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CircPSMC3 inhibits cell proliferation and induces cell apoptosis in nasopharyngeal carcinoma by downregulating ROCK1

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Abstract. – OBJECTIVE: Currently, the importance of circular RNAs in malignant tumors has attracted much attention. However, the role of circPSMC3 in nasopharyngeal carcinoma (NPC) remains unclear. The aim of this study was to investigate the function of circPSMC3 in the proliferation and apoptosis of NPC and to explore its possible underlying mechanism.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to determine the level of circPSMC3 in NPC tissues and cell lines. The ass between circPSMC3 expression and p ts prognosis was analyzed. CircPSMC3 len s was constructed and transfected into NPC c Cell growth ability and apoptosis were dete ed through Cell Counting Kit-8 (CCK-8) assa colony formation assay, and f metry, re- spectively. Western blot wa erfor to analyze the target protein o CPSMC urthermore, the function of ci MC3 w in nude mice.

RESULTS: CircPS wa expressed in NPC tissues c ared wi cent normal tissues. Low PSMC3 expre was associated with nosis of NP atients. Meanwhile, th xpr of circPSMC3 was significantly d wn-regu in NPC cell lines as well. growth abili NPC cells was marked nhibited after circ C3 was overexpre . Ove pression of circPSMC3 signifi y prom the apoptosis of NPC cells *in* OCK xpress decreased markedly *via* ov sion of PSMC3. Furthermore, tumor fo was bited after the up-regu- of cir *vivo*.

CONCLUSIONS: CircPSMC3 could suppress cell wth and promote cell apoptosis in NPC by g ROCK1.

Words:
lar RNA, CircPSMC3, Nasopharyngeal carcinoma ROCK1.

duction

N ryngeal carc a (NPC) is one of t ost common epithelial malignancy in the d and neck a sen from nasopharynx epi- lium. The m dity of NPC is particularly in Southe China and Southeast Asia[1]. W e adva in interventions and screening, is of patients with local and regional NPC has been significantly improved. However, relapse still occurs in approximately PC patients[2]. High incidence of lymph de tastasis and treatment resistance contributes to poor prognosis and cancer-related death of NPC, with a median survival of 12 months[3,4]. Thus, it is of great significance to identify the cellular and molecular mechanisms of NPC and to improve the prognosis for these patients.

Circular RNAs (circRNAs) are a class of noncoding RNAs, which are tissue-specific and ubiquitously expressed. Due to resistance to exonucleolytic degradation, circRNAs are more stable than linear RNA[5]. Recently, it has been reported that numerous circRNAs play an important role in tumorigenesis by serving as microRNA (miRNA) sponges. CircRNA 100146 functions as an oncogene in non-small cell lung cancer and enhances cell proliferation by binding to miR-615-5p and miR-361-3p[6]. Suppressing RUNX2 and stimulating miR-217 expression, the low expression of hsa_circ_0000144 restrains the progression of bladder cancer[7]. Hsa_circ_0005986 functions as a tumor suppressor gene in hepatocellular carcinoma by serving as a miR-129-5p sponge. Meanwhile, it may be a novel biomarker for hepatocellular carcinoma[8]. By sponging miR-370, the knockdown of hsa_circ_0061140 inhibits cell growth and

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cell metastasis in ovarian cancer[9]. Currently, circPSMC3 is a novel circRNA discovered in numerous cancers.

Our study first uncovered that circPSMC3 was significantly downregulated in NPC tissues and cell lines. Low circPSMC3 expression was associated with poor disease-free survival of NPC patients. Moreover, circPSMC3 significantly inhibited cell proliferation and promoted cell apoptosis in NPC *in vitro*. ROCK1 has been reported to participate in the progression of malignancies. Recent studies have shown that ROCK1 can be regulated by long noncoding RNAs, microRNAs, and circular RNAs. Here, we found that circPSMC3 overexpression decreased tumor formation and downregulated ROCK1 expression in NPC cells and nude mice.

