

Original Article

Downregulation of circ-TRPS1 suppressed prostatic cancer prognoses by regulating miR-124-3p/EZH2 axis-mediated stemness

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Abstract: Abnormal circular RNA (circRNA) expression correlates with human traits such as many kinds of cancers. Though circRNAs have links to cancer, they have less characterization in metastatic castration-resistant prostate cancer (PCa), which is main reason for PCa mortality. Therefore, high-throughput sequencing was used for selected circRNA profiles. The result showed that circ-TRPS1 was upregulated significantly in high-grade PCa tissues or cell lines. High circ-TRPS1 expression correlated to aggressive PCa phenotypes. Knockdown of circ-TRPS1 suppressed PCa proliferation and metastasis through targeting miR-124-3p/EZH2 axis-mediated stemness in PCa, which was validated by luciferase reporter assays. EZH2 overexpression or miR-124-3p inhibition reversed the inhibition of circ-TRPS1 silencing in PCa cell migration and proliferation by recovering stemness. In summary, data demonstrated that circ-TRPS1 suppressed PCa progression through functioning similar to a miR-124-3p sponge to enhance EZH2 expression and cancer stem-like cell differentiation. Thus, circ-TRPS1 might be a candidate target for PCa treatment.

Keywords: Prostatic cancer, circ-TRPS1, miR-124-3p, EZH2, cancer stem-like cell

Introduction

Prostate cancer (PCa) is amongst the most popular carcinomas in male. Organ-confined PCa is efficiently treated through radical prostatectomy [1]. For advanced PCa, androgen deprivation therapy is regarded as first-line treatment [2]. If hormone resistance occurs, advanced PCa is usually severe because of PCa metastasis [3]. Therefore, studying molecular mechanisms and characterizing new targets for the development of therapeutics for patients with PCa is indispensable.

Studies find that noncoding RNAs (ncRNAs), including circular RNAs (circRNAs) and microRNAs (miRNAs) function in cancer progression [4]. CircRNAs arise from backsplicing of a 3' splice donor to an upstream 5' splice acceptor. CircRNAs are transcripts that are closed covalently and are resistant to exoribonuclease degradation, which are usually more stable than

linear transcripts [5]. Aberrant circRNA expression has been discovered in many kinds of cancers such as breast [6], colorectal [7], gastric [8], and lung [9]. For prostate cancer, studies show that circLMTK2 acts as tumor suppressor in PCa through regulating microRNA-183 expression [10]. Silencing circular RNA circZNF609 restrains growth, invasion and migration through upregulating microRNA-186-5p in Pca [11]. Studies also find that circular RNA 17 [4], circAMOTL1L [12], circ-102004 [13], and circSMARCA5 [14] also function in PCa progression. Although some circRNA involvement is reported in PCa, investigations into biological mechanism and regulatory functions regarding circRNAs in PCa remain largely unclear. In this manner, the regulatory mechanisms regarding circRNAs in cancer need to be elucidated.

This investigation studied circRNA expression profiles in different grades of PCa tissues. We identified that circ-TRPS1 was increased signifi-

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cantly in high grade-PCa tissues and was related closely to PCa prognosis. We also discovered that circ-TRPS1 might act as an antioncogene sponge of miR-124-3p to enhance EZH2 expression and promote cancer stem-like cell-mediated PCa progression. As a result, circ-TRPS1 increases could function as biomarkers for prognosis and prediction, and a candidate PCa therapeutic target.

Materials and methods

Tissue samples

We collected 80 fresh PCa tissues after getting patient consent that informed in Renji Hospital Affiliated Shanghai Jiaotong University (Shanghai, China). Cancer tissues removed surgically from patients with PCa were frozen immediately in liquid nitrogen, which were stored at -80°C . We evaluated clinicopathological features of PCa following the revised International System for Staging Prostatic Cancer. The Ethics Committee in Renji Hospital Affiliated Shanghai Jiaotong University (Shanghai, China) approved this study.

Strand-specific high-throughput RNA-Seq library construction

We extracted total RNA from high-grade PCa (H-PCa) and low-grade PCa (L-PCa) tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). We used about total RNA of 3 μg from each sample with VAHTS Total RNA-seq (H/M/R) Library Prep kits from Illumina (Vazyme Biotech Co., Ltd, Nanjing, China) to remove ribosomal RNA, and retain RNA classes including noncoding RNAs and mRNAs. We treated RNA through 40 U RNase R (Epicenter) at 37°C for three hours, followed by TRIzol purification. We prepared RNA-seq libraries via KAPA Stranded RNA-Seq Library Prep kits (Roche, Basel, Switzerland) and used them for deep sequencing (Illumina HiSeq 4000 at Aksomics, Inc., Shanghai, China).

Animals

We utilized BALB/c nude mice at 4 weeks old weighing 15~20 g (SLARC, Shanghai, China). Ethics Committee at Renji Hospital Affiliated Shanghai Jiaotong University (Shanghai, China) approved the animal experiments.

Cell culture and transfection

We cultured PC3, LnCaP, and DU145 cells. We maintained them in RPMI 1640 medium (Gibco, Grand Island, NY, USA) with penicillin of 100 units/ml and streptomycin of 100 $\mu\text{g}/\text{ml}$. We maintained RWPE-1 cells in K-SMF medium (Gibco, Grand Island, NY, USA) supplemented with epidermal growth factor of 5 ng/mL and bovine pituitary extract of 50 $\mu\text{g}/\text{mL}$. We incubated cultures in humidified environment with CO_2 of 5% and 37°C .

We transfected small interfering RNAs for circ-TRPS1 (si-circ-TRPS1, 5'-TGCAGGACATCAATTC-TTCAAGG-3'), miR-124-3p mimics (5'-UAAGGC-ACGCGGUGAAUGCC-3'), miR-124-3p inhibitors (5'-CGUGUUCACAGCGGACCUUGAU-3'), small interfering RNA for EZH2 (siEZH2, 5'-GAGGGAAA-GUGUAUGAUATT-3'), and the EZH2 pCDNA3.1 overexpression vector, and their NCs into cultured PC3 or LnCaP cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to standard process. To verify the circ-TRPS1 effects using *in vivo* experiments, lentiviral stabilized circ-TRPS1 silenced (sh-circ-TRPS1) PC3 cells were constructed.

Quantitative real-time polymerase chain reaction (RT-qPCR)

We used TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to isolate total RNA from cultured tissues and cells. We determined RNA concentration and purity through a Nano-Drop 2000 spectrophotometer (Thermo Scientific, USA). We used PrimeScript™ RT reagent Kit (Takara, Japan) to synthesize cDNA. We determined miR-124-3p expression through miRNA qRT-PCR Starter kits (Riobobio, USA) and U6 and miR-124-3p (Riobobio) primers. We used the SYBR® Green Real time PCR Master Mix (Toyobo, Japan) to measure circ-TRPS1 and EZH2 expression. We normalized miR-124-3p, circ-TRPS1 and EZH2 expression to U6 and GADPH. We calculated relative miR-124-3p, circ-TRPS1 and EZH2 expressions using the $2^{-\Delta\Delta\text{Ct}}$ method. Primers for circ-TRPS1, miR-124-3p, and EZH2 were: circ-TRPS1, (Forward, 5'-CTAAATGGGACC-TATG-3'; Reverse, 5'-CAATTGTCTTAGAAGACGCTC-3'); miR-124-3p, (Forward, 5'-CTCAACTGGT-GTCGTGGAGTCGGCAATTCAGTTGAGGGCATT-CA-3'; Reverse, 5'-ACACTCCAGCTGGTAAGGC-ACGCGGTGAATGCC-3'); EZH2 (Forward, 5'-GC-

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CATTATGGACATCGCGGTGC-3'; Reverse, 5'-CTT-CGCGCTCGCGTGCCG-3'); U6 (Forward, 5'-CTC-GCTTCGGCAGCACA-3'; Reverse, 5'-AACGCTTC-ACGAATTTGCGT-3'); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Forward, 5'-AC-AACTTTGGTATCGTGAAGG-3'; and Reverse, 5'-GCCATCACGCCACAGTTTC-3').

