INTRODUCTION

Prostate cancer (PCa) is the disease with the highest number of new cases and the second most common cause of cancer-related death among American men.1,2 Recent years, the incidence and mortality rate of PCa have been significantly increasing in China. Substantial researches have explored the roles of androgen receptor (AR) or other important genes in development and progression of PCa.3,4 However, the molecular mechanisms regulating the tumorigenesis and progression of PCa are still unclear.

Circular RNA circFOXO3 promotes prostate cancer progression through sponging miR-29a-3p

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Abstract
Circular RNA FOXO3 (CircFOXO3, also termed as Hsa_circ_0006404) is derived from exon 2 of forkhead box O3 (FOXO3) gene, and abnormal expression is shown in different diseases. However, whether circFOXO3 plays important roles in tumorigenesis and progression of prostate cancer (PCa) remains unclear. In this study, we found that circFOXO3 was up-regulated in both PCa tissues and serum samples. Moreover, circFOXO3 was positively correlated with the Gleason score in PCa samples. CircFOXO3 was observed to be up-regulated in Gleason score > 6 PCa samples compared with Gleason score = 6 PCa samples. Knock-down circFOXO3 could remarkably inhibit PCa cell cycle, proliferation and promote cell apoptosis in vitro. Furthermore, we demonstrated circFOXO3 could act as miR-29a-3p sponge to up-regulate SLC25A15 expression by bioinformatics analysis, dual-luciferase reporter assays and biotinylated RNA pull-down assays. SLC25A15 could reverse the tumour suppressing roles of knock-down circFOXO3 in PCa. Of note, we found that miR-29a-3p was down-regulated; however, SLC25A15 was overexpressed in PCa samples compared with normal tissues. In conclusion, circFOXO3 acts as a miR-29a-3p sponge to exhibit oncogenic activity that affects the cell cycle and cell apoptosis in PCa through transcriptional up-regulation of SLC25A15. Our analysis suggests circFOXO3 could act as promising prostate cancer biomarkers.

KEYWORDS biomarker, circFOXO3, miR-29a-3p, miRNA sponge, prostate cancer (PCa)
Recent reports have showed that 80%-90% RNA molecules are non-coding RNAs (ncRNAs).\textsuperscript{5} Circular RNAs (circRNAs), a newly emerging endogenous ncRNA, are originated from its parental linear genes by RNA polymerase II and harbour covalently closed circular structure without poly(A) tail and 5’-3’ polarity.\textsuperscript{6,7} Studies have exhibited that circRNAs are conserved, stable and stage/tissue-specific expression.\textsuperscript{8,9} Recently, scholars paid more attention on circRNAs and identified that lots of circRNAs were significantly differential expression in various cancers, suggested circRNAs might have crucial effect on cancer development.\textsuperscript{10} For example, our differential expression in various cancers, suggested circRNAs might play a crucial role in carcinogenesis of cancers.\textsuperscript{8,9} In this study, we discovered the expression of circFOXO3 was highly expressed in PCa tissue samples and serum samples than controls. Therefore, we knock down circFOXO3 expression to identify its potential roles and explore possible mechanisms in carcinogenesis of PCa. Here, we demonstrated that circFOXO3 acted as a miR-29a-3p sponge to up-regulated solute carrier family 25 member 15 (SLC25A15) and played an oncogenic role in PCa.

2 | MATERIALS AND METHODS

2.1 | Tissue samples and serum samples from PCa patients

A total of 53 PCa samples and corresponding adjacent normal prostate tissues were obtained from patients at Fudan University Shanghai Cancer Center. In order to detect the expression levels of circFOXO3 in serum samples, we collected the serum samples from 26 PCa patient (among 53 PCa patients) and 19 healthy donors (n = 19), who also provided informed consent at Fudan University Shanghai Cancer Center. Those patients did not receive any pre-operation treatment. The healthy volunteers had no history of cancer until sample accumulation. Samples were centrifuged at 3000 g for 10 minutes at 4°C for isolation of serum. The clinicopathological features of the patients are summarized in Table 1 and Table S2. The study was approved by the Research Ethics Committee of Fudan University Shanghai Cancer Center. Informed consent was provided by all patients. All samples were collected and used for gene expression analysis by qRT-PCR.

