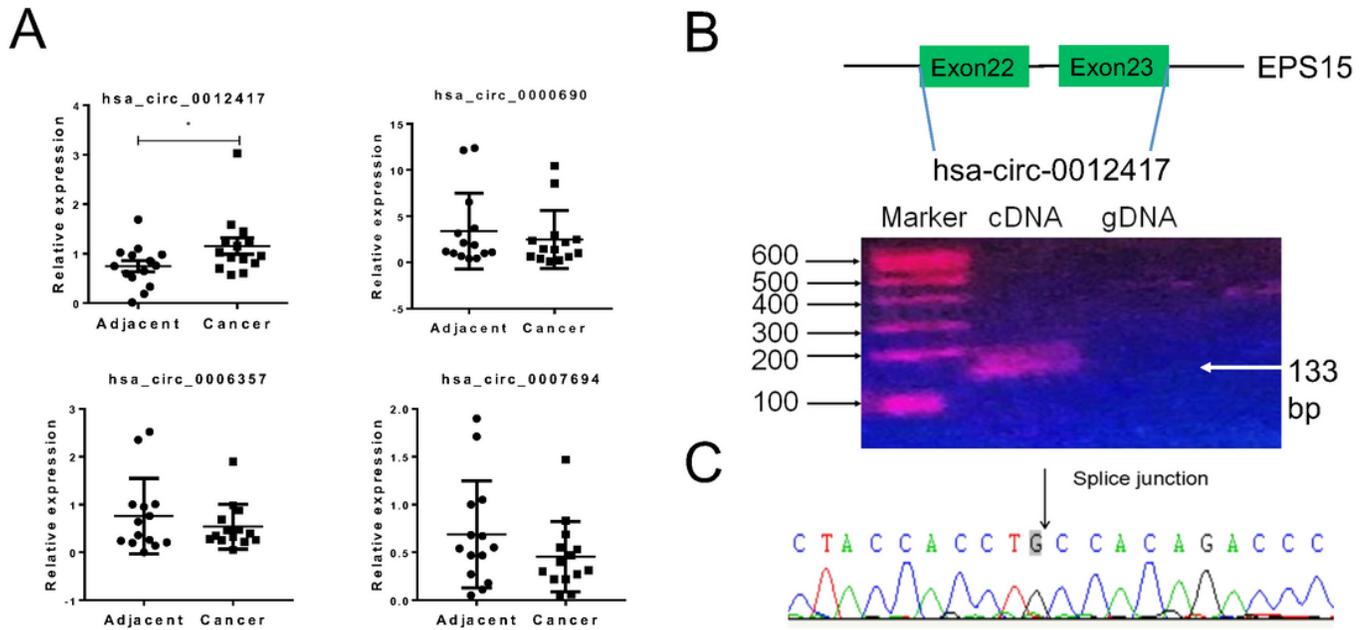


Figure 1

Hsa-circ-0012417 is overexpressed in thyroid cancer. (A) The quantitative RT-PCR analysis of hsa-circ-0012417 expression in 14 thyroid cancer tissues and their adjacent normal tissues; (B) Agarose gel electrophoresis of circ-0012417 detection from complementary DNA (cDNA) and genomic DNA (gDNA); (C) The splicing site within the circ-0012417 is shown. * $p < 0.05$ by 2-tailed t test.