Fluorescence in situ hybridization (FISH)

We obtained specific probes for circ-TRPS1 (Dig-5'-CTATGATGTGCAGATGTACAGATCAGCTC-3'-Dig) from Genesee Biotech (Guangzhou, China). Cells were immunostained with Cy3-conjugated antidigoxin and Cy3-conjugated anti-biotin antibodies (Jackson ImmunoResearch Inc., West Grove, PA, USA). We counterstained nuclei through 4,6-diamidino-2-phenylindole (DAPI). We visualized staining through Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Bioinformatic analysis

We identified circRNA/miRNA target genes with *CircularRNA Interactome*. We predicted interactive correlations between miR-124-3p and EZH2 through *TargetScanHuman*.

Tumor sphere formation assays

We harvested PC3 and LnCaP cells, and resuspended them as single cells in serum-free medium. After cell counting, we added 200 cells/well in 200 μ L serum-free medium to 96-well plates, 10 wells per group, changing medium every 2 days. We obtained images of five randomly selected regions for each group of wells with a camera-equipped micro-plate reader (Leica, Wetzlar, Germany) and calculated sphere percentage as number of spheres/200.

Cell proliferation assays

We employed Cell Counting Kit-8 assays (CCK-8; Gibco, Logan, Utah, USA) to quantify cellular proliferation. We seeded transfected cells into plates with 96 wells with 5,000 cells/well in triplicate, and determined cell viability with a CCK-8 system at 0, 24, 48 and 72 h after seeding, following given procedures.

For colony formation assays, we seeded cells that transfected into plates with 6 wells at 2,000 cells/well and maintained them in 1640

containing FBS of 10% for ten days. We imaged and counted colonies after fixing and staining.

Transwell assays

For invasion assays, we placed Transwell assay inserts (Millipore, Billerica, MA, USA) into plates with 24 wells, coating membranes in upper chambers with Matrigel (BD Biosciences). We placed 500 μ L 1640 containing 10% FBS into bottom chambers and 10,000 cells in 200 μ L serum-free 1640 in upper chambers. After 1~2 days, we utilized methanol to fix cells on membranes and stained them with crystal violet before observing them via microscope (Leica).

Western blots

We extracted protein from tissues or cells with RIPA lysis buffer and performed western blots as previously described [15]. Primary antibodies were against OCT4, Nanog and GAPDH (Cell Signaling Technology, Beverly, MA, USA) and blots were stained following standard procedures. We visualized immunoreactivity with chemiluminescence detection kits (Western Blotting Substrate, Donghuan Biotech, China).

Dual-luciferase reporter assays

We inserted binding sites for circ-TRPS1, termed circ-TRPS1-WT and circ-TRPS1-Mut, and the EZH2 3'-UTR, termed EZH2-3'UTR-WT, and EZH2-3'UTR-Mut, into HindIII and KpnI sites regarding a pGL3 promoter vector (Realgene, Nanjing, China) for dual-luciferase reporter assays. We plated cells into plates with 24 wells, and transfected 80 ng plasmid, 50 nM miR-124-3p mimics, 5 ng Renilla luciferase vector pRL-SV40, and NC reagents into cells through Lipofectamine 2000 (Invitrogen). We collected and measured cells 2 d after transfection with Dual-Luciferase Assay kits (Promega, Madison, WI, USA), following standard instructions. We independently repeated all experiments three times.

Animal studies

For xenograft assays, we subcutaneously injected 1×10^6 modified (circ-TRPS1-silenced) or NC PC3 cells into right sides of male nude mice. We calculated tumor volumes (length * width² * 0.5) at timepoints indicated and excised tumors 1 m after injection.

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For metastasis analysis, we transfected 2×10^5 NC or circ-TRPS1-silenced PC3 cells with luciferase expression vectors, and injected cells intravenously into mouse tails. After 30 d, PC3 cell metastases were analyzed by bioluminescence imaging following an intravenous injection of luciferin (150 mg luciferin/kg body weight) into tails.

Immunohistochemistry

We fixed tumor tissue samples in formalin 10% and embedded them in paraffin. We stained sections (5- μ m) with Ki67 to explore proliferation. We examined sections with an Axiophot light microscope and imaged them via digital camera.

Statistics analyses

We assessed differences among groups via paired/unpaired two-tailed *t*-tests using Pearson's correlation tests to obtain associations among groups. We denoted data by mean \pm SEM. *P*-values < 0.05 indicated significant. We performed all statistical analysis with GraphPad Prism (GraphPad Inc., San Diego, CA, USA).

Results

High Circ-TRPS1 expression predicted unfavorable prognoses for PCa

To determine correlations between circRNA expression and PCa progression, three PCa samples from H-PCa and three from L-PCa were used in circRNA high-throughput sequencing. The results showed a series differentially expressed circRNA in PCa tissues comparing (Figure 1A). RT-qPCR illustrated the six upregulated circRNA expressions in H-PCa and L-PCa tissues. The results showed that hsa_circ_0006950 (circ-TRPS1) had the highest expression in H-PCa tissues comparing with L-PCa tissues (Figure 1B). RT-qPCR also showed that circ-TRPS1 expression increased in H-PCa tissues compared to L-PCa patients (Figure 1C). FISH assays demonstrated that circ-TRPS1 expression increased in H-PCa tissues compared with L-PCa tissues. Circ-TRPS1 was localized predominantly to the cytoplasm (Figure 1D). To further understand the prognostic value of circ-TRPS1 expression, we analyzed correlations with patient characteristics. High circ-TRPS1 expression correlated with higher pathological T stage, lymph node metastasis, and

distant metastasis (Table 1). Circ-TRPS1 is derived from circularizing exons from gene *TRPS1*, which are at chr8:116631358-116635985. *TRPS1* is 4627 bp and its spliced mature circRNA is 1087 bp (Figure 1E). The findings indicated that circ-TRPS1 functioned in PCa progression.

Downregulation of circ-TRPS1 suppressed PCa proliferation in vivo and in vitro

To identify circ-TRPS1 functions in PCa proliferation, we employed RT-qPCR to detect circ-TRPS1 expression. Circ-TRPS1 expression increased in PCa cell lines PC3, LnCaP, and DU145 compared with RWPE-1 (Figure 2A). PC3 and LnCaP cells had the highest circ-TRPS1 levels, so they were selected for further experiments. Circ-TRPS1 expression decreased significantly in both PC3 and LnCaP cells after transfection with an siRNA against circ-TRPS1 (Figure 2B). CCK8 (Figure 2C, 2D) and colony formation assays (Figure 2E, 2F) showed that circ-TRPS1 silencing decreased cell proliferation in PC3 and LnCaP cells. Xenograft results validated that circ-TRPS1 knockdown suppressed PC3 tumor growth (measured as volume and weight) comparing with an NC group (Figure 2G-I). Immunohistochemical Ki67 detection demonstrated that circ-TRPS1 silencing suppressed Ki67 expression in tumor tissues (Figure 2J, 2K), supporting that circ-TRPS1 knockdown suppressed tumor growth. Together, these data suggested that knocking down circ-TRPS1 suppressed the proliferation ability of PCa cells *in vivo* and *in vitro*.

circ-TRPS1 downregulation suppressed PCa invasion in vitro and in vivo

Transwell detection showed that circ-TRPS1 knockdown suppressed cell migration in PC3 and LnCaP cells (Figure 3A, 3B). The metastasis ability of PC3 cells also decreased after circ-TRPS1 silencing by decreased metastatic foci using live imaging analysis of metastasis model mice (Figure 3C, 3D). Together, these data suggested that knocking down circ-TRPS1 suppressed invasion by PCa cells *in vivo* and *in vitro*.

miR-124-3p and EZH2 are downstream targets of circ-TRPS1 in PCa

Next, we investigated how circ-TRPS1 regulation affected PCa proliferation and invasion.

