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*Ciz*1 promotes tumorigenicity of prostate carcinoma cell

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1. ABSTRACT

Prostate cancer is the most common malignancy in men and is the second leading cause of cancer-related mortality in developed countries. Recent work has revealed the significance of CIP-interacting zinc finger protein 1 (CIZ1) in cancer cell biology, but its roles in prostatic carcinoma are unknown. Our study compared *CIZ1* gene expression in banked prostatic carcinomas versus matched paraneoplastic tissues and in tumor cell lines of varying origin. This study revealed that the expression of *CIZ1* was higher in high-grade prostate cancer than in low-grade prostate cancer and normal tissues. Among the tumor cell lines, PC-3 exhibited the highest levels of *CIZ1* expression. *CIZ1* gene silencing in PC-3 cells reduced cell proliferation and colony formation, induced cell cycle arrest in G1, inhibited tumor formation in nude mice, and suppressed the expression of genes related to prostate carcinoma. These results suggest that CIZ1 may play an important role in the progression of human prostate carcinoma and us which may be used as a therapeutic target in prostate cancer.

2. INTRODUCTION

Deregulation of the cell cycle is a hallmark of the neoplastic phenotype (1). In normal eukaryotic cells, sequential activation of various cyclin-dependent kinases (CDKs) regulates the cell cycle, and both extracellular and intracellular signals control CDKs (2). CDKs reside in complexes containing cyclin, proliferating cell nuclear antigen (PCNA), and p21CIP1/WAF1. Previous work indicated that p21 inhibits CDKs; however, p21 is absent from CDK-cyclin complexes in some transformed cells (3). A yeast two-hybrid screen with p21 as bait identified CIP-interacting zinc finger protein 1 (CIZ1) as a p21-interacting protein. CIZ1 is a predominantly nuclear protein that controls the intracellular localization of p21 (4). It also coordinates the actions of cyclins E and A by ensuring that they execute their functions in the proper spatiotemporal manner (5). In a number of cancers, missplicing of exon 4 within the *CIZ1* pre-mRNA alters the protein's spatial distribution within the nucleus, but does not affect the ability of CIZ1 to stimulate initiation of DNA replication (6).

A number of observations point to the significance of CIZ1 in cancer cell biology. For example, a novel, alternatively spliced variant of CIZ1 is over-represented in lung cancer (7). Additionally, CIZ1 was isolated from a human medulloblastoma, and was identified as a DNA-binding protein specific to the sequence ARY(G/C)$R_{(0-2)}$YYAC (8). CIZ1 activates the estrogen receptor-α (*ERα*) gene to impart estrogen hypersensitivity in breast tumorigenesis (9), and is a glucocorticoid receptor-responsive gene (10). Drug treatment studies found that the medicinal corticosteroid clobetasol and the phytoestrogen genistein (a protein tyrosine kinase inhibitor) synergize to reduce cellular CIZ1 levels (11).

Prostate cancer is the most common malignancy in men and is the second leading cause of cancer-related mortality in developed countries (12). Despite its prevalence, the molecular mechanisms underlying prostate cancer formation, maintenance, and progression are poorly understood. As noted above, the significance of CIZ1 in cancer cell biology is emerging, particularly in hormone-responsive cancers (9). However, the roles of CIZ1 in prostatic carcinoma are unknown. In this work, we found that *CIZ1* gene expression is highly elevated in some tumor cell lines and in prostate tumor samples. Moreover, silencing *CIZ1* gene expression in the PC-3 prostate carcinoma cell line severely reduced cell proliferation, colony formation *in vitro*, tumor formation in nude mice, and prostate cancer-related gene expression. Thus, these studies provide new insights into the biological effects of CIZ1 in prostatic carcinoma and may provide a useful reference for clinical diagnosis.

3. MATERIALS AND METHODS

3.1. Tissue preparation

Tissue samples were collected retrospectively from patients with prostatic carcinoma confirmed by histopathological analyses after surgical resection. Paraneoplastic tissues were also obtained from the same patients. All human tissue samples were paraffin-embedded archival remnants of tissues resected for clinical purposes, and were obtained from the Department of Urology of the First Affiliated Hospital of China Medical University. The pathological grade of tumors was defined according to the Gleason grading system. Tissue samples were collected and used for qPCR and immunohistochemistry assays to measure *CIZ1* gene expression. The research was conducted in accordance with the ethical standards of the Helsinki Declaration. The PSA value was determined with Plus-180 (Bayer, Pittsburgh, PA).

3.2. Cell culture

The prostatic carcinoma cell lines DU 145 and PC-3, bladder carcinoma cell lines T24 and 5637, renal carcinoma cell lines OS-RC-2 and 786-0, and embryonic kidney cell line HEK 293T were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The prostatic carcinoma cell lines LNCaP and 22Rv1 were cultured in RPMI-1640 (CellGro), also containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in 5% CO_2.