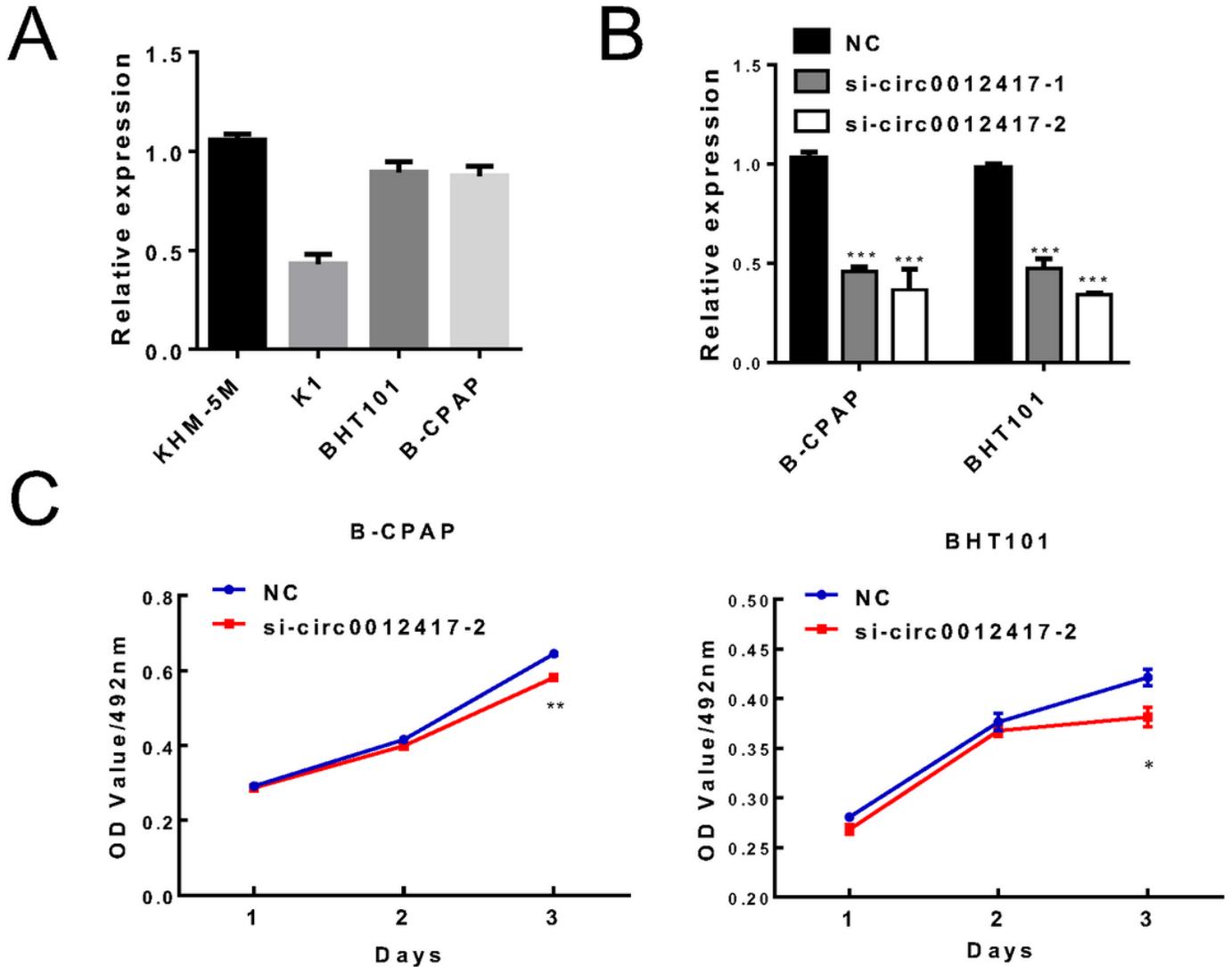


Figure 2

Transfection of si-circ0012417 inhibits the cell proliferation in thyroid cancer cells (A) The relative expression of circ-0012417 in indicated thyroid cancer cell lines was RT-PCR; (B) Quantification of circ-0012417 expression in B-CPAP, BHT101 and KHM-5M cells after transfection of its specific silence RNA (si-circ0012417); (C) CCK-8 proliferation assay of indicated cells treated with si-circ0012417. * $p < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by 2-tailed t test.

