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ORIGINAL ARTICLE

# CircBAGE2 (hsa_circ_0061259) regulates CCND1 and PDCD10 expression by functioning as an miR-103a-3p 'sponge' to alter the proliferation and apoptosis of prostate cancer cells*

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## Abstract

**Objective** The aim of the article is to explore the function of circBAGE2 (hsa_circ_0061259) in prostate cancer (PCa) cells.

**Methods** Sequencing results of circBAGE2 were verified by quantitative RT PCR (qRT-PCR) and Sanger sequencing. Agarose gel electrophoresis was used to detect the resistance of GAPDH, BAGE2, and circBAGE2 to RNase R and their expression as cDNA and gDNAin 22RV1 cells. The biological functions of circBAGE2 were investigated by CCK8 assay and flow cytometry in 22RV1 cells transfected with siRNAs. Multiple databases were used to predict the target binding sites between circRNAs, miRNAs, and mRNAs. Western blotting was used to detect the expression of CCND1 and PDCD10.

**Results** CircBAGE2 was significantly upregulated in PCa samples and PCa cells compared to that in matched normal tissues and normal cells, and CircBAGE2 knockdown inhibits cell proliferation and promotes apoptosis. Downregulation of circBAGE2 compromised the expression of CCND1 and PDCD10. The 3' UTRs of CCND1 and PDCD10 were matched by miR-103a-3p, which shared binding sites with circBAGE2.

**Conclusion** CircBAGE2 contributes to PCa progression by upregulating CCND1 and PDCD10 expression through its role as a 'sponge' of miR-103a-3p. CircBAGE2 may be a potential therapeutic target for PCa.

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**Key words:** prostate cancer (PCa); circBAGE2; CCND1; PDCD10

Prostate cancer (PCa) is the most frequently diagnosed malignancy and the second leading cause of death in men worldwide [1–2]. In Asian countries, such as China, the incidence of PCa has increased annually. Clinically, serum prostate-specific antigen (PSA) levels are currently detected in diagnostic studies. However, serum PSA has several detection flaws involving false positives, which cause overdiagnosis and subsequent overtreatment [3]. Therefore, it is necessary to develop a more accurate and sensitive biomarker to guide the diagnosis, prognosis, and therapy of PCa.

With recent developments in bioinformatics and the application of RNA sequencing, the biological activity of circular RNAs (circRNAs) is receiving increasing attention. CircRNAs are a class of noncoding RNAs that are emerging as key new members of the gene regulatory milieu, which are produced by back-splicing events within genes [4]. The expression analysis of circRNA transcripts revealed that numerous circRNAs seem to be specifically expressed in various tissues, including PCa [5]. Some reports have identified that circRNAs, such as circSMARCA5 [6], circFOXO3 [7], and circZMIZ1 [8] play

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important roles in PCa. However, many circRNAs and their mechanisms remain unclear.

In order to further explore the biomarkers of PCa and the underlying mechanisms, we previously performed a circRNA sequencing analysis and identified some significantly upregulated and downregulated circRNAs in PCa cell lines [9]. Among them, circBAGE2 (hsa_circ_0061259) is one of the most upregulated circRNAs in 22RV1 cell line compared to RWPE-1 cell line, and is therefore considered responsible for the progression of PCa. In addition, BAGE genes in its linear transcript are silent in normal cells and expressed in some tumors and cancer cell lines [10–11]. Therefore, we further explored the mechanism and function of circBAGE2 in the 22RV1 cell line. The present study aimed to investigate the role of circBAGE2 in PCa progression.

## Materials and methods

### Ethics statement

This study was approved and supervised by the Ethics Committee of the Navy Military Medical University (Shanghai, China). Human PCa tissues and adjacent normal tissues were obtained from patients undergoing surgery at the Changhai Hospital of Navy Military Medical University, Shanghai, China. All tumors and paired adjacent normal tissues were confirmed by pathologists. Written informed consent was obtained from the patients for research purposes.

### Cell culture

RWPE-1, 22RV1, PC3, and LNCaP cells were purchased from the American Type Culture Collection (ATCC), USA. 22RV1, PC3, and LNCaP cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), F12 (ham) medium (Gibco, Carlsbad, CA, USA), and RPMI-1640 medium (Gibco, Carlsbad, CA, USA), respectively. RWPE-1 cells were cultured in PEpiCM medium (ScienCell, USA). All media were supplemented with 10% FBS and 1% penicillin-streptomycin, and all cell lines were cultured at 37 °C and 5% $CO_2$. The medium was changed every 2 days, and cells were digested at room temperature with 0.5 mL 0.25% trypsin/EDTA (Gibco, Carlsbad, CA, USA) per well and grown to 70%–80% confluency.