3.3. Construction of shRNA lentiviral vectors and lentivirus packaging

This pGCSIL-GFP plasmid (GeneChem, Shanghai, China) used in this study contains a CMV-driven eGFP reporter and a U6 promoter upstream of the *Age*I and *Eco*RI restriction sites used for cloning. Oligodeoxyribonucleotides encoding two different short hairpin (sh)RNAs targeting *CIZ1* (GenBank Accession: NM 001131015) were designed and cloned into the pGCSIL-GFP plasmid vector. Briefly,

the plasmid was linearized by digestion with *Age*I and *Eco*RI, and the shRNA oligodeoxyribonucleotides were annealed, digested with *Age*I and *Eco*RI, and ligated into the *Age*I/*Eco*RI-digested plasmid. Correct insertion of the shRNA cassettes was confirmed by restriction enzyme mapping and DNA sequencing. Using previously reported methods (13), lentiviruses were packaged and purified to yield Lv-CIZ1-shRNA1, Lv-CIZ1-shRNA2, and Lv-scr-shRNA, which were used to transduce cells. The shRNA sequences are shown in the Supplementary Information.

3.4. RNA extraction and qPCR

RNA was extracted with Trizol reagent (Gibco BRL) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA with reverse transcriptase (MMLV-RT; Promega) and random hexamer primers using a standard protocol. For the negative control, MMLV was omitted. The qPCR reactions were performed using a two-step method according to the manufacturer's instructions. Amplification was performed with the following conditions: an initial denaturation cycle at 95°C for 15 s; and 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 15 s. Reactions were performed and data were analyzed with the Gene Amp Sequence Detection System (Applied Biosystems, Foster City, CA). The scrambled sequence 5′-TTC TCC GAA CGT GTC ACG T-3′ was used as a negative control, named scr-shRNA. The top-strand sequences of the deoxyribonucleotides used for cloning were: CIZ1-shRNA1, 5′-CCG GCA CTC CAA ATT TGC AAC AGT TCT CGA GAA CTG TTG CAA ATT TGG AGT GTT TTT TG-3′; and CIZ1-shRNA2, 5′-CCG GCC AAT CGA AAG GAT TCT TCT TCT CGA GAA GAA GAA TCC TTT CGA TTG GTT TTT TG-3′. The PCR primer sequences were as follows: CIZ1, 5′-GCC AAA CAA TCC TTG CGA C-3′ and 5′-CAA CCC ACA GCG TCC ACT-3′; AKT1, 5′-TGG CAC CTT CAT TGG CTA C-3′ and 5′-CCG CTC CGT CTT CAT CAG-3′; PSA, 5′-TAC CCA CTG CAT CAG GAA CA-3′ and 5′-CCT TGA AGC ACA CCA TTA CA-3′; and tubulin, 5′-CAA GAG GCT GAC GCA GAA TG-3′ and 5′-TTC ATG TAG CCA GGG TAG CG-3′.

3.5. Immunohistochemical analysis

Immunohistochemistry was performed according to the avidin-biotin complex protocol for paraffin-embedded tissue as described previously (9). Antigen retrieval was performed by pressure cooker treatment in 10 mM citrate buffer, pH 6. The antibodies used were anti-CIZ1 (ab94615, 1:100) and goat anti-rabbit IgG-H&L (HRP) (ab6721, 1:100) from Abcam. Negative controls were performed by substituting the primary antibody with rabbit IgG. To determine immunoreactivity, the level of cytosolic or nuclear staining showing yellowish or brownish granules was graded as follows: 0 for background staining, 1 for faint staining, 2 for moderate staining, and 3 for strong staining. In addition, positively stained areas in entire tissue sections were graded as follows: 0 for < 5%, 1 for 5–25%, 2 for 26–50%, 3 for 50–75%, and 4 for 75–100%. Total scores of 0–2 and ≥3 from the sum of these two parameters were considered negative and positive staining, respectively.

3.6. Western blotting

Cells were washed twice, resuspended in PBS, lysed by ultrasonication, and then centrifuged at 12,000 × *g* for 15 min at 4°C. The protein concentrations of the supernatants were measured by Bradford assay. An equal volume of 2× loading buffer was added, and the samples were boiled for 5–10 min before loading onto an SDS-polyacrylamide gel. Western blotting was performed according to standard procedures. Proteins were detected with an ECL Plus western blotting system (Amersham). The antibodies used for western blotting were anti-CIZ1 (ab94615, 1:100), anti-AKT1 (ab6076, 1:100), anti-PSA (ab68466, 1:100), and goat anti-rabbit IgG-H&L (HRP) (ab6721, 1:100) from Abcam. Tubulin was used as an internal control.