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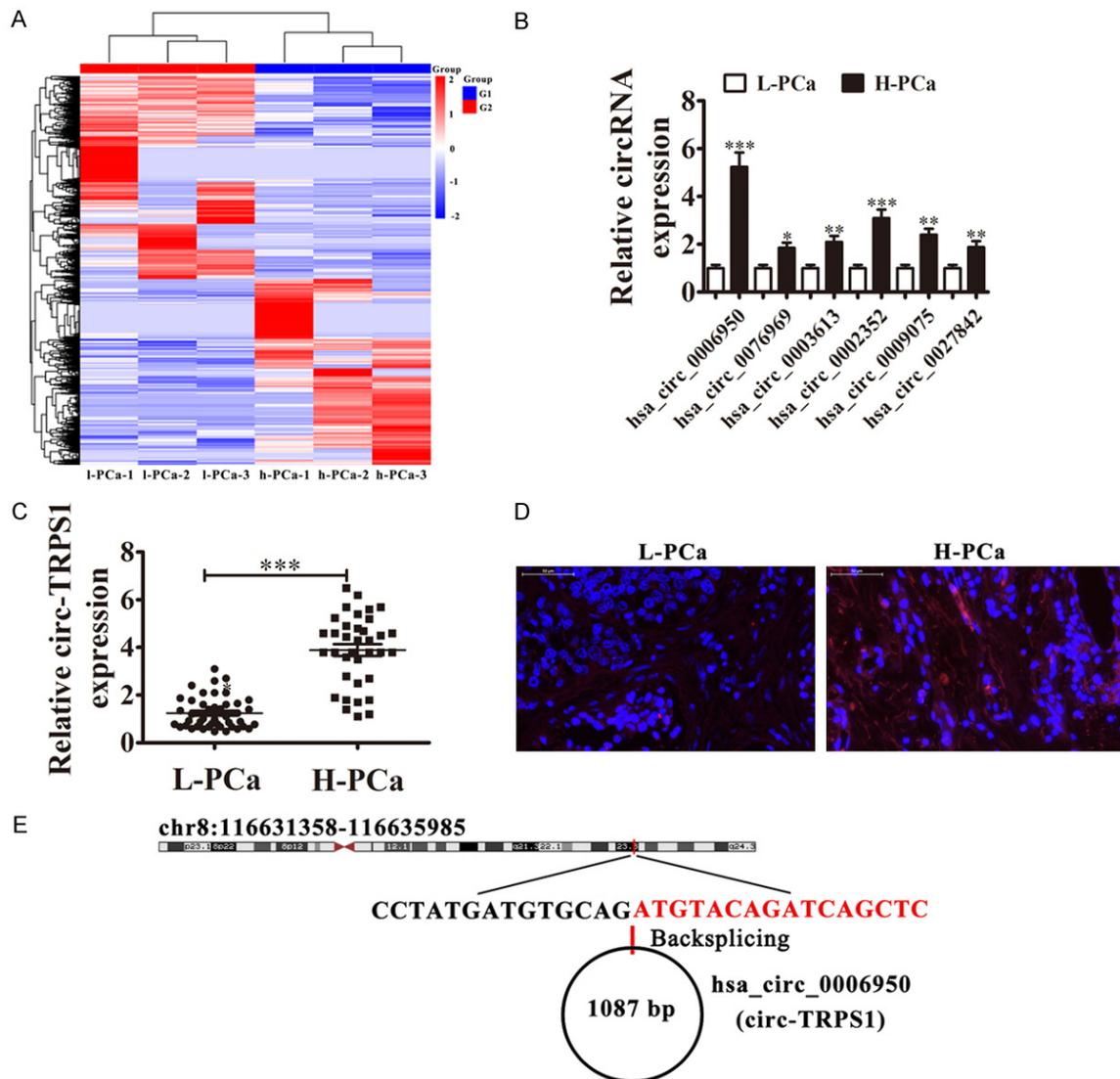


Figure 1. Analysis of circular RNA expression in human PCa tissues and cell lines. A. Heat map with expression of circRNA from high-grade PCa (H-PCa) and low-grade PCa (L-PCa) after high-throughput sequencing. B. RT-qPCR of expression of six upregulated circRNAs in H-PCa and L-PCa tissues. Data are means \pm SD; *P < 0.05, **P < 0.01, ***P < 0.001 vs. L-PCa group. C. RT-qPCR for circ-TRPS1 expression in L-PCa (n = 44) and H-PCa (n = 36). Data are means \pm SD; ***P < 0.001 vs. L-PCa group. D. Fluorescence *in situ* hybridization (FISH) of circ-TRPS1 (red) combined with nuclear DAPI staining (blue) in L-PCa and H-PCa tissues. E. Location of circ-TRPS1 on the human chromosome.

Previous studies found that circRNA acts as a miRNA sponge. Given that circ-TRPS1 is mainly localized to the cytoplasm, we hypothesized that circ-TRPS1 might regulate tumor biological behavior by acting as a sponge to miRNAs. We employed Interactome to predict miRNAs. The data showed that miR-124-3p might have circ-TRPS1 acting as sponge (Figure 4A). To validate the association between circ-TRPS1 and miR-124-3p, we prepared wild-type (WT) or mutated (MUT) circ-TRPS1 sequences that included

miR-548 binding sequences into a luciferase reporter vector (Figure 4A). We transfected this reporter vector into 293T cells with or not miR-124-3p mimic. Luciferase reporter analysis suggested that miR-124-3p inhibited luciferase activity in WT-transfected, while not MUT-transfected cells (Figure 4B), suggesting that miR-124-3p was circ-TRPS1 target. FISH assays demonstrated that both circ-TRPS1 and miR-124-3p were co-localization in the cytoplasm (Figure S1).

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Table 1. Correlation between circ-TRPS1 expression and clinicopathologic characteristics of PCa patients

Clinicopathological Features	Overall (n = 80)	High (n = 36)	Low (n = 44)	p-Value
Clinical T Stage				
T1 and T2	59	22	37	0.006
T3 and T4	21	14	7	
Lymph Node Metastasis				
Yes	16	12	4	0.01
No	64	24	40	
Distant Metastasis				
Yes	14	10	4	0.03
No	66	26	40	

Bioinformatic analyses (TargetScanHuman) discovered that EZH2 was a miR-124-3p downstream target. To detect the association between miR-124-3p and EZH2, we constructed WT or MUT 3'UTR-EZH2 sequences that included miR-548 binding sequences into a luciferase reporter vector (**Figure 4C**). We transfected this reporter vector into 293T cells with or without miR-124-3p mimic. Luciferase reporter analysis demonstrated that miR-124-3p inhibited luciferase activity in WT-transfected but not MUT-transfected cells (**Figure 4D**), suggesting that EZH2 was a miR-124-3p target.