### Total RNA and circRNA extraction

Total RNA was extracted from cultured cell lines and tissues using RNAiso Plus (Takara, Dalian, China), according to the manufacturer's instructions. To remove linear RNAs and enrich circRNAs, 3 units/μg of RNase R (Epicenter, Madison, WI, USA) was used to digest the total extracted RNA for 15 min at 37 °C. RNA quantity and quality were evaluated using a NanoDrop 2000 c spectrophotometer (Thermo, Wilmington, DE, USA), and RNA integrity was assessed by 2% agarose gel electrophoresis.

### Agarose gel electrophoresis

Agarose was dissolved in a TAE buffer in a microwave oven at a concentration of 1.5% with Gelred dye, and then placed in an electrophoresis tank by adding samples containing 6 × loading buffer after cooling. The reaction was performed at 90 V for 30 min. A gel camera system (G-box-chemi-R5, Hong Kong Gene Co., Ltd., China) was used to capture the images.

### PCR

PCR analyses of the expression levels of the circRNAs were performed using Premix Ex Taq II (Takara, Dalian, China) in 20 μL reaction volume, including 1 μL of cDNA, 10 μL of 2 × Master Mix, 0.3 μL of Forward Primer (10 μM), 0.3 μL of Reverse Primer (10 μM), and 8.4 μL of double distilled water. The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 30 s, 72 °C for 45 s, and 72 °C for 7 min in a PCR System (Bio-Rad, CA, USA). All primers are shown in Table 1.

### Quantitative RT-PCR (qRT-PCR)

cDNA was synthesized from 500 ng of total RNA using PrimeScript RT Master Mix (Takara, Dalian, China), according to the manufacturer's instructions. qRT-PCR analyses of the expression levels of the circRNAs were performed using SYBR Premix Ex Taq II (Takara, Dalian, China). qRT-PCR was performed in a 20 μL reaction volume, including 1 μL of cDNA, 10 μL of 2 × Master Mix, 0.3 μL of for wardprimer (10 μM), 0.3 μL of reverseprimer (10 μM), and 8.4 μL of double distilled water. The reaction was performed with the protocol; 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s in a real-time PCR System (ABI, CA, USA), GAPDH was used as a reference, and samples were amplified in triplicate wells, and the relative level was calculated using the $2^{-\Delta\Delta Cq}$ method. All primers are shown in Table 1.

### Gene transfection

Cells were transfected with corresponding specific siRNA or control siRNA (10 mM, 7.5 μL siRNA/125 μL OPTI-MEM) using Lipofectamine RNAiMax (5 μL in 125 μL OPTI-MEM) (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations. Briefly, the cells were incubated with siRNAs and Lipofectamine RNAiMax mixture for 15 min at room temperature. Cells were then cultured in medium without antibiotics, after washing with OPTI-MEM twice. The following siRNA sequences were used: siRNA-1 for circBAGE2 sense: 5'-CGGUCCAAACA

**Table 1** Primers of all moleculars used in PCR and qRT-PCR

| Primer name | Primer sequence (5'-3')-F | Primer sequence (5'-3')-R |
|---|---|---|
| circ-BAGE2 | AACTGGCATGGGTAAACCAG | TGTTCTGGACAAAGCAGGAA |
| BAGE2 (cDNA) | AATACAGTGAGCCCACCCTCGT | TTTCAGCTTTGACCTGCCTCGG |
| BAGE2 (gDNA) | TGTAAGCACTTTGGAGCCACTATCA | TTCAGGAGCTTGGTCAATGTGTTCT |
| CCND1 | CGCCCTCGGTGTCCTACTTCAA | GTTCCTCGCAGACCTCCAGCAT |
| PDCD10 | CGCAGGGCACTTGAACACCAA | TCGGTTGGCACTTACGAACACA |
| GAPDH | AAGAAGGTGGTGAAGCAGG | GTCAAAGGTGGAGGAGTGG GG |
| circ-GUCY1A2 | GCTCCTATGCAGACCACTCC | TTTCTGCATCCCTGTAACCA |
| circ-ETV3 | ACGGGGAATTTGTCATCAAG | AATGGGTAGTTGGGCATCAC |
| circ-KCNN2 | GGATAATTGCCGCATGGA | CTGCTCCATTGTCCACCA |
| circ-MIR663A | CTACCGTTCTGCCTCCGA | CGCGTCTCGTCTCACTCA |
| circ-KRT6A | GCGTTGGACAAGTCAACATC | GAACTGAAGCCACCTCCAAC |
| circ-CD276 | AGCTTCACCTGCTTCGTGAG | ATCCTGCCAGAACACCTCAG |
| circ-ZFP57 | TGGCCAGAATCTTTCTGCAT | TCCTGGTAAAGGACCCTCTG |
| circ-PSMA7 | CTCATCGTGGGTTTCGACTT | ATGCAGACGTTGTCATCCAA |
| circ-RPPH1 | GGGCTCTCCCTGAGCTTC | CAAGGGACATGGGAGTGG |