3.7. Cell proliferation assay

Cells were seeded in 96-well culture plates in culture medium at a density of 5×10^3 cells per well in triplicate wells for the Lv-CIZ1-shRNA1, Lv-CIZ1-shRNA2, and Lv-scr-shRNA groups. At 5 days post transduction, cellular proliferation was quantified by an MTT assay (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Absorbance was measured at 490 nm with a microtiter plate reader, and the values from the triplicate wells were averaged for each time point.

3.8. Colony formation assay

Soft agar assays were performed in 6-well plates with a 1.5-ml bottom layer and a 0.5-ml top layer in order to determine substrate-independent cell growth. Cells (1×10^4 per well) were distributed as a cell suspension in 3.4-mg/ml agar (Difco Laboratories, Detroit, MI) in assay medium and overlaid onto a bottom layer consisting of 5.1-mg/ml agar in assay medium. Cells were incubated for 2–3 h at room temperature and then transferred

to 37°C, 5% CO_2. The cell layer was covered with 400 µl of fresh assay medium every 2 days. Colony formation was quantified by Giemsa staining and counting after 10 days.

3.9. Cell cycle assay

Cells were harvested, washed twice with D-Hanks medium, and fixed in 70% ethanol at 4°C overnight. The cells were then collected by centrifugation and stained with propidium iodide (50 µg/ml). RNase A (20 µg/ml) was added and the samples were incubated for 30 min at 37°C. Cell cycle analysis was performed with a BD FACS Calibur (Becton Dickinson, San Jose, CA) and the cell cycle phases were analyzed with Flowjo and FCS3.0 software.

3.10. Tumorigenicity assay with nude mice

The tumorigenicity of Lv-shRNA-transduced PC-3 cells was evaluated with BALB/cA nude mice (Taconic). The animal care practices used and all experiments conducted were reviewed and approved by the Animal Committee of Tongji University School of Medicine, Shanghai, China (TJmed-010-10). The concentration of the cell suspensions was adjusted to 5×10^7/ml. The mice were injected with 0.2 ml of cell suspension from the different Lv-shRNA-transduced cells (n = 6 mice per group). Injections were subcutaneous in the right lateral aspect of the thoracic wall. The mice were inspected for tumor growth three times per week.

3.11. Statistical analysis

Data were analyzed by one-way ANOVA with SPSS 12.0. $P < 0.05$ was considered statistically significant. Error bars represent standard deviation.

4. RESULTS

4.1. Elevated expression of *CIZ1* in prostatic carcinoma tissues and prostatic carcinoma cell lines

CIZ1 gene expression in prostatic carcinoma tissues was compared to that in matched paraneoplastic tissues by qPCR and immunohistochemical analysis. *CIZ1* mRNA was markedly higher in 30 of 38 (78.94%, $P < 0.05$) prostatic carcinoma samples compared to the corresponding paraneoplastic tissue (Figure 1A). Likewise, immunohistochemical analyses showed higher levels of CIZ1 in tumors (Figure 1B shows a representative result) in initial experiments.

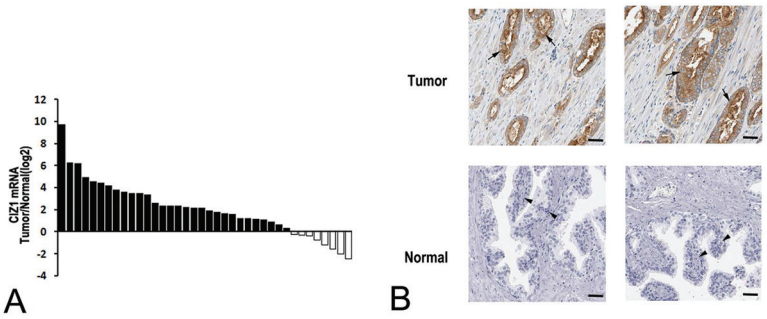
Next, we determined the CIZ1 expression levels in 83 human prostatic carcinoma samples of different grades by immunohistochemical analysis. Positive results were observed in 57.89% (22/38) of low-grade prostatic carcinoma tissues, 91.11% (41/45) of high-grade prostatic carcinoma tissues, and 7.69% (4/52) of adjacent normal tissues (Figure 1C). We also compared the expression of CIZ1 in samples with different Gleason scores, PSA values, and clinical stages. Positive results were observed in 41.70% (5/12), 65.63% (21/32), and 94.87% (37/39) of tissues with Gleason scores of 2–6, 7, and 8–10, respectively (Figure 1D). No CIZ1 was detected in prostatic carcinoma tissues with a PSA value <10, but CIZ1 was detected in 37.5% (6/16), 80% (24/30), and 100% (33/33) of prostatic carcinoma tissues with PSA values of 10–20, 20–30, and >30, respectively (Figure 1E). CIZ1 was detected in 33.33% (3/9), 66.67% (20/30), and 90.91% (40/44) of stage B, C, and D prostatic carcinoma tissues, respectively (Figure 1F).