2.2 | Cell culture

All cell lines (WPMY-1, LNCaP, 22Rv1, DU145 and PC-3) were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. WPMY-1 (the human normal prostate epithelial cell) was expanded in DMEM medium (HyClone), and the PCa cell lines (LNCaP, 22Rv1, DU145 and PC-3) were maintained in RPMI 1640 medium (HyClone). All of the medium should be added 10% foetal bovine serum before use (FBS, Biological Industries). Then, all cell lines were maintained at 37°C, 5% CO₂ incubator.

2.3 | Cell transfection

For knock-down of circFOXO3, circFOXO3-specific siRNA (sicircFOXO3) sequence was designed as Burton Yang et al’s report\textsuperscript{15} and synthesized by GenePharma. The PCa cells were separately transfected with sicircFOXO3 or negative control (NC) at a final concentration of 50 nmol/L using HilyMax.

For overexpression of miRNA, hsa-miR-143-3p, hsa-miR-221-5p, hsa-miR-23a and hsa-miR-29a-3p mimics were also designed and synthesized by GenePharma. The PCa cells were separately transfected with miRNA or negative control (NC) at a final concentration of 50 nmol/L using HilyMax. Transfected cells were used for gene expression analysis or other experiments. All the above sequences are shown in Table S1.

| Table 1 | Clinicopathologic characteristics of patient samples and expression of circFOXO3 in PCa |
|---------|---------------------------------|
| Characteristics | Number of cases |
| Age (median = 68.5) | |
| ≤69 | 26 |
| >69 | 26 |
| Serum PSA at diagnosis, ng/mL | |
| <18 | 23 |
| ≥18 | 25 |
| Median | 18 |
| SD | 32.34 |
| Mean | 28.70 |
| Gleason score | |
| ≤7 | 28 |
| >7 | 22 |
| circFOXO3 expression | |
| ≤2.82 | 26 |
| >2.82 | 27 |
| Median | 2.82 |
| SD | 29.54 |
| Mean | 49.27 |
was performed using Mut Express® II Fast Mutagenesis Kit V2 (Promega). Luciferase® Reporter Assay System (Cat. # E1910, Promega). The cell apoptosis was measured on FACSCalibur flow cytometer (BD). At 48 hours after transfection, cells were treated using the FITC‐Annexin V Apoptosis Detection Kit (Dojindo) for 15 minutes at room temperature. For each well, 100 μL CCK‐8 was added to each well and then incubated for 2 hours at 37°C. The optical density was measured at 450 nm by Microplate Reader ELx808 (BioTek).

2.5 | Cell proliferation assay

Cell proliferation was assessed using the CCK-8 (Dojindo) as our previous report. In brief, transfected cells were maintained in 96‐well plates at a density of 5000 cells per well and at 0, 24, 48 and 72 hours post‐treatment, 10 μL CCK-8 was added to each well and then incubated for 2 hours at 37°C. The optical density was measured at 450 nm by Microplate Reader ELx808 (BioTek).

2.6 | Flow cytometry analysis of cell cycle

PC‐3, LNCaP‐AI and DU145 cells were maintained in 6‐well plates and transfected with circFOXO3 or NC by HilyMax transfection reagents. After 48 hours of transfection, cells were harvested and treated with Triton X‐100 (0.03%) and propidium iodide (PI, 50 ng/mL) for 15 minutes. Cell cycle analysis was performed by FACScalibur flow cytometer (BD).