GCGAUGUGTT-3'; antisense: 5'-CACAUCGCUGUU UGGACCGTT-3'; and siRNA-2 for circBAGE2 sense: 5'-CAAACAGCGAUGUGCAUUUTT-3'; antisense: 5'-AAAUGCACAUCGCUGUUUGTT-3'. Cells were harvested 48 h after transfection and expression levels were determined using qRT-PCR.

## Cell proliferation assays

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan), according to the manufacturer's protocol. The cells were plated in 96-well plates ($3 \times 10^5$ cells/well). Cell proliferation was detected every 24 h according to the manufacturer's protocol. Briefly, 10 μL of CCK-8 solution was added to each well and incubated for 2 h at 37 °C. The solution was then spectrophotometrically measured at 450 nm.

## Cell cycle and cell apoptosis assays

Cell lines, seeded in 6-well plates ($3 \times 10^5$ cells/well), were assayed using the Cell Cycle Staining Kit (MultiSciences, Hangzhou, China) and the Annexin V-FITC/PI Apoptosis DetectionKit (MultiSciences, Hangzhou, China), according to the manufacturer's protocol. After 24 h of treatment with or without siRNAs, the cells were harvested and washed twice with cold PBS. For cell cycle analysis, the trypsin-harvested cells were incubated with 1 mL DNA staining solution and 10 μL permeabilization solution for 30 min at room temperature. For the cell apoptosis assay, cells were incubated with 5 μL Annexin V-FITC and 3 μL PI solution in 500 μL binding buffer for 5 min at room temperature. Fluorescence was measured using a MACSQuant Analyzer 10 (Miltenyi Biotec GmbH, Germany) and analyzed using FlowJo 7.6.1 software (CA, USA).

## Western blotting

Cellular protein (25 μg) was loaded onto a 12% SDS-PAGE gel and then transferred to PVDF membranes. After blocking with 5% BSA in TBST for 2 h at room temperature, membranes were incubated with primary antibodies against CCND1, PDCD10 (1:1000 dilution, CST, Beverly, MA, USA) and GAPDH (1:10000 dilution, CST, Beverly, MA, USA) at 4 °C overnight, followed by incubation with appropriate HRP-conjugated secondary antibody (1:1000 dilution, CST, Beverly, MA, USA) at room temperature for 2 h. The membranes were then exposed using a chemiluminescent detection system (Syngene G, Box, America). Quantitative densitometric analyses of immunoblots were performed using the ImageJ software (Ver. 1.48, Bethesda, MD, USA), and the relative ratios were calculated.

## Annotation and functionalprediction of circBAGE2

The circRNA-miRNA-mRNA network of circBAGE2 was constructed using CloudSeq's homemade software based on miR and a and TargetScan (CloudSeq Inc., Shanghai, China), combined with Cytoscape (http://www.cytoscape.org/).

## Statistical analysis

Each experiment was performed in triplicates. All data are represented as the mean ± SEM. Student's *t*-test and one-way analysis of variance were used to determine statistical significance. Differences were considered statistically significant at $P < 0.05$.