CIZ1 mRNA and protein were also examined in a number of human tumor cell lines, including prostatic carcinoma (LNCaP, DU 145, PC-3, 22RV1), bladder carcinoma (T24, 5637), and renal carcinoma (OS-RC-2, 786-0) cell lines. While the tumor cell lines exhibited varying levels of *CIZ1* mRNA, the level was highest in the prostatic carcinoma cell line PC-3 ($P < 0.05$). Western blot analyses indicated that protein abundance was proportional to mRNA abundance in all cell lines, and that PC-3 again had the highest levels of CIZ1 (Figure 1G, 1H). Based on these observations, we decided to employ PC-3 cells as a model to examine the effects of CIZ1 on cellular proliferation and tumorigenicity.

4.2. Effects of *CIZ1* gene silencing on cell function

We designed two shRNA expression constructs for lentiviral-mediated gene silencing to examine the biological effects of *CIZ1* silencing. A third construct was designed to express a control shRNA, named scr-shRNA. The constructs also expressed GFP, which allowed transduction efficiency to be assessed by fluorescence microscopy. At an MOI of 40, all PC-3 cells were transduced at high efficiency as observed at 72 h (Figure 2A).

Compared to mock-transduced and Lv-scr-shRNA-transduced cells, the transduction of Lv-ciz1-shRNA1 and Lv-CIZ1-shRNA2 reduced *CIZ1* mRNA levels, both by ~80% (Figure 2B; $P < 0.05$). However,



C

	normal tissue (N=52)	low grade (N=38)	high grade (N=45)	p
CIZ+	7.70%	57.90%	91.20%	<0.05

D

Gleason score	2-6 (n=12)	7 (n=32)	8-10 (n=39)	p
CIZ+	41.70%	65.70%	94.90%	<0.05

E

PSA value	<4 (n=1)	4-10 (n=3)	10-20 (n=16)	20-30 (n=30)	>30 (n=33)	p
CIZ+	0.00%	0.00%	37.50%	80%	100%	<0.05

F

Clinical stage	B (n=9)	C (n=30)	D (n=44)	P
CIZ+	33.40%	66.70%	90.90%	<0.05

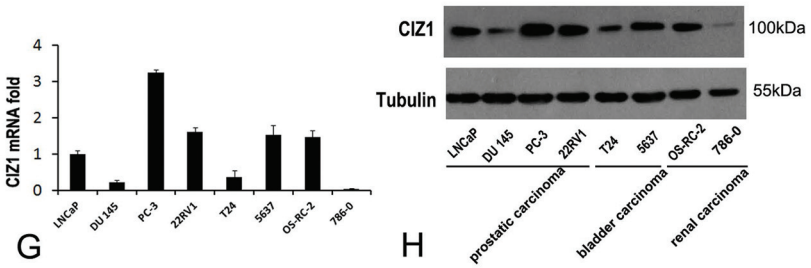


Figure 1. Expression analyses of *Ciz1* in prostatic carcinoma tissue and tumor cell lines. Relative abundance of *Ciz1* mRNA was evaluated in prostatic carcinoma tissues and matched paraneoplastic tissues by qPCR (n = 38). Data are plotted as the log2 of the ratio of tumor/normal Ciz1 mRNA (A). Levels of *Ciz1* were evaluated by immunohistochemical analysis in prostatic carcinoma tissues and normal tissues (B), in normal tissues, low-grade and highgrade prostatic carcinoma tissues(C), in prostatic carcinoma tissues with different Gleason scores (D), with different PSA values (E), and with clinical stages (F). *Ciz1* gene expression was evaluated in prostatic tumor cell lines (LNCaP, DU 145, PC-3, 22RV1), bladder tumor cell lines (T24, 5637), and renal tumor cell lines (OS-RC-2, 786-0) by qPCR (G) and Western blotting (H), showing Ciz1 gene expression was markedly higher in PC-3 cells compared to the others (n = 5). B, scale bar = 20um. In panel (G), qPCR data are plotted as Ciz1 mRNA fold, i.e., each cell line was compared to LNCaP, which was set to 1.