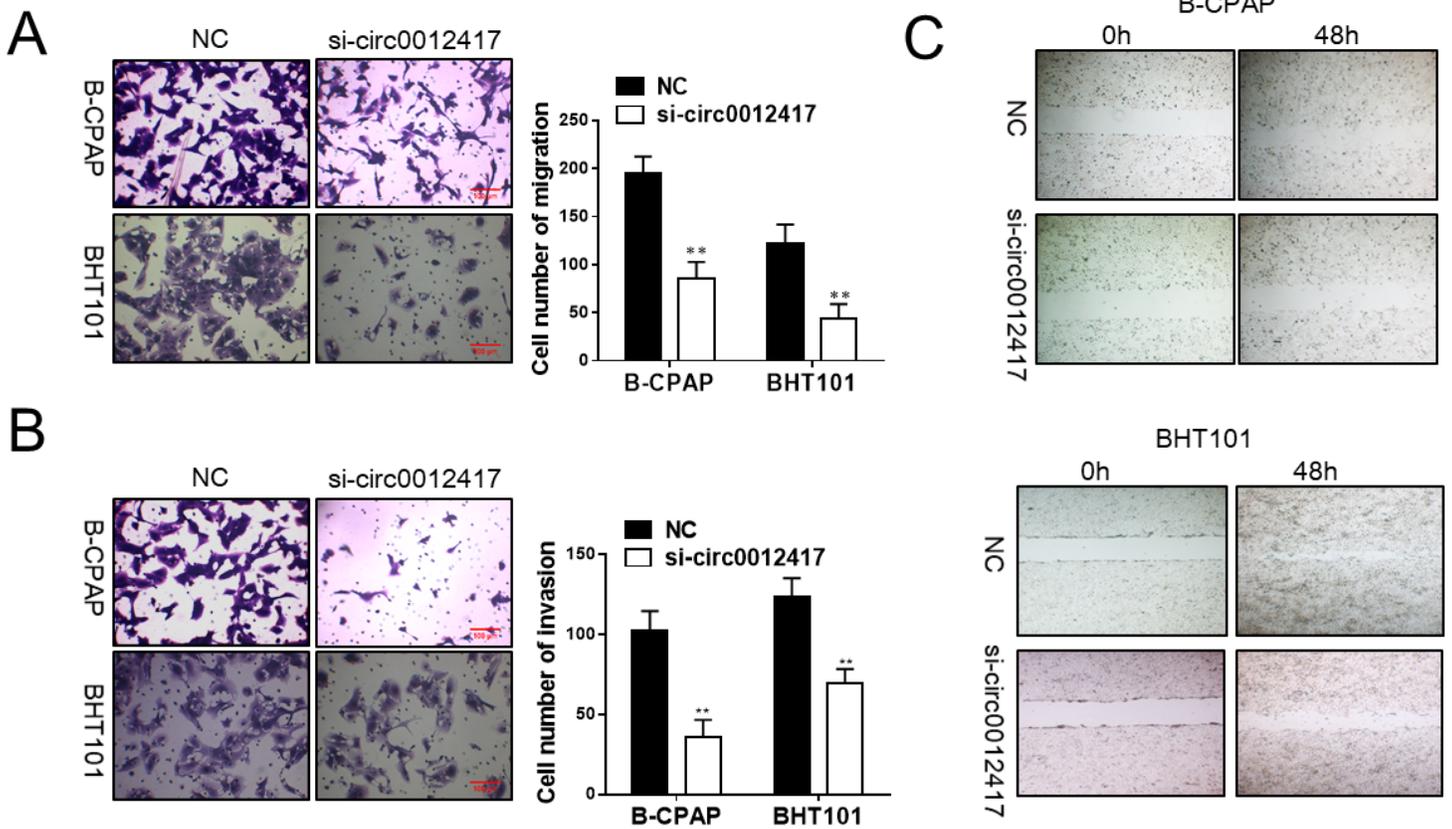


Figure 3

Transfection of si-circ0012417 inhibits the cell migration, invasion and wound healing capacity in thyroid cancer cells (A) and (B) The invasive capacity and migratory ability of B-CPAP and BHT101 ,after silencing si-circ0012417, were assessed by transwell assay with (invasion) or without (migration) Matrigel; (C) The migratory ability of indicated cells after silencing si-circ0012417 was assessed by the scratch wound assay. ** P < 0.01 by 2-tailed t test.