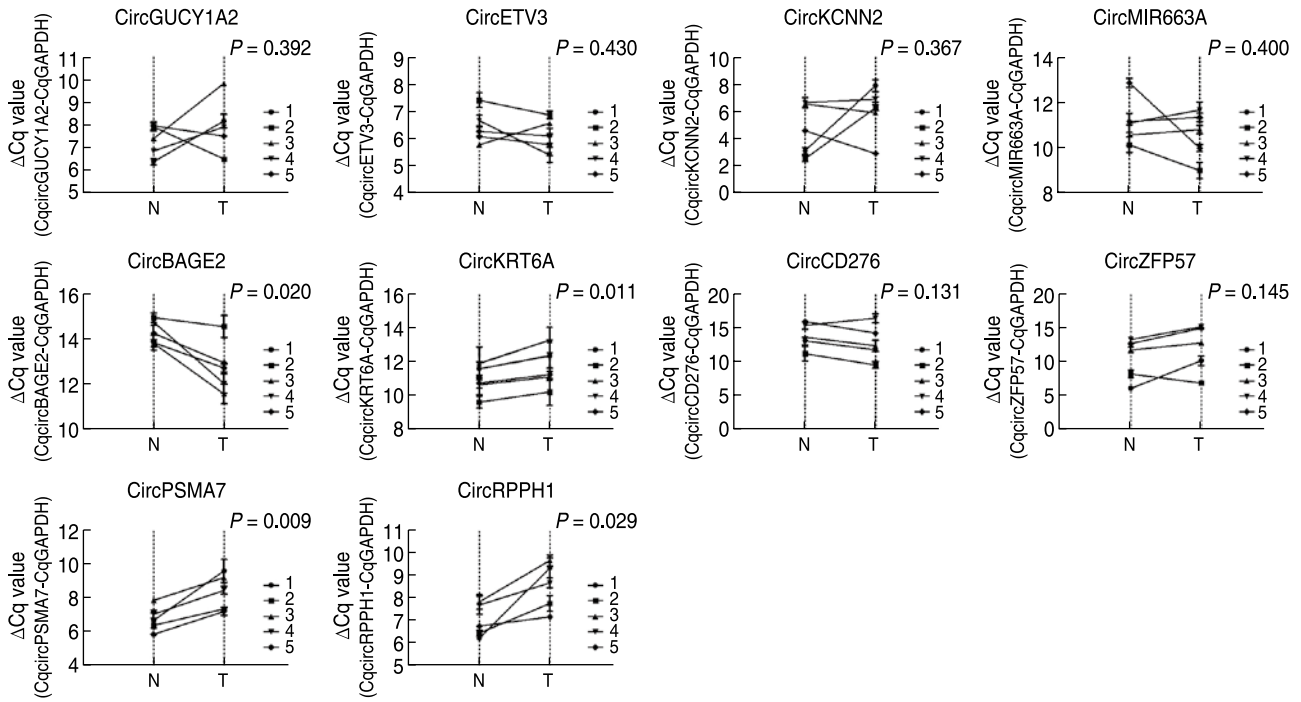


**Fig. 1** The expression levels of candidate circRNAs for validation in 5 pairs of human prostate cancer tissue and adjacent normal tissue samples via qRT-PCR: circGUCY1A2, circETV3, circKCNN2, circMIR663A, circBAGE2, circKRT6A, circCD276, circZFP57, circPSMA7 and circRPPH1. N: normal tissues; T: tumor tissues

# Results

## Expression of circBAGE2 in human PCa tissues

The expression levels of five significantly upregulated and downregulated circRNAs in 22RV1 cell line compared to RWPE-1 cell line [9] were detected in five samples of human PCa tissues and their paired adjacent normal tissues by qRT-PCR using GAPDH as the internal standard, targeting circGUCY1A2, circETV3, circKCNN2, circMIR663A, circBAGE2, circKRT6A, circCD276, circZFP57, circPSMA7, and circRPPH1. As shown in Fig. 1, circBAGE2 was identified as the only upregulated circRNA in cancer tissues compared to the corresponding normal tissues, with statistical significance ($P < 0.05$). Meanwhile, circKRT6A, circPSMA7, and circRPPH1 were downregulated in cancer tissues compared to normal tissues.

## Characterization of circBAGE2 in human PCa cell lines

CircBAGE2 was significantly upregulated in 22RV1 cells compared to RWPE-1 cells when normalized to GAPDH by qRT-PCR and nucleic acid electrophoresis (Fig. 2a and 2b). The distinct product of the expected size amplified using outward-facing primers was confirmed by Sanger sequencing (Fig. 2c). Using PCR and nucleic acid electrophoresis, circBAGE2 was confirmed to be more resistant to RNase R treatment than the line control BAGE2 (Fig. 2d). As expected, PCR assays alone did not produce amplification in genomic DNA with circBAGE2 primers using cDNA and genomic DNA of 22RV1 as templates, which ruled out the possibility of genomic rearrangement (Fig. 2e).

## Silencing circBAGE2 inhibited proliferation and increased apoptosis of human PCa cell lines *in vitro*

The expression of circBAGE2 was suppressed by the two siRNAs (Fig. 3a). CircBAGE2 knockdown significantly decreased the viability of 22RV1 cells (Fig. 3b). As shown in Fig. 3c and 3d, 22RV1 cells transfected with siRNAs were arrested in the G1-phase. In addition, downregulation of circBAGE2 led to a substantial increase in cell apoptosis (Fig. 3e and 3f).