2.7 | Annexin V‐FITC apoptosis detection

PC‐3, LNCaP‐AI and DU145 cells were maintained in 6‐well plates and transfected with circFOXO3 or NC by HilyMax transfection reagents. At 48 hours after transfection, cells were treated using the FITC‐Annexin V Apoptosis Detection Kit (Dojindo) for 15 minutes at room temperature. The cell apoptosis was measured on FACScalibur flow cytometer (BD).

2.8 | Dual‐luciferase reporter assay

A portion of human SLC25A15 3′‐UTR (621 bp) and circFOXO3 including the seed sequence of miR‐29a‐3p and miR‐221‐5p (570 bp) were separately amplified (Table S1) and inserted into psiCHECK™‐2 firefly/Renilla luciferase reporter vector (Promega). Mutagenesis was performed using Mut Express® II Fast Mutagenesis Kit V2 (Vazyme). After transfection for 48 hours, the Renilla luciferase activity and firefly luciferase activity were measured by Dual‐Luciferase® Reporter Assay System (Cat. # E1910, Promega).

2.9 | Biotinylated RNA pull‐down assays

Biotin‐coupled circFOXO3 probe was designed according to the junction of circFOXO3 (Table S1). Cellular protein was extracted using lysis buffer [100 mmol/L KCl, 5 mmol/L MgCl₂, 10 mmol/L HEPES (pH 7.0), 0.5% NP‐40 supplemented with fresh 200 U RNase inhibitor (Yeasen Biotech Co Ltd), 1 mmol/L DTT, 20 mmol/L EDTA, EDTA‐free protease inhibitor cocktail (Roche) and PMSF] and incubated with 3 μg biotin‐coupled probes at 4°C for 2 hours. Then, 30 μL streptavidin‐conjugated magnetic beads (11205D, Invitrogen) were added into the cell lysate and incubated at 4°C for 1 hour. The retrieved RNA was detected by qRT-PCR as described above.

2.10 | Statistical analysis

All data are reported as the mean ± standard deviation (SD) and represent average of at least three independent experiments. Statistical comparisons between two groups are carried out using Student’s two‐tailed unpaired t test, and P < .05 is considered statistically significant.
after treatment with actinomycin D, a transcription inhibitor, by qRT-PCR. The result showed that circFOXO3 was much more stable than FOXO3 mRNA (Figure 1H). Furthermore, we performed subcellular fractionation and detected the cellular localization of circFOXO3 by qRT-PCR in LNCaP, LNCaP-AI, PC-3 and DU145 cells. The result revealed that circFOXO3 was predominantly cytoplasmic (Figure 1I). Taken together, our results show that circFOXO3 is a stable, predominantly cytoplasmic circRNA and up-regulated in PCa.

3.2 | Knock-down of circFOXO3 inhibited proliferation and promoted apoptosis of PCa cells

To investigate the biological roles of circFOXO3 in PCa, we performed loss-of-function experiments in PCa cell lines. First, we used siRNA reported in Burton Yang et al’s study specifically targeting circFOXO3 to knock down its expression. Compared to linear RNA, back-spooling site was the circRNA-specific sequence. SicircFOXO3 was designed to target the back-splicing site (Figure 2A). Compared to treatment with NC, sicircFOXO3 substantially decreased circFOXO3 levels in LNCaP-AI, PC-3 and DU145 cells (Figure 2B).

Cell proliferation assay showed that knock-down of circFOXO3 could suppressed cell proliferation compared with the cells transfected with the NC in LNCaP-AI, suggesting endogenous circFOXO3 might be involved in the cell proliferation of PCa (Figure 2C). In DU145 cells, we also found circFOXO3 silencing suppressed cell proliferation compared with the NC (Figure 2D).