Patients and Methods

Tissue Samples

A total of 48 NPC tissues and para-cancer tissues were collected from patients who received treatment in People's Liberation [illegible] Hospital 960 Ziboyuan District. The rela[illegible] between circPSMC3 expression and the p[illegible]o-sis of patients was analyzed. No radiothera[illegible] chemotherapy was performed before the surg[illegible] The research was approved by the Ethics Co[illegible] mittee of People's Liberation [illegible]pital 960 Ziboyuan District. Signed w[illegible]n in[illegible]ed consents were obtained from [illegible]particip[illegible] before the study.

Cell Culture

Human NPC c[illegible]nes (CNE2, [illegible]1, 5-8F, and 6-18B) and [illegible]asopharyngea[illegible]pithelial cell line (NP[illegible] were [illegible]ased from the American Type [illegible]ulture Colle[illegible]ATCC; Manassas, VA, USA[illegible]l cells were cu[illegible]in Dulbecco's Modifi[illegible]agle's Medium (DM[illegible]M) containing 10% [illegible] bovin[illegible]um (FBS; Life Technologies, Ga[illegible]burg[illegible], USA)[illegible]an incubator with 5% CO_2 a[illegible]

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A[illegible] 24 h o[illegible]ture on 6-well plates, NPC cells[illegible]re transfected with lentivirus targeting sp[illegible]geting circPSMC3 or scramble [illegible]or (GenePharma; Shanghai, China) accord-[illegible]to the instructions of Lipofectamine 3000 ([illegible]gen, Carlsbad, CA, USA). GFP-positive cells[illegible]re chosen for the following experiments.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The TRIzol RNA isolation [illegible]Invitrogen, Carlsbad, CA, USA) was u[illegible]d to extract total RNA in tissues and cells.[illegible]quently, the extracted RNA was[illegible]verse [illegible]ibed into complementary d[illegible]ribose nuc[illegible]ids (cDNAs) through [illegible]Revers[illegible]Transcri[illegible] Kit (TaKaRa Biote[illegible]logy C[illegible]Ltd., Dalian, China). QRT-PCR [illegible]on [illegible]itions[illegible]e as follows: 94°C f[illegible]0 s,[illegible]or 30 [illegible]72°C for 90 s, for [illegible]al of 4[illegible]s.[illegible] relative expression l[illegible]of the target g[illegible]s calculated by the [illegible]hod. Primer [illegible]quences used for RT-[illegible]R we[illegible]follows: circPSMC3, forward: [illegible]3′-GTTTAC[illegible]CCTGCCCTTTG-5′; circ[illegible], reverse:[illegible]GTGTTGGGCTG-C[illegible]CCATC-5′; glyceraldehyde 3-phosphate [illegible]ydrogenase (GAPDH) primers forward: [illegible]CCAAAATC[illegible]ATGGGGCAATGCTGG-3′ reverse 5′[illegible]ATGGCATGGACTGTGGT-C[illegible]CA-3′. G[illegible]raldehyde 3-phosphate dehydrog[illegible]DH) was used as an internal reference.

[illegible]liferation Assay

A [illegible]l of 2 ×10^3 transfected cells were first seeded into 96-well plates. Cell proliferation was assessed in accordance with the Cell Proliferation Reagent Kit I (MTT; Roche, Basel, Switzerland) at 0, 24, 48, and 72 h, respectively. Absorbance at 490 nm was detected using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay

NPC cells were seeded into 6-well plates and cultured for 10 days. Subsequently, formed colonies were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA) was used for data analysis.

Flow Cytometry

Harvested cells were washed twice using ice-cold and a flow cytometry binding buffer (100 µL) was added. The cells were stained in the dark for 15 min using a mixture containing 5 µL Annexin V/FITC (fluorescein isothiocyanate) and 5 µL Propidium Iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA). Then, 400 µL binding buffer

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was added to the cells. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze cell apoptosis.