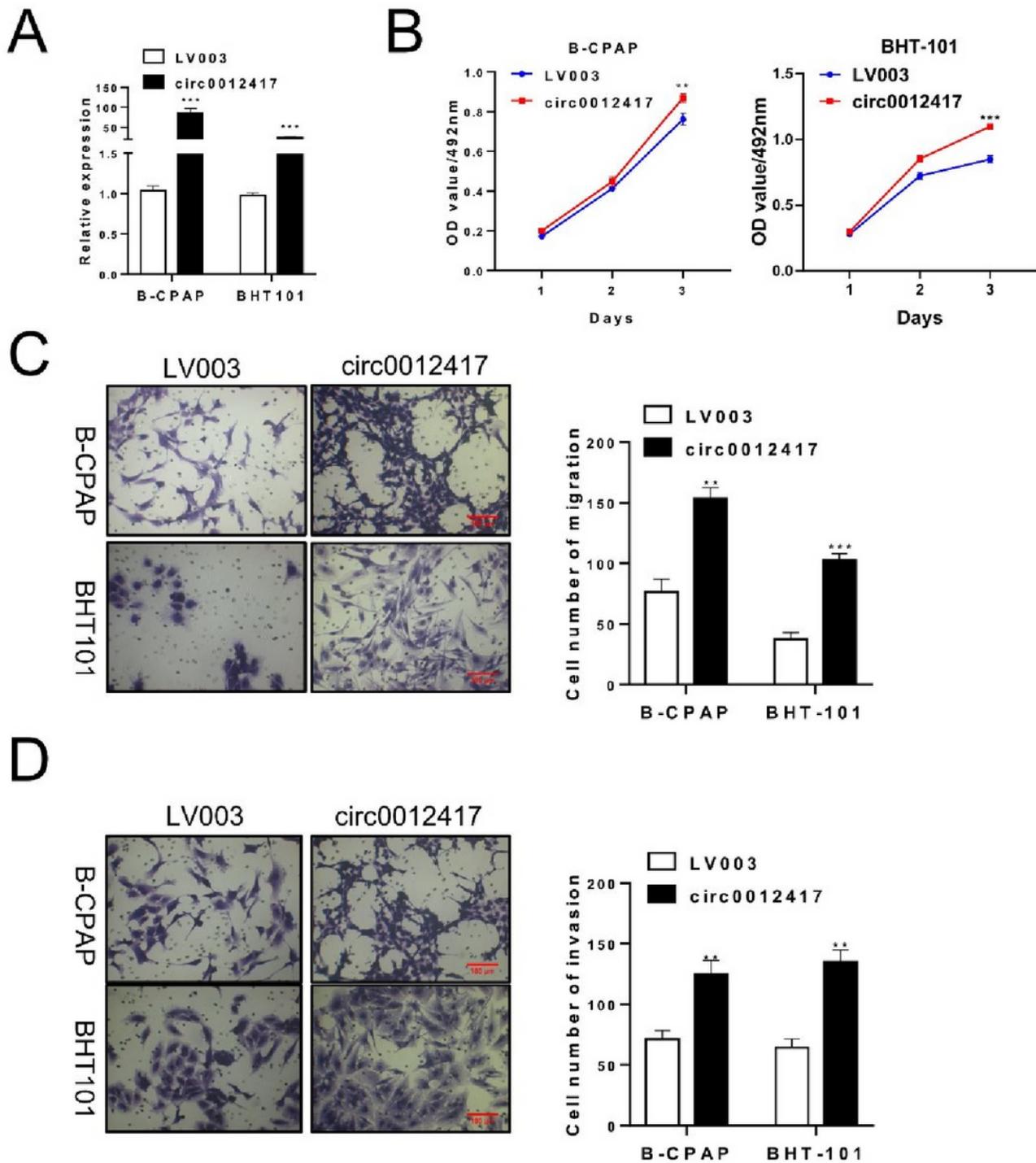


Figure 4

Enforced overexpression of ectopic circ-0012417 stimulates the cell migration and invasion in thyroid cancer cells (A) Quantification of circ-0012417 expression in B-CPAP and BHT101 cells after transfection of its specific overexpression lentivirus (LV003-circ0012417); (B) CCK-8 proliferation assay of indicated cells treated with ectopic circ0012417 expression; (C) and (D) The invasive capacity and migratory ability

of B-CPAP and BHT101, with overexpressed circ0012417, were assessed by transwell assay with (invasion) or without (migration) Matrigel. ** P < 0.01; ***P < 0.001 by 2-tailed t test.