We next investigated the effect of circFOXO3 in cell apoptosis of PCa. DU145, PC-3 and LNCaP-AI cells were separately treated with sicircFOXO3 or NC, and at 48 hours after transfection, we analysed cell apoptosis by flow cytometry. The apoptosis rates of the sicircFOXO3 and NC were 11.74 ± 0.68% and 7.61 ± 0.48% in DU145 cells, 11.55 ± 0.27% and 8.86 ± 0.16% in PC-3 cells, and 12.18 ± 0.22% and 6.94 ± 0.32% in LNCaP-AI, respectively (Figure 2E-G). The result indicates that knock-down of circFOXO3 expression could significantly promote apoptosis of PCa cells.

3.3 | circFOXO3 knock-down affected cell cycle progression of PCa cells

Next, we detected the effect of circFOXO3 on the PCa cell cycle progression using flow cytometry. Knock-down of circFOXO3 increased the percentage of cells in G0/G1 phase (58.75% to 72.24% in LNCaP-AI, 58.56% to 62.55% in DU145 and 52.66% to 60.5% in PC-3 cells) and decreased the percentage of S phase (26.58% to 20.12% in LNCaP-AI, 27.74% to 24.87% in DU145 and 29.11% to 24.87% in PC-3 cells) (P < .05; Figure 3). Taken together, these results indicate that the effect of silencing circFOXO3 on PCa cell proliferation could be attributed to its promotion of apoptosis and cell cycle arrest.

3.4 | Identification of circFOXO3 mediated ceRNA and confirmation of the sponging effect between circFOXO3 and miR-29a-3p

As mentioned in previous reports, natural circRNAs could function as efficient microRNA sponges to regulate protein-coding genes. To explore potential mechanism of circFOXO3 regulating PCa progression, we constructed circFOXO3-mediated competing endogenous RNA (ceRNA) networks. By using RegRNA 2.0 database, we identified 14 miRNAs targeted to circFOXO3 (Figure 4A-B).

Except as RNA sponge, mounting evidence suggested circRNAs could sequester proteins. Here, we constructed a circFOXO3 interaction protein network. By using RBPDB database, eight RNA binding proteins (MBNL1, NONO, SFRS9, RBM4, SFRS1, FUS, EIF4B and RBMX) with relative score ≥ 0.99 were identified to interact with circFOXO3 (Figure S1A). Next, we analysed the interaction proteins of these RNA binding proteins got from NCBI interaction protein database. As shown in Figure S1A, the circRNA-related interaction protein network contained 481 nodes and 674 edges. Bioinformatics analysis showed circFOXO3-related network was mainly associated with regulating RNA splicing (including RNA splicing, nuclear mRNA processing, alternative splicing and RNA–protein interactions).

### TABLE 2 Correlation between circFOXO3 expression and clinicopathologic features in PCa patients

| Features                  | circFOXO3 expression | P-value |
|---------------------------|----------------------|---------|
|                           | Low      | High    |         |
| Age at surgery (n = 52)   |          |         |         |
| Median (range)            | 69.88    | 67.96   | .352    |
|                           | (68.29-71.47)      | (66.67-69.25) |
| Mean                      | 69.88    | 67.96   |         |
| Pre-operation PSA level (ng/mL) (n = 48) |          |         |         |
| Median (range)            | 21.19    | 36.86   | .1017   |
|                           | (16.41, 25.97)      | (28.85, 44.88) |
| Mean                      | 21.19    | 36.86   |         |
| Gleason score (n = 50)**  |          |         |         |
| Gleason < 7               | 12       | 2       | .0004   |
| Gleason ≥ 7               | 13       | 23      |         |

***P < .01.
splicing, mRNA processing, mRNA transport and mRNA catabolism) and cell proliferation (including cell cycle, anti-apoptosis, DNA replication, cell cycle arrest, DNA repair and mitosis) (Figure S1B).