Western Blot Analysis

Total protein in cells was extracted by reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). Subsequently, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies of rabbit anti-GAPDH and rabbit anti-ROCK1 (Cell Signaling Technology, CST, Danvers, MA, USA) overnight. On the next day, the membranes were incubated with goat anti-rabbit secondary antibody (Cell Signaling Technology, CST, Danvers, MA, USA). Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of protein expression.

Xenograft Model

After circPSMC3 overexpression, NPC cells were replanted into NOD/SCID mice (old). Tumor volume was calculated every ys as the formula (volume = length × width^2 × Tumors were extracted after 4 weeks. The search was approved by the Animal Ethics Committee of People's Liberation ital 960 Ziboyuan District.

Statistical Analysis

Statistical Prod and S Solutions (SPSS) 18.0 (SPS c., Chicago, A) was used for all st analysis. Gra ad 5.0 (GraphPad S are, La Jolla, CA, USA) was applie for image The difference between t wo groups was red by the Kaplan-M method and Student's *t*-test. $p<0.05$ was dered stically significant.

Results

The ease-f Survival Rate of NP atients

were divided into two groups, ding low circPSMC3 expression group high circPSMC3 expression group. The Meier method was used to analyze the disea free survival of patients after surgery. Compared with patients in high circPSMC3 expression group, the disease-free survi in low circPSMC3 expression grou s significantly worse (Figure 1).

Expression of CircPSMC3 in Tissues and Cells

RT-qPCR was used t tect circPS pression in 48 NPC p ts' tis s and sponding tissues. Ci MC3 e ssion was significantly lower in t su an tha adjacent tissues (Fig 2A) while, SMC3 was lowly exp d in NP (C E, CNE1, 5-8F, and 6 when com th normal nasophary thelial cell (NP69) as well (Fi 2B). sults suggested that dysregulated circPSMC associated with NPC pro .

cPSMC3 Overexpression Inhibited ll Proliferat and Induced Cell optosis in

cover her circPSMC3 affected NPC prol apoptosis, MTT assay, colony formation assay, and flow cytometry were conducted. CNE2 cells were chosen for overexpression cPSMC3 *in vitro*. RT-qPCR was used to measure the transfection efficiency (Figure 3A). As shown in Figure 3B, the MTT assay detected that the growth ability of CNE2 cells was significantly repressed after circPSMC3 overexpression. Colony formation assay indicated that the number of formed colonies was significantly reduced after circPSMC3 was overexpressed

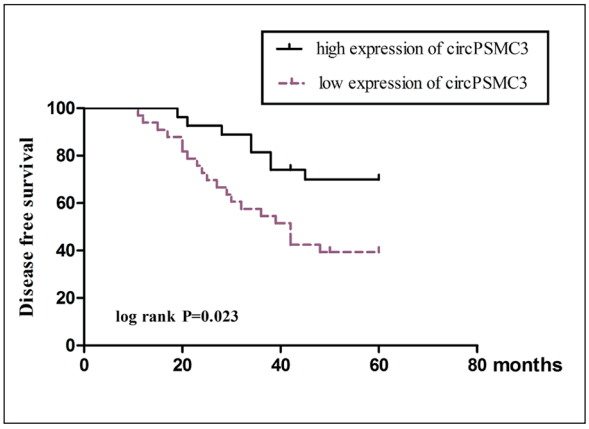

Figure 1. The association between circPSMC3 expression and the prognosis of NPC patients. Low level of circPSMC3 was associated with poor disease-free survival of NPC patients. Data were presented as mean ± standard error of the mean. *$p<0.05$.