To identify the regulatory relationships among circ-TRPS1, miR-124-3p and EZH2, we transfected PC3 and LnCaP cells with si-circ-TRPS1, miR-124-3p inhibitor or EZH2 overexpression vector, either singly or in combination. RT-qPCR verified that circ-TRPS1 expression decreased significantly after transfection with siRNA against circ-TRPS1. MiR-124-3p downregulation or EZH2 overexpression did not reverse circ-TRPS1 expression in PC3 and LnCaP cells (**Figure 4E, 4F**). Circ-TRPS1 downregulation increased miR-124-3p expression in miR-124-3p cells. Treating cells with an miR-124-3p inhibitor suppressed miR-124-3p expression, but EZH2 overexpression did not reverse miR-124-3p expression (**Figure 4G, 4H**). We discovered that silencing circ-TRPS1 decreased EZH2 expression in LnCaP and PC3 cells, while treatment with an miR-124-3p inhibitor partially recovered EZH2 expression. Transfecting with an EZH2 overexpression vector increased EZH2 expression (**Figure 4I, 4J**). The results demonstrated that miR-124-3p and EZH2 were downstream targets of circ-TRPS1 and that

EZH2 was a miR-124-3p downstream target.

Downregulating miR-124-3p or overexpressing EZH2 reversed inhibition by si-circ-TRPS1 of PCa proliferation and migration via cancer stem cell differentiation

Colony formation assays (**Figure 5A-C**) validated that silencing circ-TRPS1 suppressed PC3 and LnCaP cell proliferation. Further silencing miR-124-3p or overexpressing EZH2 rescued PC3 and LnCaP cell proliferation. Transwell assays to measure cell migration (**Figure 5D-F**) also found that silencing miR-124-3p or overexpressing EZH2 rescued migration of both PC3 and LnCaP cells.

We conducted tumor sphere formation assays. Downregulation of circ-TRPS1 reduced cancer stem cell differentiation by inhibiting tumor sphere formation. These data implied that downregulating miR-124-3p or overexpressing EZH2 enhanced the sphere percentage in LnCaP and PC3 cells (**Figure 5G-I**). Western blots showed that downregulating miR-124-3p or overexpressing EZH2 promoted expression of stemness markers OCT4 and Nanog in both PC3 and LnCaP cells even after circ-TRPS1 silencing (**Figure 5J, 5K**). These results suggested that circ-TRPS1 promotion enhanced PCa progression via regulating miR-124-3p/EZH2 axis-mediated stemness.

Overexpressing EZH2 restored cancer stem cell-mediated invasion and PCa cell proliferation after miR-124-3p overexpression

RT-qPCR illustrated that miR-124-3p expression increased significantly in PC3 and LnCaP cells after transfection with a miR-124-3p mimic. Upregulating EZH2 did not reverse miR-124-3p levels (**Figure 6A, 6B**). RT-qPCR demonstrated that miR-124-3p upregulation suppressed EZH2, but after transfecting with an EZH2 overexpression vector, expression of EZH2 significantly increased (**Figure 6C, 6D**). Colony formation (**Figure 6E-G**) assays validated that upregulating miR-124-3p suppressed PC3 and LnCaP cell proliferations. Overexpressing EZH2 rescued PC3 and LnCaP cell proliferation. Transwell assays to measure cell

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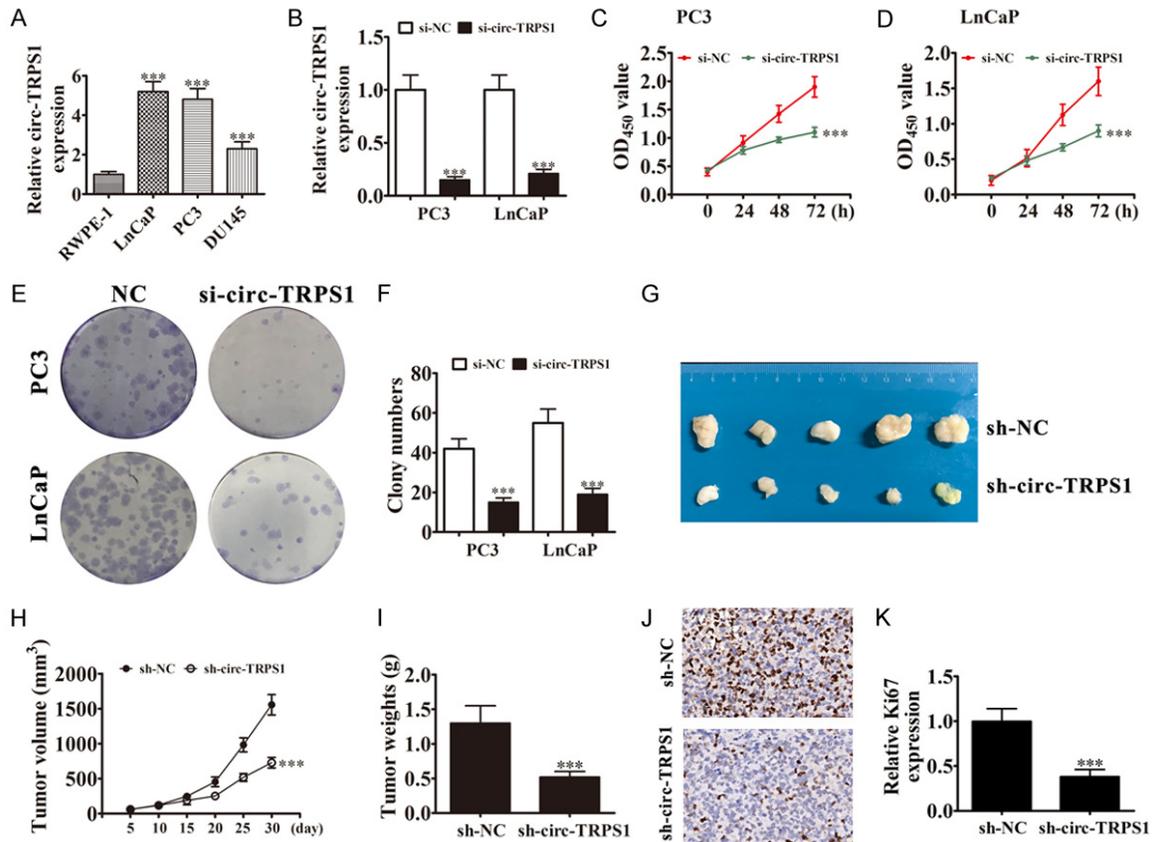


Figure 2. Downregulation of circ-TRPS1 suppressed PCa proliferation *in vitro* and *in vivo*. (A) Expression of circ-TRPS1 in noncancer and cancer cell lines analyzed by RT-qPCR. Data are means \pm SD; ***P < 0.001 vs. RWPE-1 group. (B) RT-qPCR detected circ-TRPS1 in PC3 and LnCaP after transfecting with negative control (NC) or siRNA against circ-TRPS1 (si-circ-TRPS1). Data are means \pm SD; ***P < 0.001 vs. si-NC group. (C and D) CCK8 detection showed proliferation of PC3 and LnCaP cells. Data are means \pm SD; ***P < 0.001 vs. si-NC. (E and F) Colony formation assays showing proliferation of PC3 and LnCaP cells. Data are means \pm SD; ***P < 0.001 vs. si-NC. (G) PC3 cells transfected with NC or sh-circ-TRPS1 and subcutaneously injected into nude mice. (H) Tumor growth curves, (I) tumor weight. Data are means \pm SD; ***P < 0.001 vs. sh-NC. (J and K) Immunohistochemistry for the percentage of Ki67-positive cells. The relative Ki67 expression was calculated. Data are means \pm SD; ***P < 0.001 vs. sh-NC.

migration (**Figure 6H-J**) found that overexpressing EZH2 rescued migration of LnCaP and PC3 cells after upregulation of miR-124-3p.