To test the hypothesis that circFOXO3 promoted PCa proliferation by regulating these miRNAs, we analysed these miRNA expression patterns in public data GSE21036, which contained 28

FIGURE 2 CircFOXO3 silencing suppressed cell proliferation but promoted cell apoptosis in PCa cell lines. A, A siRNA was designed to specifically target circFOXO3. B, The efficiency of si-circFOXO3 was confirmed by qRT-PCR. (C-D) Cell proliferation analysis was performed with CCK-8 assay in LNCaP-AI, DU145. Cells transfected with si-circFOXO3 and NC were seeded into 96-well plate at 5000 cells/well and examined at time points of 0, 24, 48 and 72 h. Knock-down of circFOXO3 inhibited cell proliferation in LNCaP-AI and DU145. (E-G) Cell apoptosis assay was performed in DU145, PC-3 and LNCaP-AI cells. Cells were transfected with NC or si-circFOXO3, and stained with PI and FITC. Knock-down of circFOXO3 increased the percentage of cell apoptosis. Data are presented as the mean ± SD (n = 3). Significance is defined as $P < .05$ ($^*P < .05$; $^{**}P < .01$; $^{***}P < .001$)

FIGURE 3 CircFOXO3 knock-down affects cell cycle progression in PCa cell lines. (A-C) Cell cycle assay was performed in LNCaP-AI, DU145 and PC-3 cells. Cells were transfected with si-circFOXO3 or NC for 48 h, stained with PI and evaluated with a FACScalibur flow cytometer. Knock-down of circFOXO3 increased the number of cells in G1 phase and decreased the number of cells in S and G2 phases. Data are presented as the mean ± SD (n = 3). Significance is defined as $P < .05$ ($^*P < .05$).
corresponding adjacent normal prostate tissues and 113 PCa samples. Our results showed 10 of 14 miRNAs (including hsa-let-7e-3p, hsa-miR-136-3p, hsa-miR-143-3p, hsa-miR-221-5p, hsa-miR-23a, hsa-miR-23b-3p, hsa-miR-29a-3p, hsa-miR-361-5p, hsa-miR-647 and hsa-miR-99b) were down-regulated, and only 4 miRNAs (including hsa-miR-141-3p, hsa-miR-148a-5p, hsa-miR-148b and hsa-miR-939) were up-regulated in PCa (Figure 4B-C). These results are consistent with our conclusion that circFOXO3 may act as an oncogenic circRNA in PCa.

In order to validate our ceRNA network analysis, we selected three of the most significantly down-regulated miRNAs (including hsa-miR-143-3p, hsa-miR-221-5p and hsa-miR-23a) and hsa-miR-29a-3p identified tumour-suppressive miRNAs in PCa for further study. In our previous reports, we found hsa-miR-29a-3p inhibited proliferation and induced apoptosis of PCa cells. Furthermore, we detected circFOXO3 expression level after overexpression of hsa-miR-143-3p, hsa-miR-221-5p, hsa-miR-29a-3p and hsa-miR-23a in PC-3 cells. The result showed that only overexpression of hsa-miR-29a-3p or hsa-miR-221-5p inhibited circFOXO3 expression level (Figure 4D), which suggested that circFOXO3 might interacted with hsa-miR-29a-3p and hsa-miR-221-5p.

To validate the targeting relationship between hsa-miR-29a-3p and circFOXO3, we performed dual-luciferase assay. Bioinformatics prediction showed the predicted miR-29a-3p binding site in circFOXO3, and then, we constructed psiCHECK™-2 luciferase reporter plasmid, containing the fragment of circFOXO3 with the predicted hsa-miR-29a-3p binding site (circFOXO3 Wild Type (29a)) or mutant hsa-miR-29a-3p target site (circFOXO3 Mutant (29a)) (Figure 4E). Then, we transfected both miR-29a-3p mimic and luciferase reporter plasmid into DU145 cells. Compared with miR-NC, miR-29a-3p significantly reduced the luciferase activity of circFOXO3 at ~ 50%, while miR-29a-3p had no significant effect on luciferase activity of circFOXO3 with the mutated target site (Figure 4F). Similarly, we also detected whether miR-221-5p directly interacted with circFOXO3 by dual-luciferase reporter assays. The result showed that miR-221-5p did not affect the luciferase reporter activity of circFOXO3 with either wild-type or mutated target site (Figure 4G-H). Taken together, circFOXO3 directly interacted with miR-29a-3p but not miR-221-5p. Furthermore, biotin-coupled circFOXO3 probe pull-down assay showed that miR-29a-3p was detected in the circFOXO3 pull-down pellet compared with NC group (Figure 4I). These results indicate that circFOXO3 acts as a sponge for miR-29a-3p.