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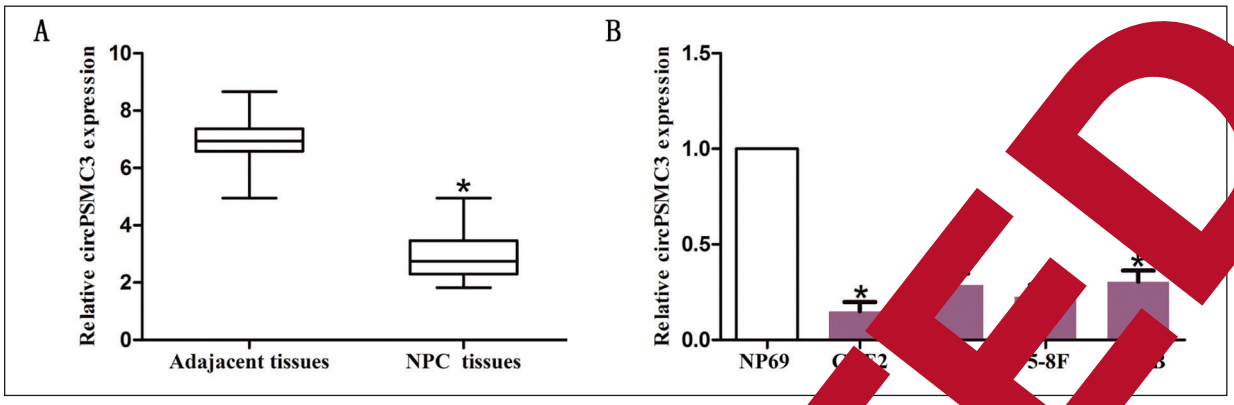
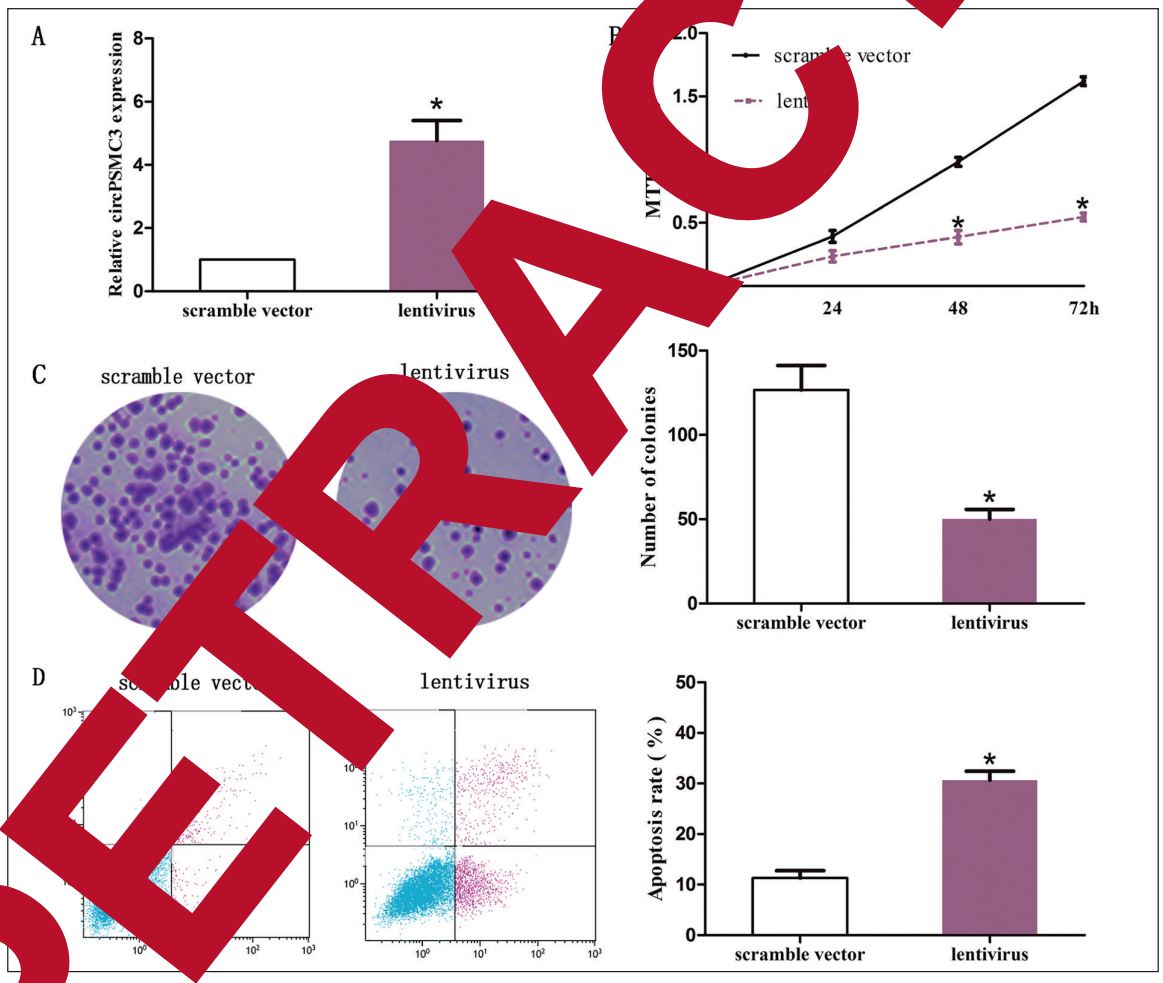