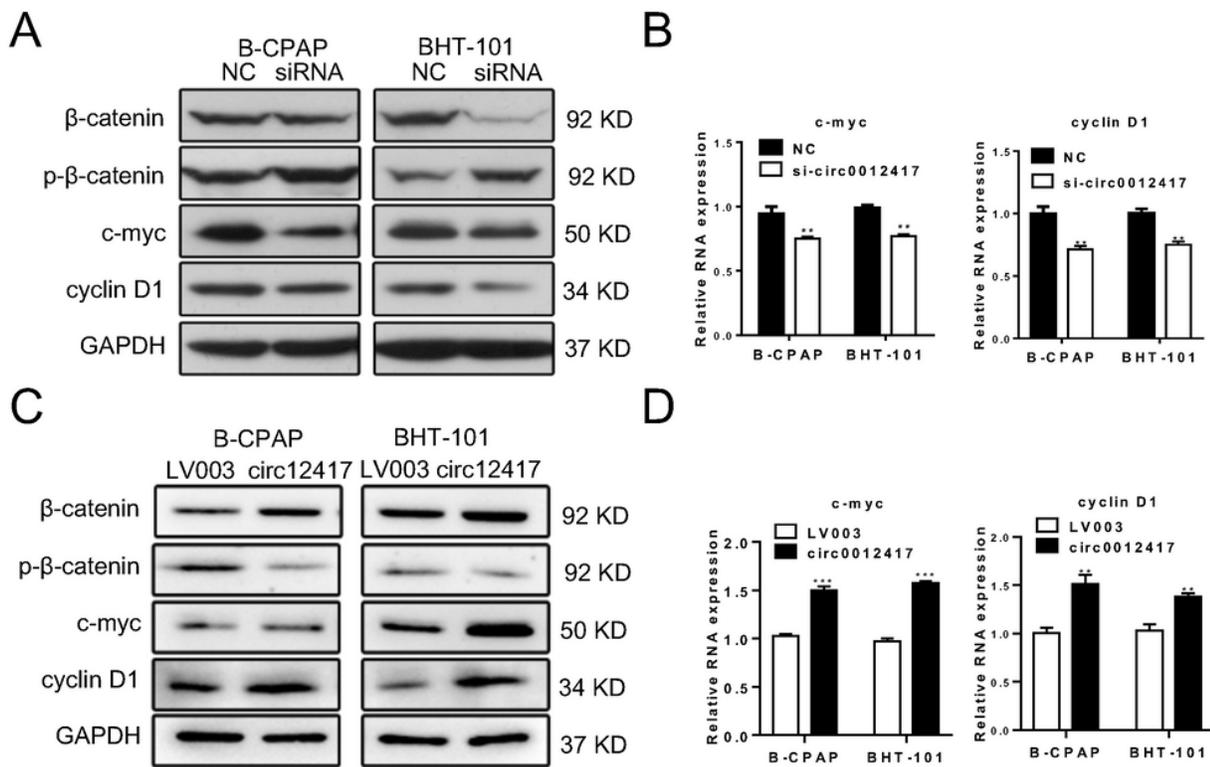


Figure 5

Transfection of circ0012417 induces cell division in thyroid cancer cells (A-B) Western blots identified β-catenin, p-β-catenin, c-myc and cyclin D1 proteins expressions changes after transfected with or without siRNA; (C-D) Western blots identified β-catenin, p-β-catenin, c-myc and cyclin D1 proteins expressions changes after transfected with or without lentivirus (LV003-circ0012417); GAPDH was used as a control. ** P < 0.01; ***P < 0.001 by 2-tailed t test.