We conducted tumor sphere formation assays. Upregulating miR-124-3p decreased cancer stem cell differentiation by inhibiting tumor sphere formation. These results indicated that overexpressing EZH2 increased the sphere percentage in LnCaP and PC3 cells (**Figure 6K-M**). Western blots showed that overexpressing EZH2 increased expression of stemness markers OCT4 and Nanog expression in LnCaP and PC3 cells even after upregulating miR-124-3p (**Figure 6N, 6O**). The results suggested that miR-124-3p decreased PCa progression by regulating EZH2-mediated stemness.

Downregulation of miR-124-3p or overexpression of EZH2 restores the growth ability of PCa after silence circ-TRPS1 in vivo

Xenograft results validated that circ-TRPS1 knockdown suppressed PC3 tumor growth (measured as volume and weight) comparing with an NC group. Downregulation of miR-124-3p or overexpression of EZH2 restores the growth ability of PCa after silence circ-TRPS1 (**Figure 7A-C**). Immunohistochemical Ki67 detection demonstrated that circ-TRPS1 silencing suppressed Ki67 expression in tumor tissues. Downregulation of miR-124-3p or overexpression of EZH2 restores Ki67 expression (**Figure 7D, 7E**), supporting that circ-TRPS1 knockdown suppressed tumor growth by regulation miR-124-3p/EZH2 axis.

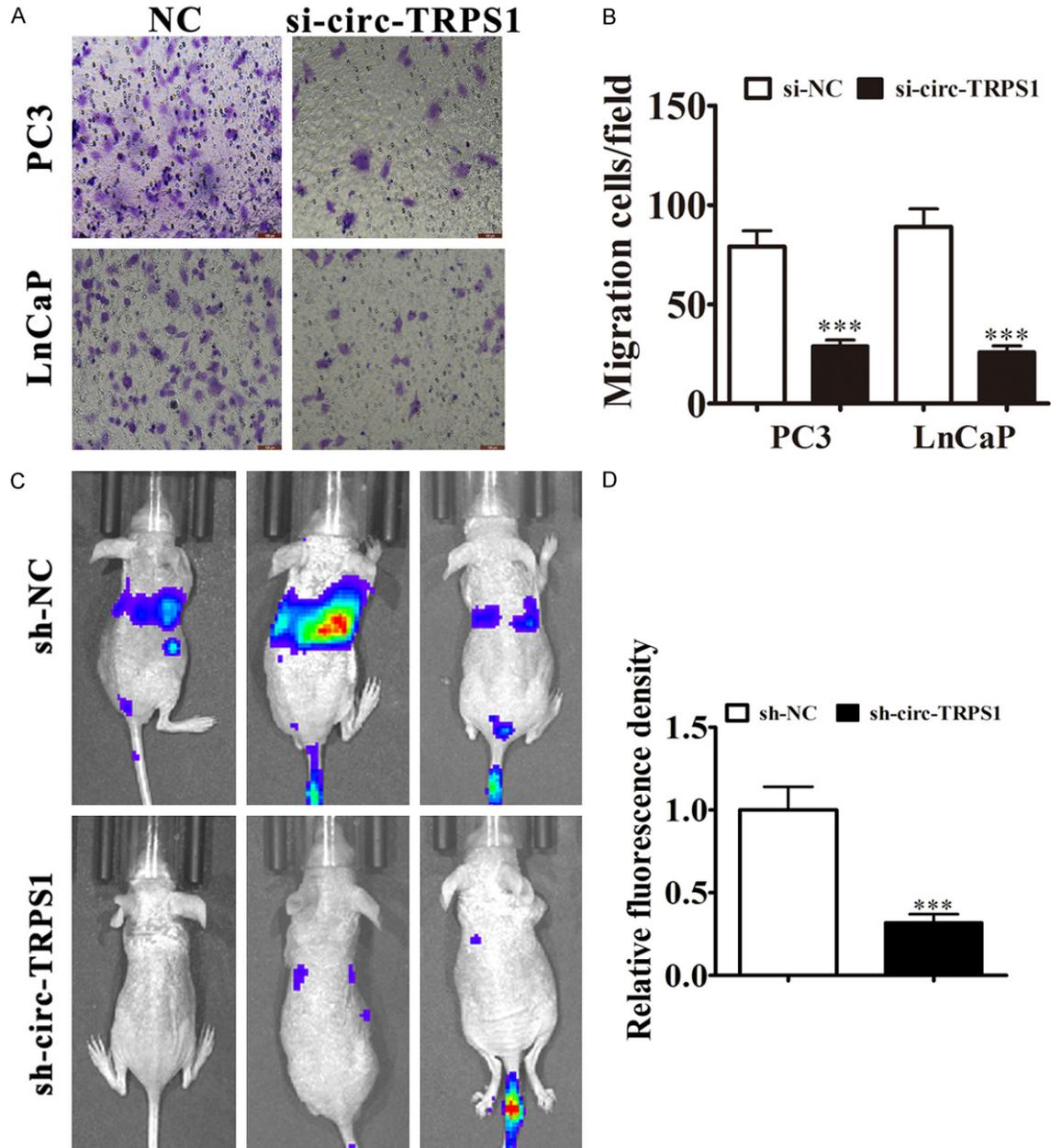


Figure 3. Downregulation of circ-TRPS1 decreased PCa invasion *in vitro* and *in vivo*. A, B. Cell migration assessed in PC3 and LnCaP cells using Transwell assays. Data are means \pm SD; ***P < 0.001 vs. NC. C, D. Live imaging showing the effects of circ-TRPS1 on metastasis of PC3 cells 30 d after intravenous tail injection. The relative fluorescence density was calculate. Data are means \pm SD; ***P < 0.001 vs. sh-NC.

Discussion

Increasingly, studies find that abnormal expression of circRNA is associated with human traits such as cancers [2]. Studies have found that silencing circZNF609 restrains growth, invasion and migration through upregulating microRNA-186-5p in Pca [11]. Upregulated circ-ZMIZ1 enhances PCa cell proliferation and is a

valuable plasma marker [16]. In this study, we investigated expression of circRNA using H-PCa and L-PCa tissues and high-throughput sequencing. The results showed that circ-TRPS1 significantly increased in H-PCa samples compared with L-PCa. High expression of circ-TRPS1 indicated poor progress, high pathological T stage, lymph node metastasis, and distant metastasis. This result suggested that circ-