## Prediction of target genes related to circBAGE2

Based on circRNA-miRNA-mRNA network analysis tools, 12 miRNAs containing binding sites with circBAGE2 were predicted: miR-450a-2-3p, miR-7-5p, miR-766-5p, miR-103a-3p, miR-107, miR-377-3p, miR-1236-5p, miR-301b-3p, miR-603, miR-520f-3p, miR-301a-3p, and miR-134-5p (Fig. 4a). CircBAGE2 had two binding sites for miR-103a-3p (Fig. 4b). Further analysis showed that miR-103a-3p matched the 3'UTR of CCND1 and

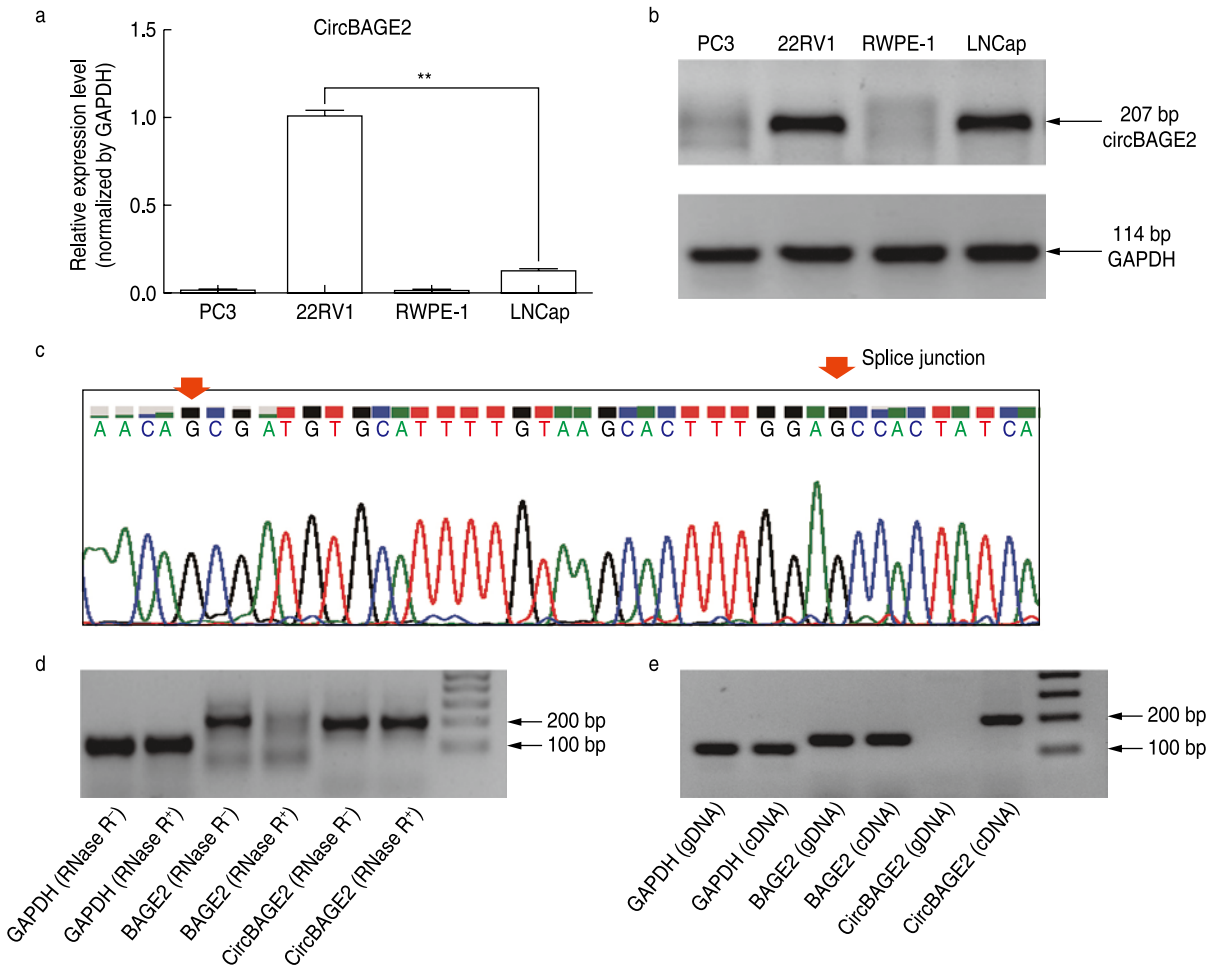


**Fig. 2** Characterization of circBAGE2 in human prostate cancer cells. (a and b) The expression levels of circBAGE2 in 4 human prostate cancer cell lines by qRT-PCR and agarose gel electrophoresis. (c) Sanger sequencing of the product amplified by primers of circBAGE2. (d) The resistance to RNase R of GAPDH, BAGE2 and circBAGE2 in 22RV1. (e) The expression levels of GAPDH, BAGE2 and circBAGE2 using cDNA and genomic DNA (gDNA) of 22RV1 as templates. ** $P < 0.01$

PDCD10 (Fig. 4c). CCND1 and PDCD10 expression was significantly decreased by co-transfection with siRNAs in 22RV1 at the mRNA (Fig. 4d) and protein levels (Fig. 4e and 4f), as detected by qRT-PCR and western blotting.

## Discussion

CircRNAs have recently been identified as a natural family that may regulate gene expression in mammals [12–13], which are important regulators of many cellular processes, such as embryonic development, cell cycle control, cellular senescence, cell signaling, and response to cellular stress [4]. All these functions are critical for cancer progression. Therefore, circRNAs may have a vital relationship with cancer, including PCa.

In our previous studies, we successfully constructed circRNA libraries of PCa cell lines and identified many differentially expressed circRNAs [9]. circBAGE2 was one of the most upregulated circRNAs in 22RV1 compared with those in the RWPE1 cell line. In this study, we found that the expression level of circBAGE2 was significantly upregulated in human PCa tissues compared to the corresponding normal tissues.

BAGE family genes code for tumor-specific antigens that are highly expressed in different histological types of tumors. BAGE2 is expressed in a significant number of tumors, including melanoma, bladder cancer, lung cancer, mammary and prostatic carcinoma, and neuroblastoma [10]. Many published studies have indicated that BAGE2 may play an important role in cancer progression and could therefore be a good candidate as a new, highly informative epigenetic biomarker for cancer diagnosis [11, 14–15]. These studies imply that circBAGE2, which is highly expressed in PCa as the corresponding circRNA of BAGE2, may also perform key functions in PCa. In this