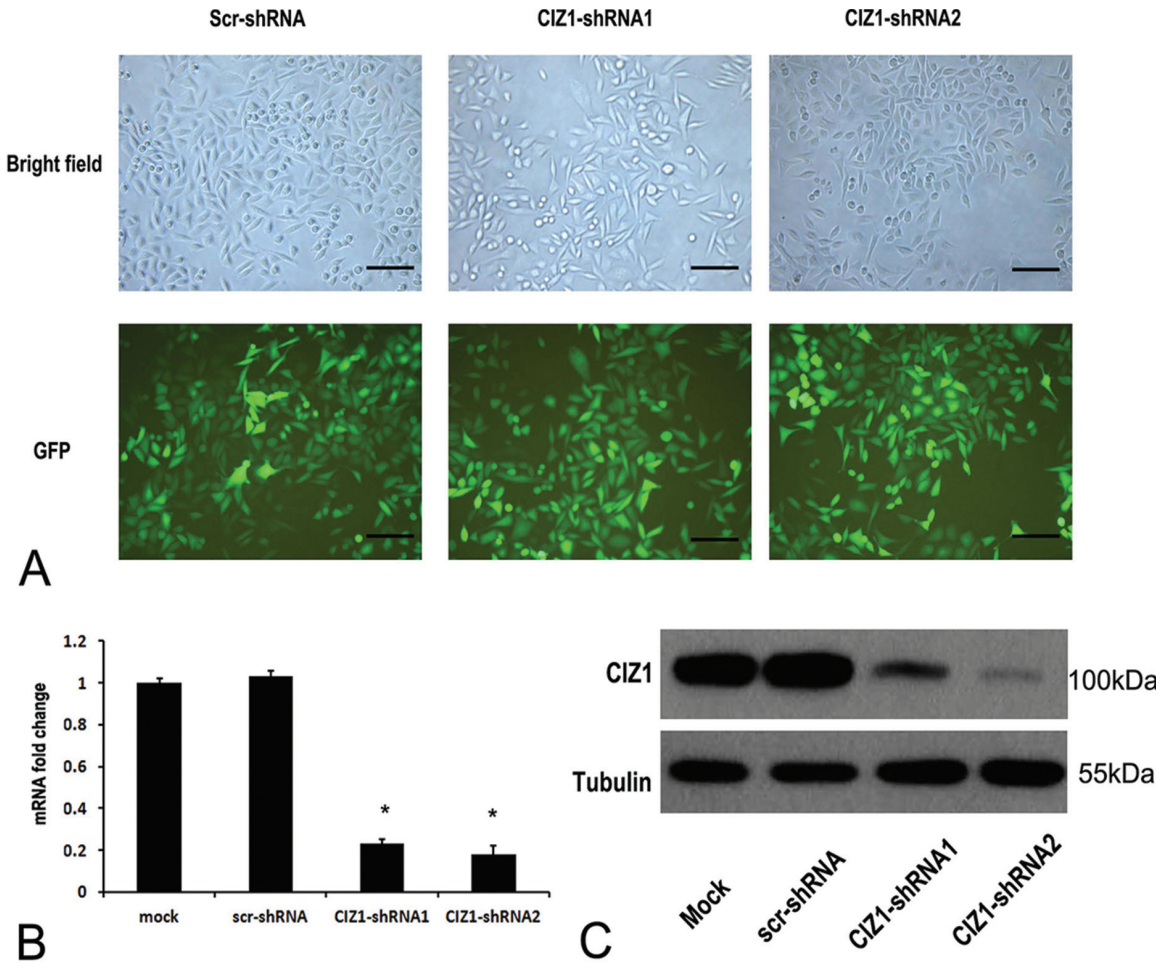


Figure 2. Evaluation of *Ciz1* gene silencing by transduced lentiviruses expressing shRNA. Two lentiviruses were prepared, Lv-Ciz1-shRNA1 and Lv-Ciz1-shRNA2, each expressing a different shRNA against *Ciz1*. A control lentivirus expressing a scrambled shRNA, Lv-scr-shRNA, was also prepared. The vector also contains a GFP expression cassette to allow assessment of transduction efficiency. PC-3 cells were transduced with a lentivirus or mock-transduced and Ciz1 gene expression was evaluated after 72 h. A. GFP expression was examined in transduced PC-3 cells by immunofluorescence (bottom row) (n = 5). Phase contrast images are also shown (top panels). Scale bar=10um. B, C. *Ciz1* expression was evaluated in transduced and mock-transduced PC-3 cells by qPCR (B) and Western blotting (C) (n = 5), where tubulin serves as a gel-loading control. In panel (B), qPCR data are plotted as mRNA fold change compared to mock-transduced cells, which was set to 1.

at the protein level, Lv-CIZ1-shRNA2 appeared to be slightly more effective (Figure 2C). These results demonstrated the efficacy of the lentiviral vectors for *CIZ1* silencing; thus, they were next used to assess the resulting biological effects in PC-3 cells.