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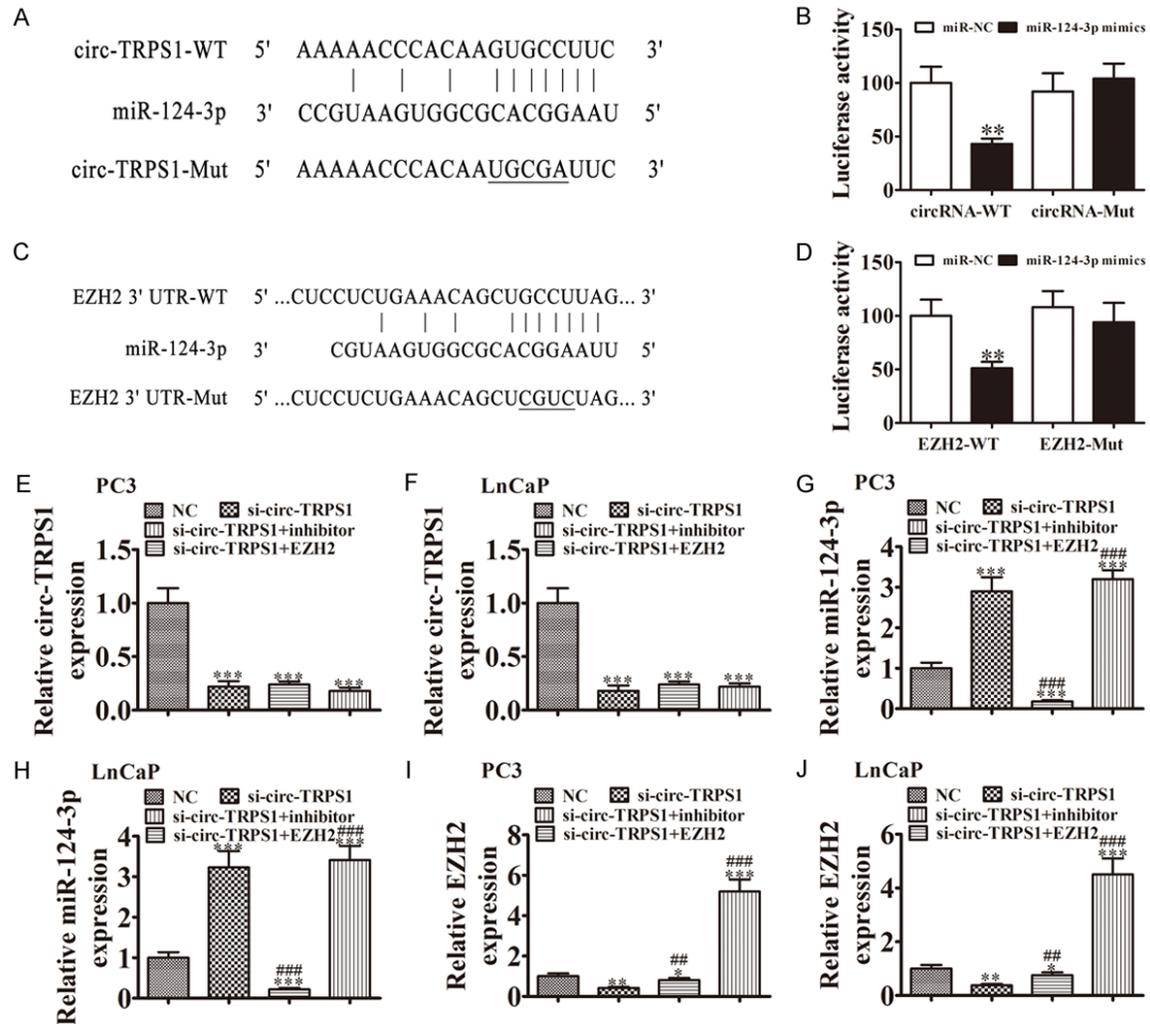


Figure 4. MiR-124-3p and EZH2 were downstream targets of circ-TRPS1. (A) Bioinformatics analysis predicting binding sites of miR-124-3p in circ-TRPS1. Mutant version of circ-TRPS1 is shown. (B) Relative luciferase activity determined 48 h after transfection of HEK293T cells with miR-124-3p mimic/NC or circ-TRPS1 wild-type/Mut. Data are means \pm SD. ** $P < 0.01$. (C) Prediction of miR-124-3p binding sites in the 3'-UTR of EZH2. Mutant version of 3'-UTR-EZH2 is shown. (D) Relative luciferase activity 48 h after transfection of HEK293T cells with miR-124-3p mimic/NC or 3'-UTR-EZH2 wild-type/Mut. Data are means \pm SD. ** $P < 0.01$. (E-J) RT-qPCR showing expression of (E and F) circ-TRPS1, (G and H) miR-124-3p, (I and J) EZH2 in PC3 and LnCaP cells. Data are means \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC. ### $P < 0.01$, #### $P < 0.001$ vs. si-circ-TRPS1.

TRPS1 was involved in the progress of PCa. FISH validated that circ-TRPS1 was distributed mainly in the cytoplasm, similar to other circRNAs [17].

Further study found that circ-TRPS1 expression increased in PCa cell lines. Downregulation of circ-TRPS1 significantly decreased invasion and proliferation in *in vitro* and *in vivo*, suggesting that circ-TRPS1 had oncogenic characteristics. CircRNAs are reported to interact with miRNAs sponging or stabilizing them [18, 19]. The CircInteractome web tool was developed to

map RNA-binding protein- and miRNA-binding sites on human circRNA [20]. We used this website for analysis and found that circ-TRPS1 interacted with many miRNAs. The combination with miR-124-3p is the most conservative. Luciferase reporting assays confirmed that miR-124-3p interacted with circ-TRPS1. RT-qPCR showed that silencing circ-TRPS1 promoted miR-124-3p expression, but miR-124-3p overexpression did not reverse circ-TRPS1 level after circ-TRPS1 silence. This result suggested that miR-124-3p was a circ-TRPS1 downstream target. Research shows that miR-124-3p ex-

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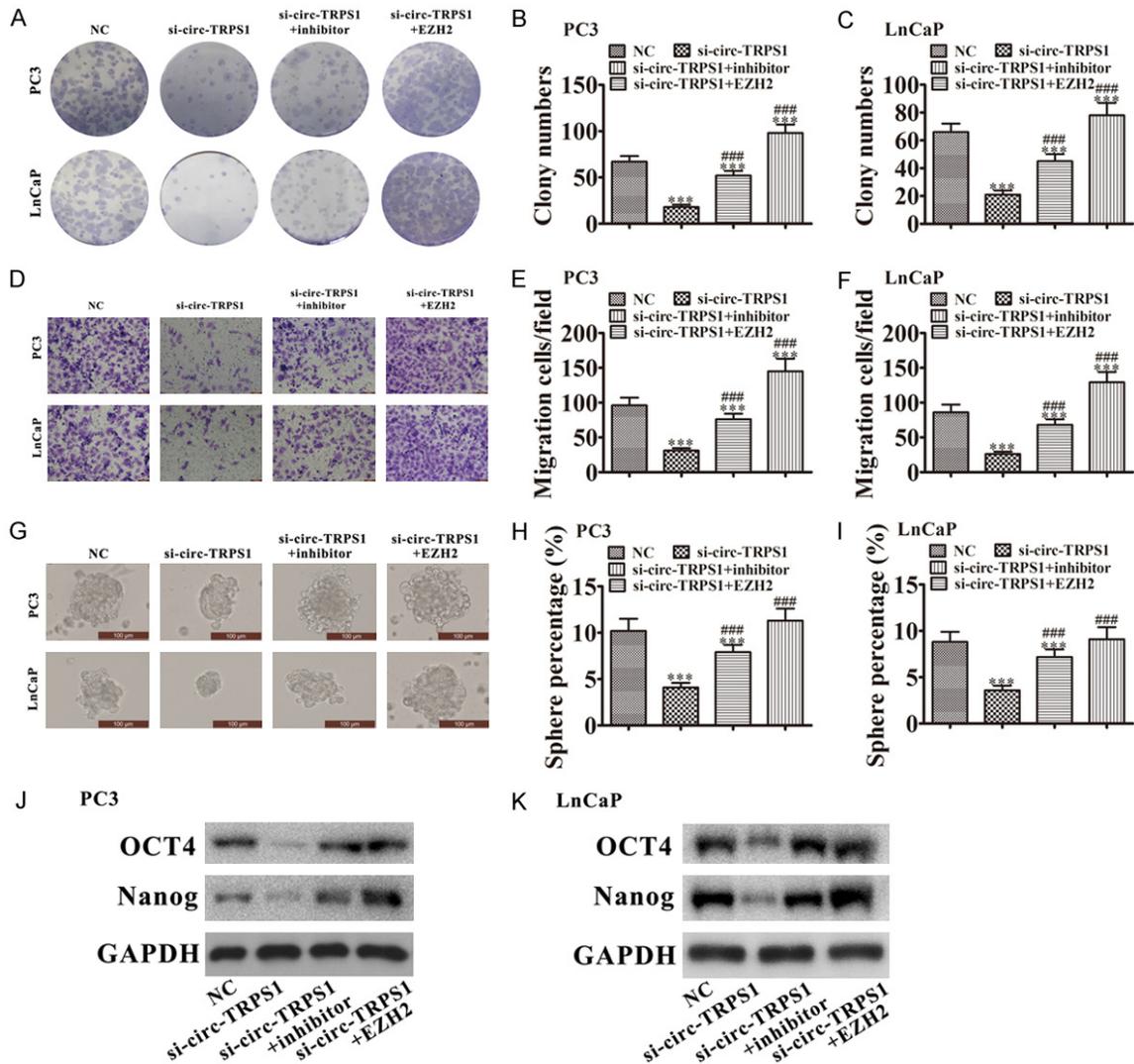