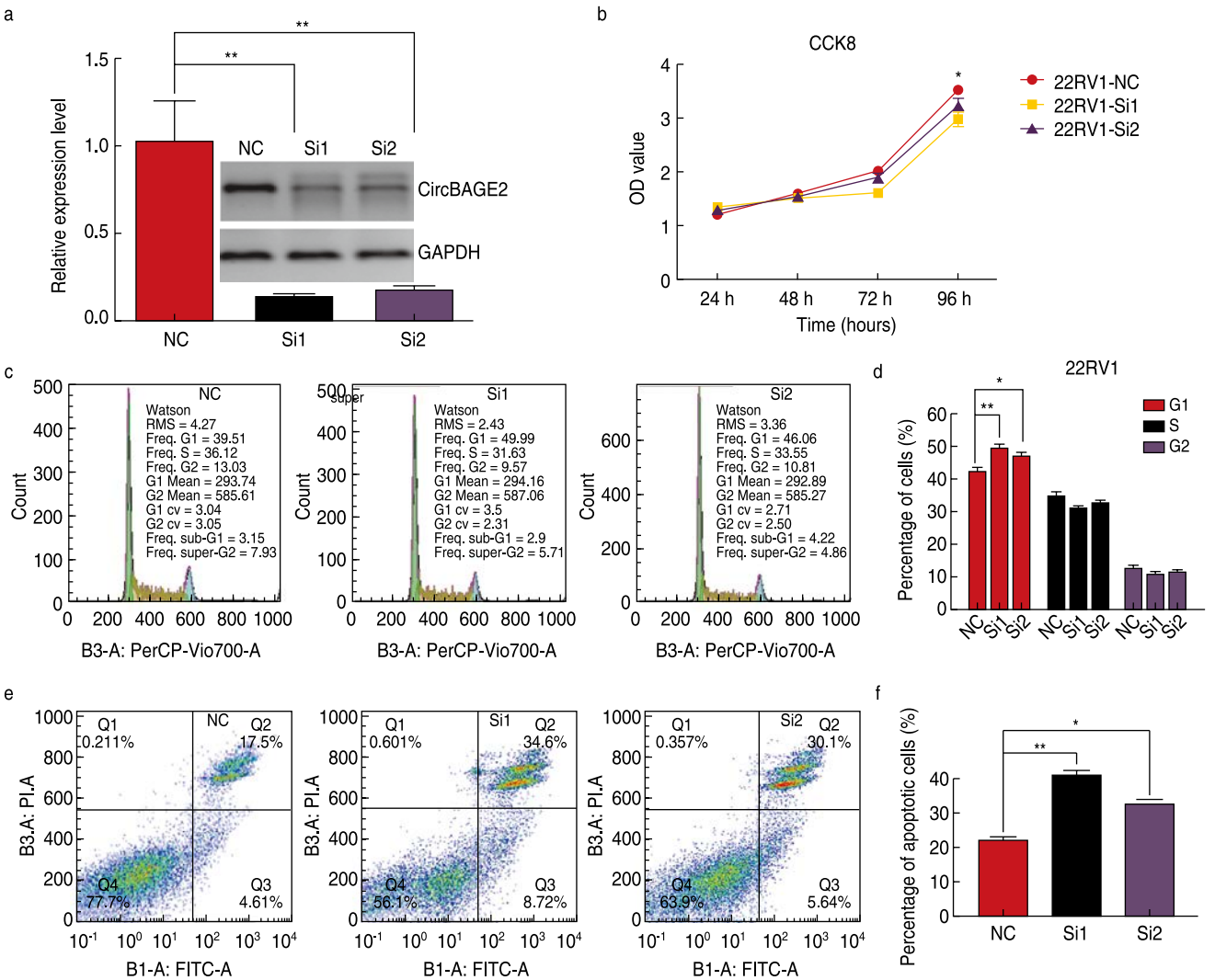


**Fig. 3** Effects of circBAGE2 on the biological functions of 22RV1. (a) Effects of two different small interfering RNAs (siRNAs) against circBAGE2 analyzed by qRT-PCR. (b) CCK-8 assay of 22RV1 transfected with two siRNAs of circBAGE2. Cell cycle assay (c and d) and cell apoptosis assay (e and f) of 22RV1 transfected with two siRNAs of circBAGE2 by flow cytometry. $^{**}P < 0.01$, $^{*}P < 0.05$, NC: negative control, Si1: siRNA1, Si2: siRNA2

study, we found that circBAGE2 knockdown inhibited cell proliferation and promoted cell apoptosis, which verified our hypothesis.

CircRNAs have been proposed to act through several mechanisms, including as miRNA sponges [16], binding with proteins or affecting translation [17], and as splicing modifiers of transcription [18]. Among them, the most important mechanism is the binding of circRNAs to specific miRNAs or groups of miRNAs, to sequester them and suppress their function in a phenomenon termed the competitive endogenous RNA hypothesis [16, 19]. Thus, we constructed a targeted circRNA-miRNA-mRNA network based on sequence-pairing prediction to explore the underlying mechanisms.

CircBAGE2 was predicted to have two binding sites with miR-103a-3p, which has been shown to be upregulated in several types of cancer, including colorectal [20] and endometrial cancer [21], and is hypothesized to induce the proliferation, migration, and invasion of cancer cells. Early reports have demonstrated that the expression level of miR-103 was significantly decreased in PCa cells [22] and could suppress PCa proliferation and migration by downregulating the oncogene PDCD10 [23]. CCND1 plays a critical role in promoting the G1-S transition of the cell cycle in many cell types [24–25]. Mutations and overexpression of CCND1 are known to lead to alterations in cell cycle progression, and are frequently observed in a variety of tumors and could thus contribute to tumor progression in cancers such as non-small cell lung cancer [26] and PCa [27]. Considering our target analyses of miR-