3.5 circFOXO3 regulates miR-29a-3p target expression

To identify novel downstream targets of miR-29a-3p, we first conducted bioinformatics analysis by using TargetScan (www.targetscan.org), miRDB (mirdb.org) and starBase v2.0 (starbase.sysu.edu.cn). A total of 64 candidate targets of miR-29a-3p were identified to be overexpressed in PCa samples using TCGA data set (Figure 5A). In the present study, 7 genes (including SLC25A15, SUV420H2, STRN4, KCTD15, SPPL2B, TET3 and ZNF282) were selected as ceRNA targets of circFOXO3/miR-29a-3p cascade. Next, we separately overexpressed miR-29a-3p in DU145, PC-3 cells (Figure 5B-C) and LNCaP, 22Rv1 cells (Figure 5D-E) and observed SLC25A15, SUV420H2, STRN4 and ZNF282 were significantly suppressed by miR-29a-3p in PCa cell lines. Interestingly, knock-down of circFOXO3 could also inhibit SLC25A15, STRN4 and TET3 expression (Figure 5D-E and Figure S1E-F). Hence, we consider SLC25A15 is a direct target of circFOXO3/miR-29a-3p.

To further confirm whether SLC25A15 is a direct target gene of miR-29a-3p, SLC25A15-3'UTR with miR-29a-3p wild-type or mutant binding site was separately cloned into downstream of the luciferase reporter gene (Figure 5F). Luciferase reporter assay results showed miR-29a-3p mimics significantly decreased the luciferase activity of SLC25A15-3'UTR vectors compared with miR-NC, while miR-29a-3p mimics had no significant effect on luciferase activity SLC25A15-3'UTR with the mutated target site (Figure 5G). Furthermore, apoptotic results showed that overexpression of SLC25A15 significantly suppressed apoptosis in LNCaP-AI cell; however, this effect was significantly abrogated by co-transfection with sicircFOXO3 (Figure 5H). These results indicate circFOXO3 acts as a miR-29a-3p sponge to regulate SLC25A15 expression.

3.6 The expression of miR-29a-3p was down-regulated in PCa

Our previous studies had showed that miR-29a-3p played as a tumour suppressor in PCa. However, the expression pattern of miR-29a-3p in PCa remained unclear. Therefore, we detected the expression of miR-29a-3p in PCa tissue. The result showed that miR-29a-3p was lowly expressed in PCa tissue sample compared with corresponding adjacent normal prostate tissues (Figure 6A-B). However, we did not observe a significant correlation between the miR-29a-3p expression...
and Gleason score, which may be due to the limited sample size (Figure 6C). Very interestingly, we observed a significantly negative correlation between miR-29a-3p and circFOXO3 or SLC25A15 (Figure 6D-E), suggested the existence of circFOXO3/miR-29a-3p/SLC25A15 in PCa samples.