Figure 2. Expression levels of circPSMC3 increased significantly in NPC tissu d cell lines. **A,** 3 expression was significantly down-regulated in NPC tissues compared with adjacent tissu ression levels o SMC3 relative to GAPDH were determined in human NPC cell lines and normal nasopha al l cell line (NP69) by RT-qPCR. GAPDH was used as an internal control. Data were presented as mean ± sta d error ean. *p<0.05.



Fi ession of circPSMC3 inhibited cell proliferation and induced cell apoptosis in NPC. **A,** CircPSMC3 expression C cells transfected with circPSMC3 lentivirus and scramble vector was detected by RT-qPCR. **B,** MTT assay showed that expression of circPSMC3 significantly repressed the growth ability of NPC cells. **C,** Colony formation assay showed that ber of colonies was significantly reduced *via* overexpression of circPSMC3 (magnification × 40). **D,** Cell apoptosis assay sh t the apoptosis rate of NPC cells increased markedly after circPSMC3 overexpression. The results represented the averag three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells.

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(Figure 3C). Furthermore, the apoptosis rate of cells increased remarkably after upregulation of circPSMC3 in CNE2 cells (Figure 3D).

CircPSMC3 Overexpression Inhibited ROCK1 in NPC

Circular RNA Interactome (https://circinteractome.nia.nih.gov/) was used to search for target microRNAs of circPSMC3. Numerous evidence has shown that ROCK1 promotes the progression of various cancers, including NPC. We explored the interaction between ROCK1 and circPSMC3. RT-qPCR was first used to detect ROCK1 expression in CNE2 cells transfected with circPSMC3 lentivirus or vector. Results uncovered that circPSMC3 overexpression significantly decreased the mRNA expression of ROCK1 (Figure 4A). The protein level of ROCK1 was measured through Western blot assay. The results indicated that circPSMC3 overexpression reduced the protein of ROCK1 (Figure 4B). To detect the function of circPSMC3 *in vivo*, tumor formation assay was conducted in nude mice. Tumor size in circPSMC3 lentivirus group was markedly less than that in scramble vector group (Figure