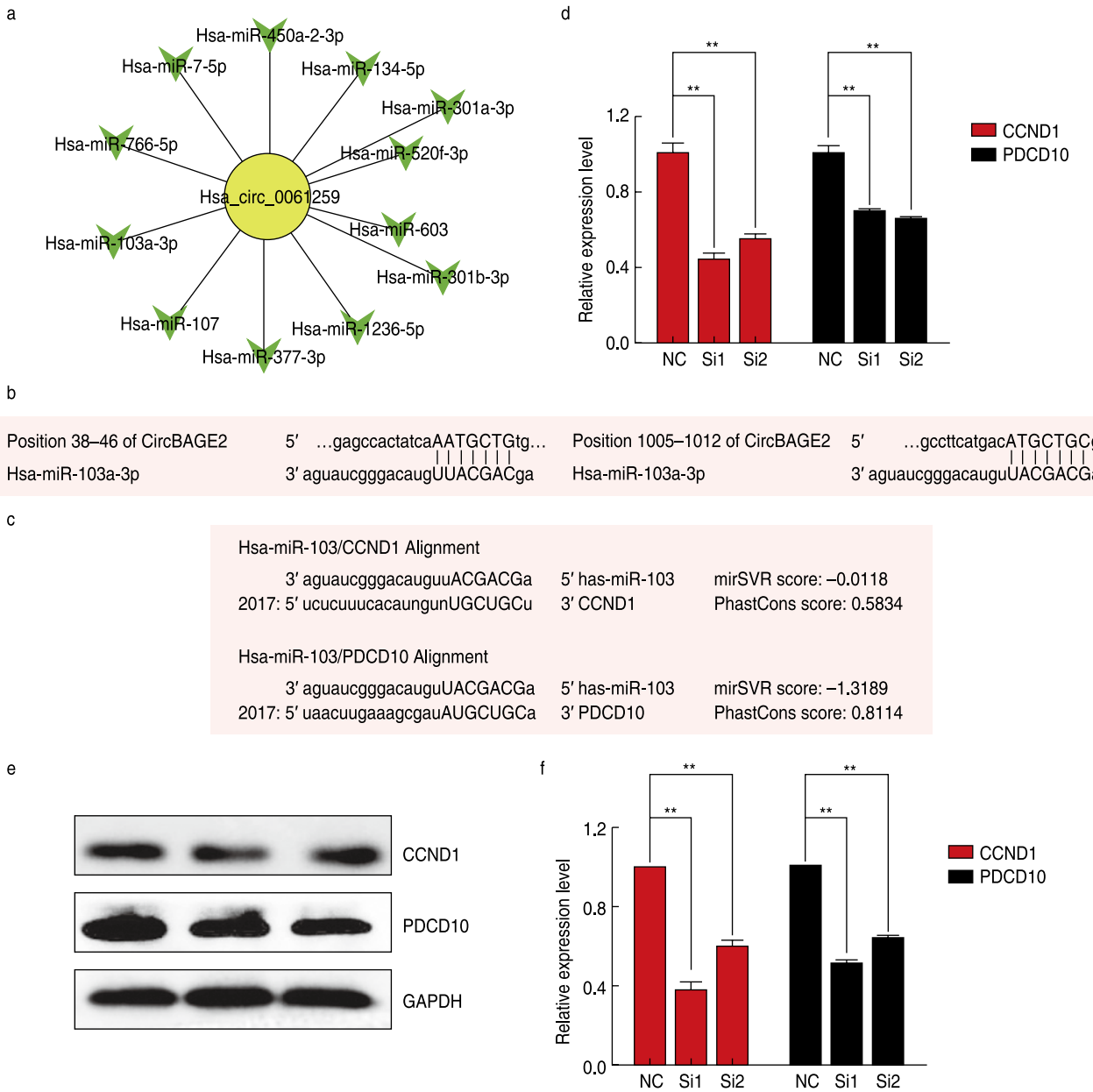


**Fig. 4** Prediction of the targets of circBAGE2 based on circRNA-miRNA-mRNA network. (a) Network of circBAGE2 and targeted miRNAs. (b) Binding sites shown by solid lines of circBAGE2 with miR-103a-3p. (c) 3' UTRs of mRNAs (CCND1 and PDCD10) matched by miR-103a-3p. (d) Expression levels of mRNAs (CCND1 and PDCD10) detected by qRT-PCR following knockdown of circBAGE2 with siRNAs. Protein expression levels of CCND1 and PDCD10 analyzed by western blotting (e) and quantified with Image J (f) following knockdown of circBAGE2 using siRNAs. GAPDH was used as a control. $^{**}P < 0.01$, NC: negative control, Si1: siRNA1, Si2: siRNA2

103a-3p using miR and a and TargetScan, CCND1 and PDCD10 may be candidate genes. In the present study, the downregulated expression of PDCD10 and CCND1 after circBAGE2 knockdown confirmed the underlying mechanism by which circBAGE2 might regulate CCND1 and PDCD10 expression by functioning as an miR-103a-3p 'sponge' to alter the proliferation and apoptosis of the 22RV1 cell line.

To the best of our knowledge, this study is the first to systematically analyze the role of circBAGE2 in PCa progression. Therefore, this study may provide a therapeutic strategy or predictor for patients with PCa. Nevertheless, deeper mechanisms or animal experiments *in situ* still need to be studied.

## Conflicts of interest

The authors indicated no potential conflicts of interest.

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