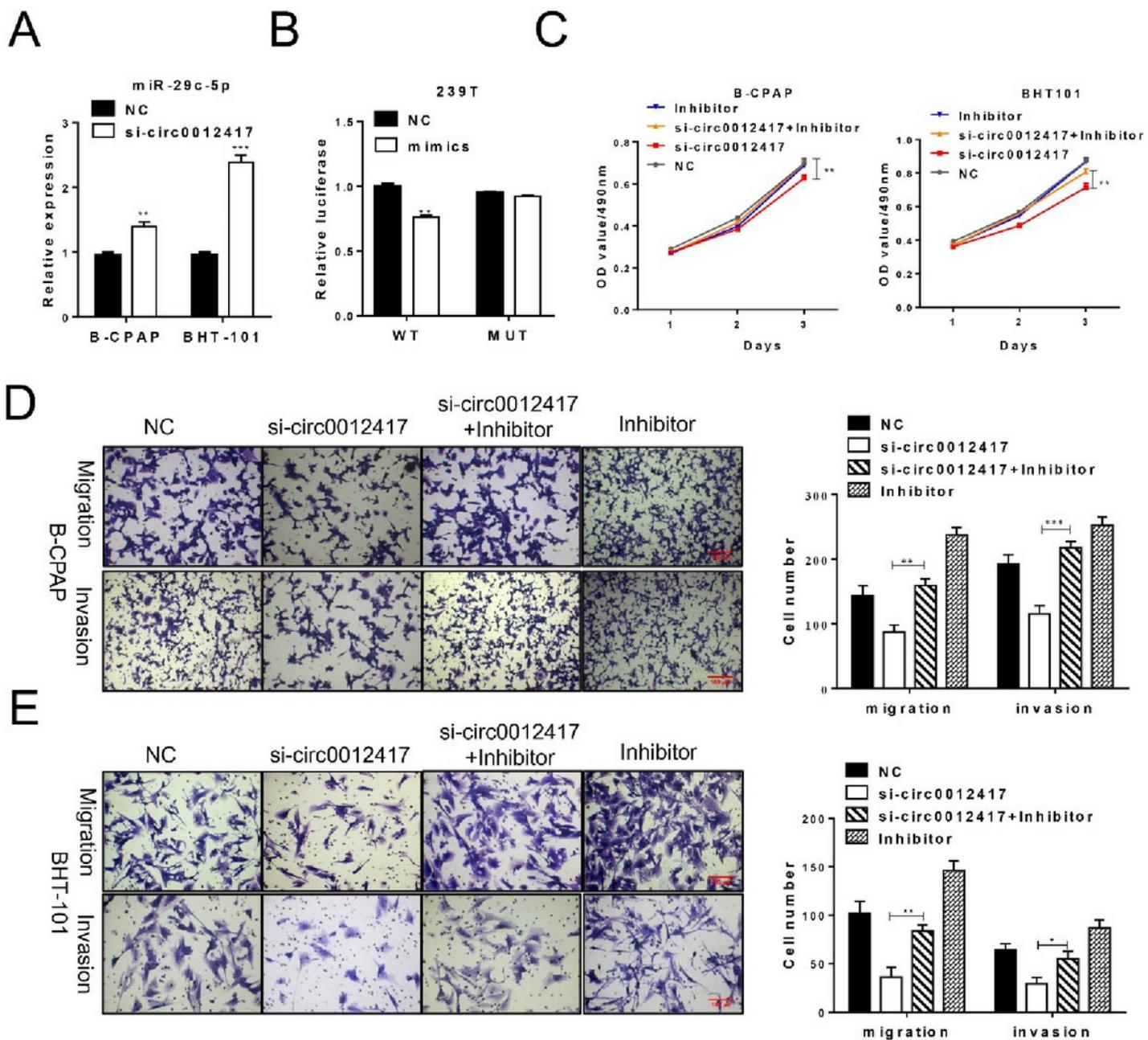


Figure 6

Inhibiting microRNA-29c reverses the tumorigenic effects of circ-0012417 in thyroid cancer cells A: Quantification of miR-29c-5p expression in B-CPAP and BHT101 cells after silencing circ-0012417; B: Luciferase activity of indicated groups in 293T cells; C: CCK-8 proliferation assay of B-CPAP cells treated with ectopic circ0012417 expression; D: The invasive capacity and migratory ability of B-CPAP and BHT101, after indicated treatment, were assessed by transwell assay with (invasion) or without (migration) Matrigel. * $p < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by 2-tailed t test.