Figure 5. Downregulation of miR-124-3p or overexpression of EZH2 reversed the inhibitory effect of si-circ-TRPS1 on Pca proliferation and migration. A-C. Colony formation assays showing the proliferation of PC3 and LnCaP cells. Data are means \pm SD; ***P < 0.001 vs. NC. ###P < 0.001 vs. si-circ-TRPS1. D-F. Cell migration was assessed in PC3 and LnCaP cells using Transwell assays. Data are means \pm SD; ***P < 0.001 vs. NC. ###P < 0.001 vs si-circ-TRPS1. G-I. Images of tumor sphere formation assays in PC3 and LnCaP cells (200 cells/well); scale bar, 100 μ m. Data are means \pm SD; ***P < 0.001 vs. NC. ###P < 0.001 vs. si-circ-TRPS1. J, K. Western blots for the expression of stemness markers OCT4 and Nanog in PC3 and LnCaP cells.

pression inhibits cancers such like gastric [21] and bladder cancer [22], osteosarcoma [23], and glioma [24]. Previous investigations also validated that miR-124-3p expression is down-regulated in Pca [25], suggesting that high expression of circ-TRPS1 promotes the progress of Pca by adsorption of miR-124-3p.

Bioinformatic and luciferase reporting experiments confirmed that EZH2 was a direct miR-124-3p target. circ-TRPS1 downregulation or miR-124-3p overexpression suppressed EZH2 expression. However, overexpression of EZH2

did not reverse circ-TRPS1 and miR-124-3p levels. *In vitro* experiments confirmed that miR-124-3p downregulation or EZH2 overexpression recovered proliferation and migration after circ-TRPS1 silencing. Overexpression of EZH2 reversed miR-124-3p inhibitory effects on Pca cell proliferation and migration, suggesting that downregulation of circ-TRPS1 suppressed Pca progress by acting as a sponge to miR-124-3p and enhancing EZH2 expression.

Enhancer of Zeste 2 (EZH2) is a gene that is highly conserved as well as expressed in multi-

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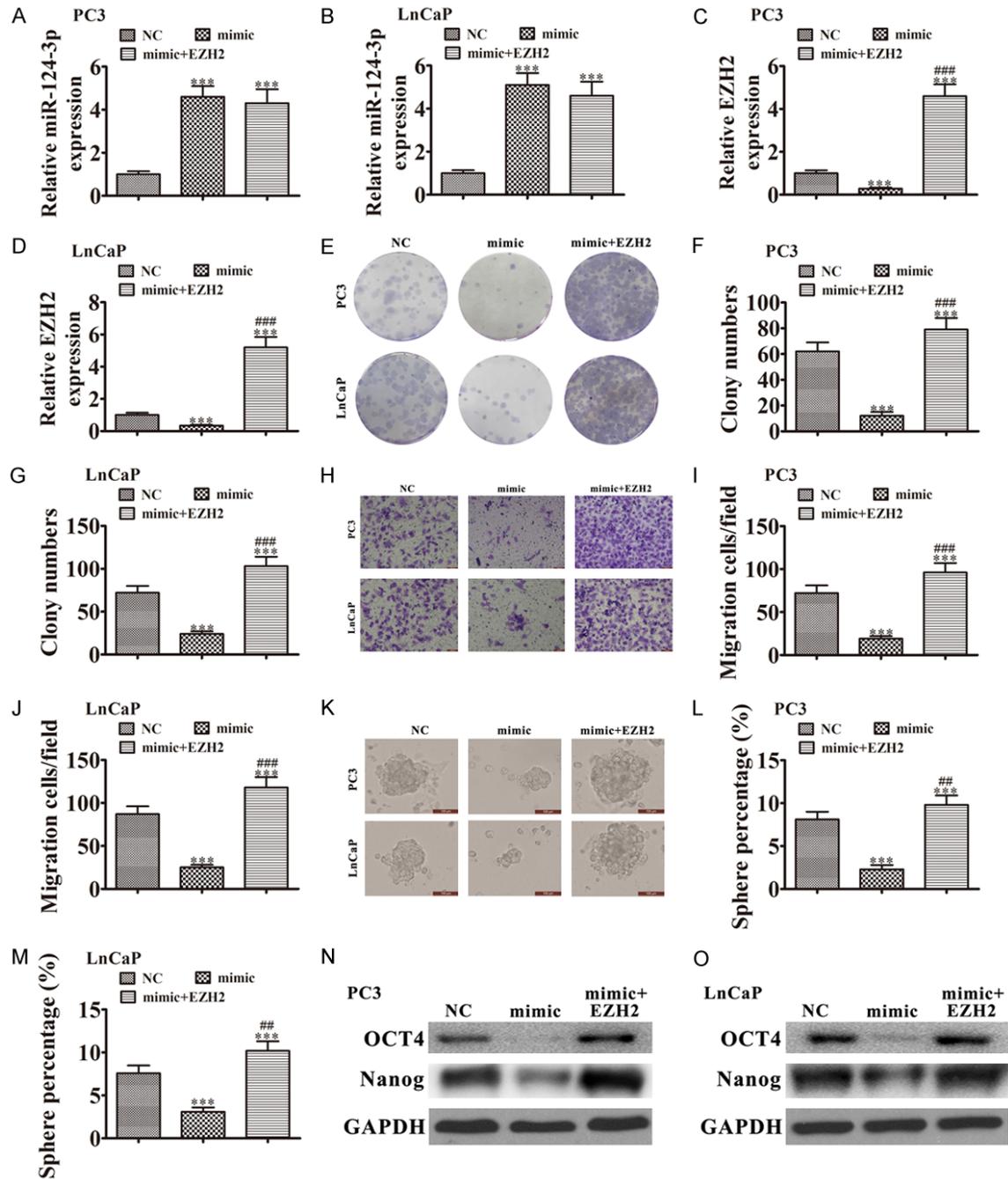