3.7 | SLC25A15 was up-regulated in PCa tissues

We compared SLC25A15 expression levels in 53 PCa tissue samples and corresponding adjacent normal prostate tissues by qRT-PCR. Our analysis revealed that SLC25A15 mRNA expression was up-regulated in PCa samples compared with corresponding adjacent normal prostate tissues (Figure 7A-B). However, we did not observe a significant correlation between the SLC25A15 expression and Gleason score (Figure 7C). To further compare SLC25A15 protein levels in PCa and normal tissues, we analysed SLC25A15 expression in Human Protein Atlas (https://www.proteinatlas.org/). We also observed SLC25A15 was overexpressed in PCa samples compared with normal samples (Figure 7D). More importantly, TCGA data set analysis revealed SLC25A15 was up-regulated in PCa samples (Figure 7E) and increased SLC25A15 expression in PCa tissues was significantly correlated with shorter 5-year overall survival time of PCa patients, shown as Kaplan-Meier survival curve (Figure 7F). Together, these data suggest that SLC25A15 is up-regulated in PCa tissues.

4 | DISCUSSION

Circular RNAs (circRNAs), a covalently closed circular structural RNA, are originated from parental linear genes by RNA polymerase II and synthesized by alternative splicing. Numerous circRNAs may act as diagnostic and therapeutic biomarkers for various diseases due to their high biological stability. Recently, reports have shown that differently expressed circRNAs in blood could act as diagnostic biomarkers. Zhao et al. reported that hsa_circ_0054633 in blood acted as a novel biomarker of pre-diabetes and type 2 diabetes mellitus. Guo et al. also identified hsa_circ_0000190 in blood may be a novel non-invasive biomarker for the diagnosis of gastric cancer. Here, we detected the expression of circFOXO3 in PCa serum samples. The results showed the levels of circFOXO3 expression in PCa serum samples were significantly higher than those in normal samples, which suggested circFOXO3 could act as a diagnostic biomarker of PCa. Of note, our results for the first time demonstrated that circFOXO3 was overexpressed in tumour tissues and positively correlated with Gleason score in PCa. The cellular localization determines the potential function of a gene. Here, RNA fractionation analyses revealed that circFOXO3 was predominantly cytoplasmic in PCa cells.

Accumulating studies showed that circFOXO3 was involved in progression of diseases, such as cardiac senescence and non-small-cell lung cancer. Moreover, Burton Yang et al. reported circFOXO3 interacted with p21 and CDK2, and retarded cell cycle progression. They also detected that circFOXO3 induced tumour apoptosis though enhancing FOXO3 activity. However, whether circFOXO3 plays a role in PCa remains unclear. Therefore, we knocked down circFOXO3 and then explored the potential biological function in PCa development. Intriguingly, our data are not consistent with previous studies. In this study, we found circFOXO3 silencing significantly inhibited the growth of PCa cells by affecting cell cycle progression (LNCaP-AI and DU145 cells) and cell apoptosis. These results suggested that circFOXO3 played an oncogenic role in the development of PCa. Importantly, this is the first study to investigate the molecular function and mechanism of circFOXO3 in PCa.