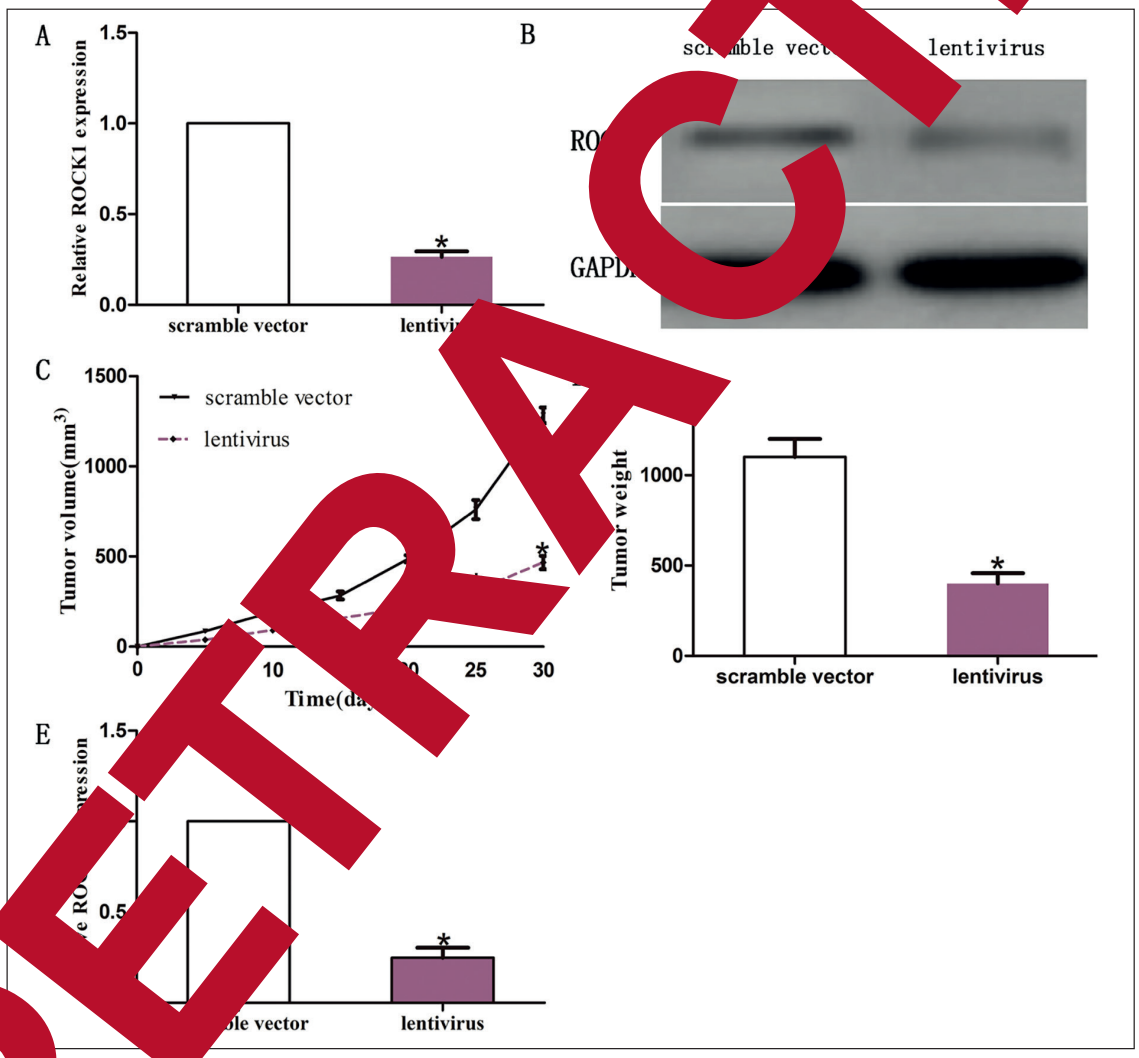

Figure 4. CircPSMC3 overexpression inhibited ROCK1 in NPC. **A,** RT-qPCR results showed that ROCK1 expression decreased in circPSMC3 lentivirus group compared with scramble vector group in NPC cells. **B,** Western blot assay showed that ROCK1 expression was down-regulated in circPSMC3 lentivirus group compared with scramble vector group. **C,** Tumor size in circPSMC3 lentivirus group and scramble vector group. **D,** Weight of dissected tumors in circPSMC3 lentivirus group was smaller than in scramble vector group. **E,** ROCK1 expression in dissected tumors of circPSMC3 lentivirus group and scramble vector group. The results represented the average of three independent experiments. Data were presented as mean ± standard error of the mean. *p<0.05.

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4C). Meanwhile, the weight of dissected tumors in circPSMC3 lentivirus group was remarkably smaller than scramble vector group (Figure 4D). Besides, the expression of ROCK1 was significantly lower in circPSMC3 lentivirus group than that of scramble vector group (Figure 4E).