Figure 6. Overexpression of EZH2 reversed the inhibitory effect of miR-124-3p on PCa proliferation and migration. (A-D) RT-qPCR showing expression of (A and B) miR-124-3p and (C and D) EZH2 in PC3 and LnCaP cells. Data are means \pm SD; *** P < 0.001 vs. NC. (E-G) Colony formation assays showing proliferation of PC3 and LnCaP cells. Data are means \pm SD; *** P < 0.001 vs. NC. ### P < 0.001 vs. mimic. (H-J) Cell migration was assessed in PC3 and LnCaP cells using Transwell assays. Data are means \pm SD; *** P < 0.001 vs. NC. ### P < 0.001 vs. mimic. (K-M) Images of tumor sphere formation assays in PC3 and LnCaP cells (200 cells/well); scale bar, 100 μ m. Data are means \pm SD; *** P < 0.001 vs. NC. ## P < 0.01, ### P < 0.001 vs. mimic. (N and O) western blots for expression of stemness markers OCT4 and Nanog in PC3 and LnCaP cells.

ple human cancers [26]. EZH2 is aberrantly expressed in glioma and has strong effects on PCa proliferation and metastasis [27-29]. Stu-

dies show that EZH2 functions in the maintenance of cancer stem-like cell (CSC) properties [30]. CSCs take part in tumor proliferation,

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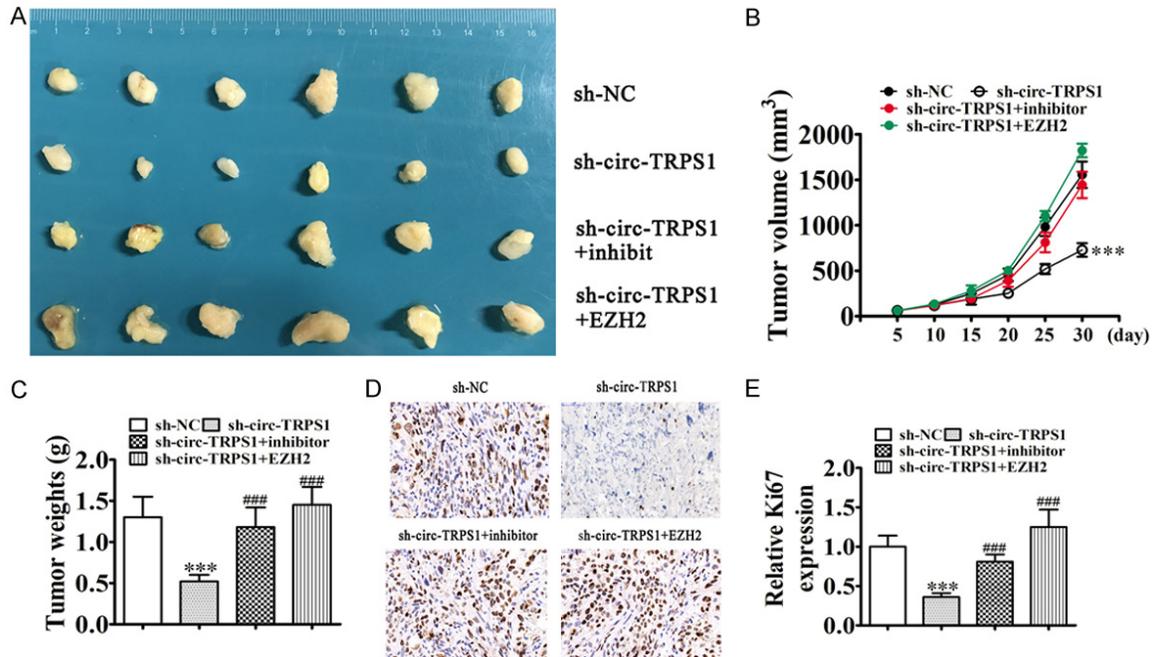


Figure 7. Downregulation of miR-124-3p or overexpression of EZH2 restores the growth ability of PCa after silence circ-TRPS1 in *in vivo*. (A) PC3 cells transfected with NC, sh-circ-TRPS1, miR-124-3p inhibitor or EZH2 overexpression vector single or combine, then subcutaneously injected into nude mice. (B) Tumor growth curves, (C) tumor weight. Data are means \pm SD; *** P < 0.001 vs. sh-NC. ### P < 0.001 vs. sh-circ-TRPS1. (D and E) Immunohistochemistry for the percentage of Ki-67-positive cells. The relative fluorescence density was calculated. Data are means \pm SD; *** P < 0.001 vs. sh-NC. ### P < 0.001 vs. sh-circ-TRPS1.

recurrence, invasion, and chemoradioresistance, which makes a new concept of tumorigenicity and progression [31]. In this study, we found that circ-TRPS1 silencing suppressed CSC differentiation using tumor sphere formation assays and western blots. Overexpression of EZH2 recovered the CSC properties of PCa after circ-TRPS1 knockdown or miR-124-3p upregulation, suggesting that downregulation of circ-TRPS1 suppressed PCa progress via regulating miR-124-3p/EZH2 axis-mediated stemness.

Conclusions

In conclusion, our investigation revealed that circ-TRPS1 was increased in PCa patient samples and cell lines, and upregulation was correlated to aggressive PCa phenotypes. In addition, circ-TRPS1 silencing suppressed PCa metastasis and proliferation *in vivo* and *in vitro*. We also discovered that circ-TRPS1 downregulation suppressed PCa migration and proliferation through targeting the miR-124-3p/EZH2 axis. We identified circ-TRPS1 as a potential therapeutic target in PCa. Further studies re-

garding the circ-TRPS1/miR-124-3p/EZH2 axis could provide a basis to develop new potential PCa therapeutic strategies.

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Disclosure of conflict of interest

None.

Abbreviations

FISH, Fluorescence *in situ* hybridization; EZH2, Enhancer of Zeste 2; CSC, cancer stem-like cells; circRNA, circular RNA; RNA-seq, high-throughput RNA sequencing; RT-qPCR, quantitative reverse transcription polymerase chain reaction; CCK-8, Cell Counting Kit-8; miRNAs, microRNA; PCa, prostate cancer.

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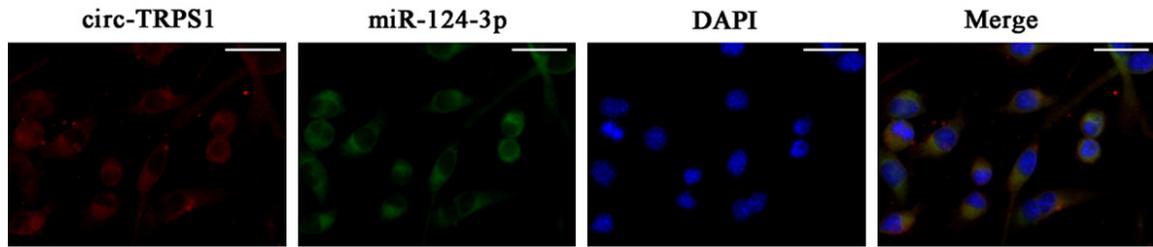


Figure S1. FISH showed the subcellular location of miR-124-3p and circ-TRPS1. Nuclei were stained with DAPI. miR-124-3p was stained green, nuclei labeled blue, circ-TRPS1 was labeled red. Scale bar, 20 μ m.