Cell proliferation, colony formation in soft agar, and cell cycle distribution were examined. PC-3 cells were transduced with Lv-scr-shRNA, Lv-CIZ1-shRNA1, or Lv-CIZ1-shRNA2. Cell proliferation was measured by an MTT assay 3 days post transduction. Transduction with either of the two Lv-CIZ1-shRNAs markedly reduced cell proliferation compared with Lv-scr-shRNA-transduced cells (Figure 3A, $P < 0.05$). Furthermore, *CIZ1* gene silencing strongly inhibited the ability of PC-3 cells to form colonies in soft agar (Figure 3B). Flow cytometry was used to measure the DNA content in transduced cells and the total fraction of cells in G1, S, and G2/M. Upon *CIZ1* gene silencing in Lv-CIZ1-shRNA1- and Lv-CIZ1-shRNA2-transduced cells, the fraction of cells in G1 phase increased to 68% and 69%, respectively, compared with 47% of Lv-scr-shRNA-transduced control cells in G1 (Figure 3C,

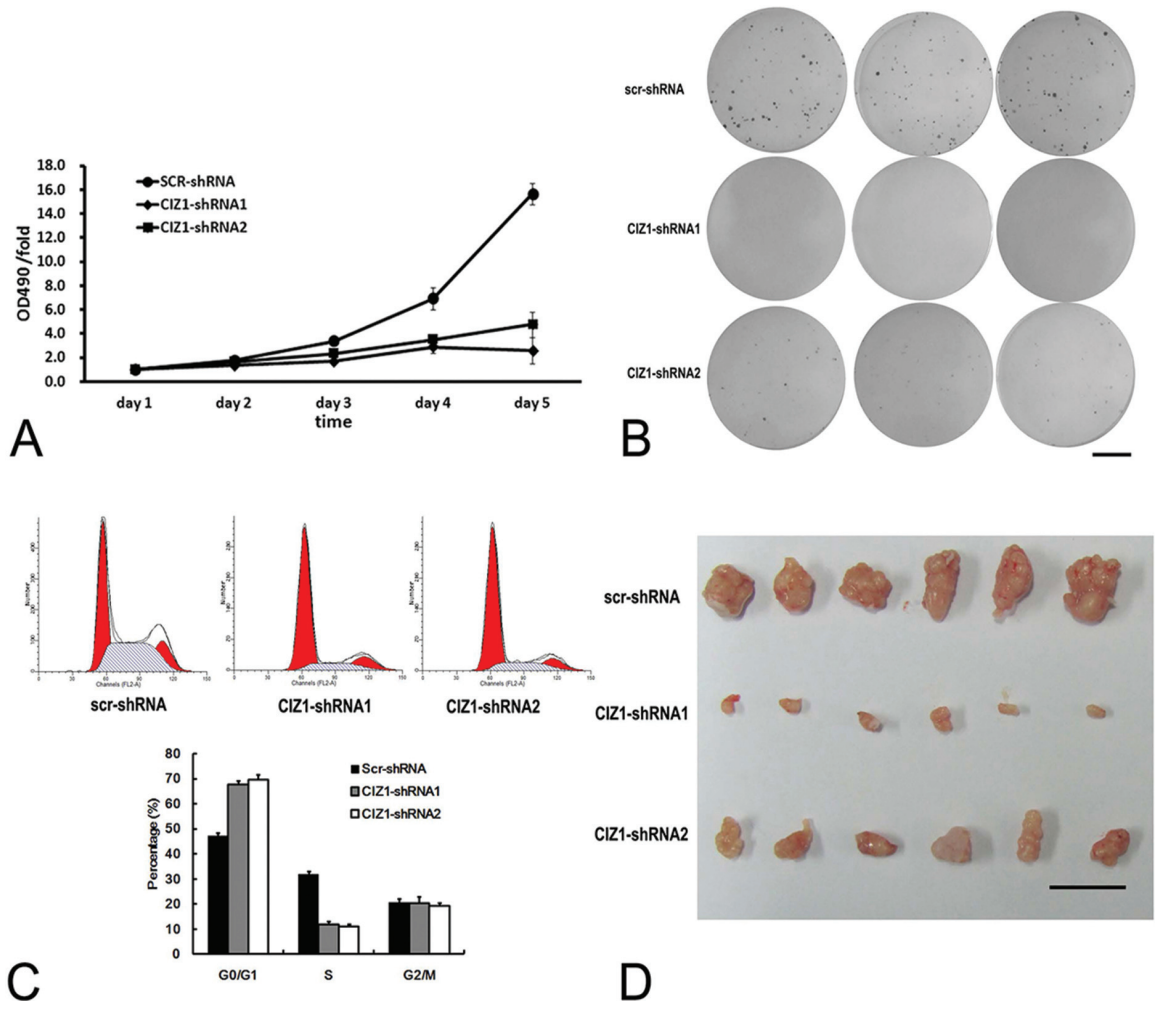


Figure 3. Effects of *Ciz1* gene silencing on cell proliferation and tumorigenicity. PC-3 cells were transduced with Lv-Ciz1-shRNA1, Lv-Ciz1-shRNA2, or control Lv-scr-shRNA. Biological assays were performed after 72 h. A. Cell proliferation was evaluated by MTT assay. Data are plotted as OD490-fold versus time over the ensuing five days, where values at day 1 are set to 1. Data points are the means ± S.D. (n = 5). Cell proliferation was significantly reduced upon *Ciz1* gene silencing. B. Tumorigenicity was evaluated *in vitro* by colony-forming assays. Giemsa-stained plates are shown. Reduced *Ciz1* expression severely impaired colony formation (n = 3). Scale bar = 1cm. C. Transduced cells were analyzed by flow cytometry to determine their distribution in cell cycle phases. The data from the flow cytometry analyses (top panel) were quantified (bottom panel). Reduced *Ciz1* expression induced cell cycle arrest in the G1 phase (n = 5). D. Tumorigenicity was evaluated in vivo in nude mouse assays. Transduced cells were used for xenographs with nude mice. Mice were evaluated for tumor development three times a week. After three weeks, tumors were removed, measured, and photographed as shown. *Ciz1* gene silencing significantly reduced tumor size (n = 6). Scale bar = 1cm.

lower panel, $P < 0.05$). The fraction of cells in S phase decreased upon *CIZ1* gene silencing. These results are consistent with the notion that CIZ1 normally functions to promote cell proliferation.

4.3. *CIZ1* gene silencing inhibits tumor formation in nude mice