To explore the potential mechanisms of circFOXO3 regulating PCa progression, we constructed a circRNA-mediated ceRNA network in PCa by using bioinformatics analysis. Our results showed circFOXO3 may act as a miRNA sponge to bind miRNAs. Interestingly, more than 71 per cent of these miRNAs (including hsa-miR-136-3p, hsa-miR-213a-3p, hsa-miR-23b-3p, hsa-miR-29a-3p, hsa-miR-361-5p, hsa-miR-647 and hsa-miR-99b) were down-regulated and only 4 miRNAs (including hsa-miR-141-3p, hsa-miR-148a-5p, hsa-miR-148b and hsa-miR-939) were up-regulated in PCa. Of note, previous reports had shown that hsa-miR-143-3p, hsa-miR-213-3p, hsa-miR-23b-3p, hsa-miR-29a-3p and hsa-miR-361-5p acted as tumour-suppressive roles in PCa. Furthermore, we performed dual-luciferase assay and biotinylated RNA pull-down assay and validated circFOXO3 acted as a miR-29a-3p sponge to regulate its target genes and progression of PCa. In addition, MiR-29a-3p was reported as a tumour suppressor in PCa. In our previous study, we also found hsa-miR-29a-3p suppressed cell proliferation and induced apoptosis in PCa. We further validated SLC25A15 was the target of miR-29a-3p. Therefore, we investigated if, in our system, circFOXO3 played significant roles in PCa development through circFOXO3/miR-29a-3p/SLC25A15 axis. Our data showed that overexpression of SLC25A15 significantly suppressed apoptosis; however, this phenomenon was remarkably
The expression of miR-29a-3p was negatively correlated with circFOXO3 and SLC25A15. (A-B) qRT-PCR analysis of miR-29a-3p expression in 53 prostatic adenocarcinoma tissue samples and corresponding adjacent normal prostate tissues. C, The expression levels of circFOXO3 in PCa according to biopsy Gleason scores. (D-E) The Pearson correlation coefficient analyses of miR-29a-3p and circFOXO3 (D), SLC25A15 (E). Data are presented as the mean ± SD (n = 3). Significance is defined as P < .05 (***P < .001)
abrogated by co-transfection with sicircFOXO3. By detecting circFOXO3/miR-29a-3p/SLC25A15 axis expression in PCa samples, our results also showed a significantly negative correlation between miR-29a-3p and circFOXO3 or SLC25A15 in PCa tissues. These results could explain, at least in part, how circFOXO3 promotes PCa progression. Very interestingly, miR-29a-3p belonged to miR-29 family.

**FIGURE 7** The expression of SLC25A15 in PCa. (A-B) qRT-PCR analysis of SLC25A15 mRNA expression level in 53 prostatic adenocarcinoma tissue samples and corresponding adjacent normal prostate tissues. C. The expression levels of circFOXO3 in PCa according to biopsy Gleason scores. D. SLC25A15 protein levels were overexpressed in PCa samples compared with normal samples by analysing Human Protein Atlas. E, TCGA data set analysis showed SLC25A15 mRNA levels were overexpressed in PCa samples. F. Kaplan-Meier curves for survival time in patients with PCa according to expression of SLC25A15. Significance was defined as $P < .05$ ($**P < .01$; $***P < .001$)
and shared the similar seed region with miR-29b/c. Despite this study focused on the effect of circFOXO3 on miR-29a, we could reasonably hypothesize that circFOXO3 could also sponge miR-29b/c. miR-29b is a miRNA that regulates both osteoblastogenesis and osteoclastogenesis. Furthermore, serum miR-29b is down-regulated in PCa patients and this increases the formation of osteolytic lesions, influencing osteoblast and osteoclast differentiation and activities. These results suggested that overexpression in serum of circFOXO3 could influence osteolytic lesions formation through these processes in PCa. However, the further validation is still needed.

SLC25A15 transports ornithine across the inner membrane of mitochondria from the cytosol to the mitochondrial matrix and plays an important role in regulating the urea cycle. However, the roles of SLC25A15 in cancers remain largely unclear. In this study, we found SLC25A15 was a downstream target of circFOXO3 and up-regulated in PCa samples compared with corresponding adjacent normal prostate tissues. Public data sets analysis also showed SLC25A15 mRNA, and protein levels were overexpressed in PCa samples. More important, we found highly expressed SLC25A15 in PCa tissues was significantly associated with poor prognosis in patients with PCa. We consider this study provides useful information for exploring potential therapeutic and prognostic targets for PCa intervention.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORS’ CONTRIBUTIONS

Zhe Kong, Xuechao Wan, Yali Lu, Liang Li and Yao Li conceived and designed the study; Zhe Kong, Xuechao Wan and Yali Lu developed the methodology; Zhe Kong, Xuechao Wan, Yingyi Zhang, Yan Huang, Yi Xu, Peiqing Zhao and Xinxin Xiang analysed and interpreted the data; Yingyi Zhang and Yi Xu collected the serum and tissue samples. Zhe Kong, Xuechao Wan and Yajuan Liu wrote, reviewed and revised the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.