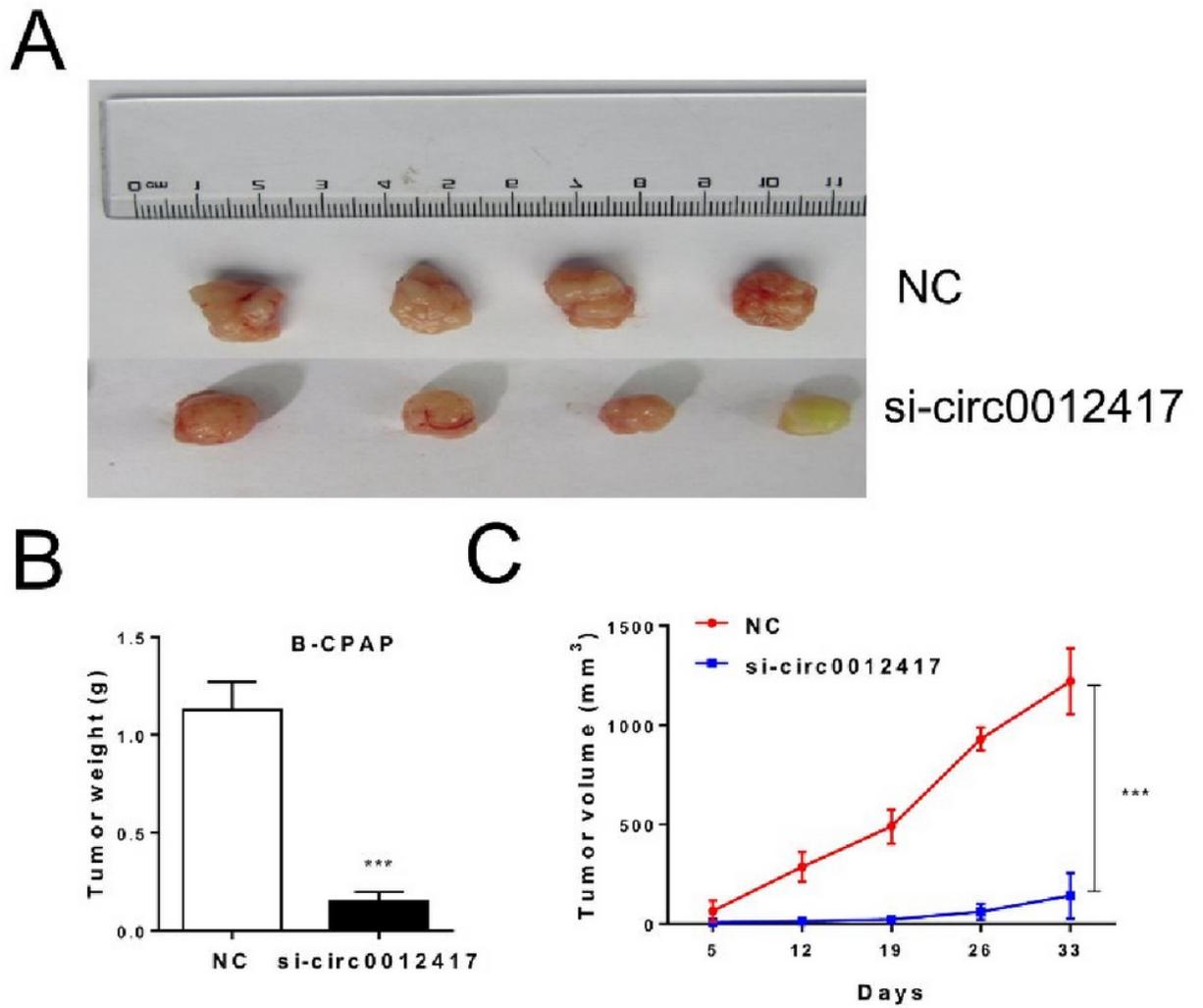


Figure 7

Silencing circ-0012417 inhibits the growth of B-CPAP xenografts in vivo.. A: compared with NC group, silencing circ-0012417 significantly inhibited tumor growth by the measurement of tumor size once a week; B: Tumor weight in silencing circ-0012417 treatment group is significantly decreased; C: At the end of the treatment, the mice were sacrificed to remove tumors. ***:P < 0.001 by 2-tailed t test.