We examined the effect of *CIZ1* gene silencing on tumor formation by PC-3 prostatic carcinoma cells. Cells were transduced with the lentiviruses expressing the control shRNA or shRNAs against *CIZ1*. Tumorigenicity assays were then performed with BALB/cA nude mice. Mice injected with the control Lv-scr-shRNA-transduced cells developed sizable tumors at the site of injection (Figure 3D, top row). Mice injected with cells expressing shRNA against *CIZ1* had significantly smaller tumors (Figure 3C, second and third rows;

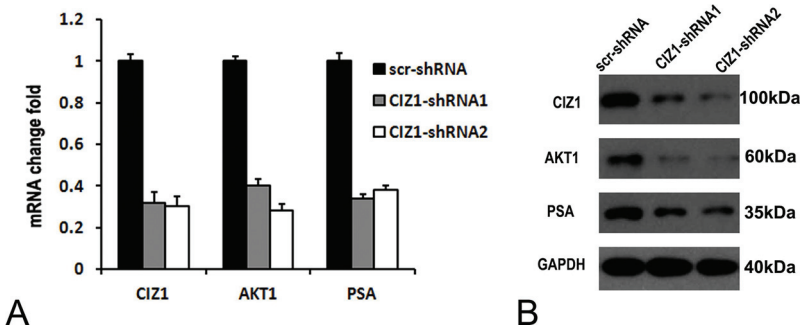


Figure 4. Effects of *Ciz1* gene silencing on expression of prostate carcinoma-related genes. PC-3 cells were transduced with lentiviruses expressing shRNAs as described in the legend for Figure 3 (n = 5). *Ciz1*, AKT1, and *PSA/KLK3* mRNA and protein were assayed by qPCR and Western blots, respectively. A. For qPCR assays, data are plotted as mRNA fold change relative to control scr-shRNA for each gene. *Ciz1* silencing decreased *AKT1* and *PSA/KLK3* mRNA levels by 60-70% (P < 0.0.5). B. Western blotting analyses indicated that *Ciz1* silencing also decreased AKT1 and PSA/KLK3 protein levels.

P < 0.05). Thus, the *in vitro* and *in vivo* data indicate that *CIZ1* gene expression contributes significantly to the cell proliferation and tumor-forming ability of PC-3 cells.

4.4. *CIZ1* gene silencing suppresses prostate cancer-related gene expression in PC-3 cells

Genes encoding the serine-threonine protein kinase AKT1 and prostate-specific antigen (PSA; also known as kallikrein-related peptidase 3, KLK3) have been implicated in prostate carcinomas (14-20). Therefore, Lv-CIZ1-shRNAs or control Lv-scr-shRNA was used to transduce PC-3 cells to determine whether *CIZ1* gene silencing affects the expression of these genes. The levels of *AKT1* and *PSA/KLK3* gene expression were examined by qPCR and western blotting. Silencing of *CIZ1* reduced levels of both *AKT1* and *PSA/KLK3* mRNAs by 60–70% compared to the levels in cells transduced with the control shRNA (Figure 4A, *P* < 0.05). However, *CIZ1* silencing seemed to affect the level of AKT1 protein slightly more than that of PSA/KLK3 protein (Figure 4B). Nonetheless, it is worth considering the idea that the reduced cell proliferation and tumorigenicity observed upon *CIZ1* gene silencing may be partially a consequence of reduced AKT1 and PSA/KLK3 abundance. Other genes affected by *CIZ1* gene silencing await future identification.