Discussion

CircRNAs have been reported as potential prognostic biomarkers and therapeutic targets for many cancers, including NPC. This may offer a clinical tool for predicting treatment response and assessing disease status and clinical outcome. For instance, circHIPK3 functions as an oncogene in NPC and promotes cell proliferation and invasion *via* depressing miR-4288-induced ELF3 inhibition[10]. By competing with microRNA-150-5p, circRNA ZNF609 enhances the growth and metastasis of NPC[11]. CircRNA_0000285 is overexpressed in patients with radioresistant NPC, serving as a prognostic biomarker[12]. CircRNA_000543 decreases irradiation sensibility of NPC by targeting miR-9/platelet-derived growth factor re[illegible]/er B axis[13].

As a novel circRNA, circPSMC3 has r[illegible]ly been reported[14] to function as a tumor suppr[illegible] in gastric cancer by serving as a competi[illegible] endogenous RNA of miR-296-5p. To determi[illegible] the function of circPSMC3 in [illegible]feration, circPSMC3 lentivirus was t[illegible]lecte[illegible]o NPC cells. Function assays sh[illegible]d that [illegible]PSMC3 overexpression significa[illegible]hibite[illegible] ability of NPC cells. [illegible]rth[illegible]e explored the effect of circPS[illegible]on the [illegible]sis of NPC cells. The results [illegible]onstrated th[illegible]PSMC3 overexpression [illegible] promoted th[illegible]optosis of NPC cells. [illegible]hese [illegible]ndicated that circPSMC3 inhib[illegible]ed cell pro[illegible]n and promoted cell apop[illegible]in NPC.

The [illegible]ted pr[illegible]teins of circ[illegible]SMC3 was further [illegible]ored th[illegible]h Circular RNA Interactome (ht[illegible]ircin[illegible]tome.n[illegible]nih.gov/). Rho-associat[illegible]1 (R[illegible]1) was predicted as th[illegible] targe[illegible]in c[illegible]cPSMC3 in NPC. Cur[illegible] ROC[illegible]own as a protein serine/thre[illegible]e kinas[illegible]at has been found to play an i[illegible]rtant role in a variety of biological and pa[illegible]cesses, including cell motility, [illegible]r metastasis and so on[15]. By sponging miR-[illegible] circular RNA HIPK3 enhances the pro-g[illegible] of gallbladder cancer *via* ROCK1 and CDK[illegible]pathway[16]. Mst1 regulates cell apoptosis in non-small cell lung cancer through ROCK1/Factin pathways induced mitochondri[illegible] Silencing of URG11 represses the [illegible]ferat[illegible] and EMT in benign prostatic h[illegible]plasia cells through RhoA/ROCK1 pathw[illegible]. In addition, lncRNA LOC441178 inhibits ce[illegible]ion and migration in oral squamou[illegible]rcinom[illegible]rget ing ROCK1[19].

The potential intera[illegible] betw[illegible]ROCK[illegible] circPSMC3 was fir[illegible] explor[illegible]n our study. Results showed t[illegible]rcP[illegible]3 ov[illegible]pres-sion significantl[illegible]decr[illegible]OCK1 [illegible]ression *in vitro*. *In vi*[illegible]xperime[illegible]mo[illegible]ated that circPSMC3 [illegible]expression de[illegible] tumor formation an[illegible]ulated ROCK[illegible]expression in nude mi[illegible]. All th[illegible]e findings indicated that circPSMC3 function[illegible] tumor suppressor in NPC[illegible]gh downregu[illegible]ROCK1.

Conclusions

[illegible]n togeth[illegible]he above data indicated that circ[illegible] remarkably downregulated in NPC tissues and was correlated with poor prog-[illegible]sis of patients. Moreover, circPSMC3 inhibited [illegible]iferation and induced cell apoptosis in [illegible]PC[illegible] downregulating ROCK1. Our findings suggested that circPSMC3 might contribute to therapy for NPC as a prospective target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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