5. DISCUSSION

Tumorigenesis is generally driven by the altered expression of critical genes, particularly those involved in the cell cycle (3,21). p21CIP1/WAF1 inhibits cell cycle progression by binding to G1 cyclin/CDK complexes and PCNA (4). Studies indicate that CIZ1 is a unique nuclear protein that regulates the intracellular localization of p21CIP1/WAF1, perhaps by binding p21CIP1/WAF1 via a 150-amino-acid region in CIZ1 (4). CIZ1 also interacts with dynein light chain 1 (DLC1) and participates in cell cycle regulation by increasing CDK2 kinase activity and inducing the G1-S transition (4,22). CIZ1 contains two functional domains: an N-terminal domain essential for DNA replication activity and a C-terminal domain that immobilizes the protein in the nucleus by acting as a nuclear matrix anchor (23).

Previous work has provided detailed insights into the role of the CIZ1 protein in tumorigenesis of the lung (7) and in hormone-responsive breast cancer (9). However, the roles of CIZ1 in tumorigenesis of prostate tissues remain poorly defined. Therefore, we first compared *CIZ1* gene expression in banked prostatic carcinomas versus matched paraneoplastic tissues and in various tumor cell lines. *CIZ1* expression was clearly elevated in carcinoma samples, indicating the clinical significance of CIZ1 and suggesting that it might play a role in tumor formation, maintenance, and/or progression (Figure 1). Of the eight tumor cell lines investigated, five expressed significant levels of *CIZ1* mRNA and protein. The three exceptions included one bladder tumor cell line (T24) and one renal tumor cell line (786-0). The prostate carcinoma cell line DU 145 expressed CIZ1, albeit at very low levels. Nonetheless, our survey of tumor cell lines revealed that the prostate carcinoma cell line PC-3 expresses high levels of CIZ1.

We thus employed a strategy to silence *CIZ1* in PC-3 cells using lentiviral vector-based RNAi and assessed the biological consequences. Consistent with the role of CIZ1 as a p21-binding protein, *CIZ1* gene silencing slowed cell proliferation, most likely by arresting the cell cycle in G1 (Figure 3). However, the contribution of the DNA replication function of CIZ1 on the arrest in G1 is not currently clear, and future work will be required to resolve this issue. In any event, additional assays demonstrated that CIZ1 plays a role in tumorigenesis: *CIZ1* silencing in PC-3 cells reduced colony formation in soft agar, and most importantly, significantly reduced tumor size in xenographs in nude mice. This latter result is quite striking for two reasons: (i) tumors arising from xenographs with reduced CIZ1 were typically 10–30% the size of controls; and (ii) tumors arising from PC-3 cells expressing CIZ1-shRNA1 were uniformly the smallest (Figure 3D). Given that the silencing efficiency of CIZ1-shRNA2 appears to be higher than that of CIZ1-shRNA1 at the protein level (Figure 2C and 4B), it is tempting to speculate that cells require a baseline amount of CIZ1, and expression levels above or below this favor tumor growth. However, future work will be required to fully address this possibility.

Though PSA is not a prostate cancer-specific marker, elevated PSA levels may suggest the presence of prostate cancer (24). In addition, genetic evidence strongly supports a role for AKT1 activation in prostate cancer (18). Our data showed that silencing of *CIZ1* gene expression significantly decreased *PSA* and *AKT1* mRNA and protein. This result is consistent with the reduced tumorigenicity of PC-3 cells upon *CIZ1* gene silencing. However, the mechanisms that reduce AKT1 and PSA levels await further studies.

In summary, we utilized *CIZ1* gene silencing to unveil roles for CIZ1 in the proliferation and tumorigenicity of prostatic carcinoma cells. Thus, CIZ1 may prove to be an effective therapeutic target or perhaps serve as a clinical diagnostic or prognostic marker for human prostate cancer.

6. ACKNOWLEDGEMENTS

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Abbreviations: Ciz1, Cip-interacting zinc finger protein 1; CDKs, cyclin-dependent kinases; DMEM, Dulbecco's modified Eagle's medium; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CDKN1A/p21/Cip1, Cyclin-dependent kinase inhibitor 1A; PSA, Prostate-specific antigen

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