Asian Journal of Urology 10 (2023) 50–57

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Original Article

Deleted in liver cancer 1 suppresses the growth of prostate cancer cells through inhibiting Rho-associated protein kinase pathway

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Received 25 May 2021; received in revised form 24 September 2021; accepted 13 October 2021
Available online 12 January 2022

KEYWORDS
Cell cycle;
Deleted in liver cancer 1;
Proliferation;
Prostate cancer;
Rho-associated protein kinase

Abstract *Objective:* Deleted in liver cancer 1 (DLC1) is a GTPase-activating protein that is reported as a suppressor in certain human cancers. However, the detailed biological function of DLC1 is still unclear in human prostate cancer (PCa). In the present study, we aimed to explore the function of DLC1 in PCa cells.
Methods: Silencing and overexpression of DLC1 were induced in an androgen-sensitive PCa cell line (LNCaP) using RNA interference and lentiviral vector transduction. The Cell Counting Kit-8 assay was performed to determine cell proliferation. The cell cycle was examined by performing a propidium iodide staining assay.
Results: Our results indicated that DLC1 overexpression markedly suppressed the proliferation and cell cycle progression of LNCaP cells. Moreover, DLC1 expression was negatively correlated with Rho-associated protein kinase (ROCK) expression in LNCaP cells. Importantly, this study showed that the ROCK inhibitor Y27632 restored the function of DLC1 in LNCaP cells and reduced the tumorigenicity of LNCaP cells *in vivo*.
Conclusion: Our results indicated that DLC1 overexpression markedly suppressed the proliferation and cell cycle progression of PCa cells and negatively correlated with ROCK expression in PCa cells and tissue.

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Peer review under responsibility of Tongji University.
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https://doi.org/10.1016/j.ajur.2021.12.007

1. Introduction

Prostate cancer (PCa) is a heterogeneous tumor with various properties and a common cancer for males worldwide [1, 2]. Although traditional strategies for PCa have progressed in recent years, the five-year survival rate of PCa patients is far from satisfactory. Therefore, gaining deep insight into the pathogenesis of PCa is a critical step for developing novel therapies.

Deleted in liver cancer 1 (DLC1) belongs to the Rho guanosine triphosphatase (GTPase) activating protein (GAP) family and is located on human chromosome 8p21-22 [3, 4]. A previous study demonstrated that downregulation of *DLC1* was associated with poor prognosis in patients with gastric cancer [5]. Moreover, DLC1 suppresses the progression of hepatocellular carcinoma by inhibiting the Rho and Rho-associated protein kinase (ROCK) signaling pathway [3]. Further, DLC1 markedly suppresses the invasion of PCa cells via regulating the Rho pathway [6]. However, the underlying molecular network of DLC1 in PCa cells still needs to be further explored.

DLC1 catalyzes the transformation of Rho GTPase from the GTP-bound active state to the GDP-bound inactive state [7]. ROCK is one of the most well-known downstream effectors of RhoA. ROCK1 and ROCK2 are two homologs of RhoA in humans that have been mapped to chromosomes 18q11 and 2p24, respectively [8]. Moreover, ROCK is upregulated in human PCa and breast cancer [9, 10]. A previous study has reported that DLC1 possesses the activity of Rho GAP, which is specific for RhoA [11]. However, the detailed correlation between *DLC1* and *ROCK* is still unclear in PCa cells.

β-catenin has been identified as a transcriptional coactivator, which is induced by Wnt and recognized as a target for cancer therapy [12,13]. Dysregulation of Wnt/β-catenin signaling is identified as a biomarker for several human malignancies [14—17]. Moreover, DLC1 interacts with α-catenin to enhance its anti-oncogene activity [18]. However, the role of DLC1 in the Wnt/β-catenin signaling pathway has not been identified in PCa cells.

The key protein in G0/G1 phase of cell cycle—cyclin D1 has been reported as an essential regulator in the process of the cell cycle [19]. Abnormal expression of cyclin D1 influences the progression of the cell cycle. The upregulation of cyclin D1 contributes to tumorigenesis [20]. Hence, cyclin D1 has functioned as a useful prognostic marker for cancers. Moreover, previous report indicated that β-catenin positively correlated with cyclin D1 [21]. However, the detailed molecular network of cyclin D1 is still unclear in PCa cells.

To further investigate the biological function of DLC1 and its possible targets in PCa cells, we induced *DLC1* silencing and overexpression in PCa cells using RNA interference (RNAi) and lentiviral vector transduction, respectively.

2. Materials and methods

2.1. Bioinformatics analysis

Data were collected from RNA-sequencing data of the PCa cohort of the Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/tcga/), and microarray gene expression profile dataset GSE55945 (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo) were used to determine the expression of *DLC1*. A gene set enrichment analysis algorithm was used to identify the pathways that were significantly different between DLC1-high and -low PCa samples.

2.2. Tissue specimens and cell culture

A total of 25 paired PCa samples and adjacent-matched noncancerous tissue were obtained from Shanghai East Hospital, Shanghai, China. Samples were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. The PCa cell line used in this study was LNCaP cells. Human normal prostate epithelial cell line (RWPE-1 cells) was used as control. Both of them were purchased from the Cell Bank of Shanghai Biology Institute (Shanghai, China). All culture media were mixed with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA) containing 2 mM glutamine and 1% penicillin and streptomycin (Solarbio, Beijing, China). LNCaP and RWPE-1 cells were grown in Dulbecco's modified Eagle's medium (Trueline, Corning, NY, USA) and maintained in a 5% CO_2 atmosphere at 37 °C. All patients were informed and gave their written consents. This study and the experimental procedures were approved by the independent ethics committee of Shanghai East Hospital, School of Medicine, Tongji University, Shanghai, China (No: 2013-DF21), and strictly obeyed the Declaration of Helsinki. The handling of mice and all animal experiments were performed according to the Institutional Animal Care and Use Committee guidelines and the institute's guidelines of Shanghai East Hospital, School of Medicine, Tongji University, Shanghai, China.

2.3. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from different samples was extracted using TRIzol reagent (Invitrogen, CA, USA). Then, RNA was reverse transcribed into complementary DNA (cDNA) using a cDNA synthesis kit (Fermentas, Ontario, Canada) according to the manufacturer's instructions. qRT-PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s, and then normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$

method [22]. All data represented the average of three replicates. The primers used in this study are listed in Supplementary Table 1.

2.4. Lentiviral-mediated RNA interference and overexpression of *DLC1*

Three short-interfering RNAs (siRNA) targeting the human gene *DLC1* (NM_001164271.1; *siDLC1-1*, *siDLC1-2*, and si*DLC1-3*) were synthesized (Major, Shanghai, China). A nonspecific scramble siRNA sequence was treated as a negative control (*siNC*). Moreover, lentiviral plasmid (pLVX-puro) containing the full length of human *DLC1* cDNA sequence (*oeDLC1*) was synthesized by Genewiz Company (Suzhou, China). The mock plasmid was functioned as a negative control (oeNC). All of the constructs were transiently transfected into LNCaP cells by Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Assays were performed 48 h after transfection. Detailed information about *siDLC1* sequences is provided in Supplementary Table 2.

2.5. Western blot analysis

Whole protein lysates of the indicated samples were extracted by RIPA lysis buffer (JRDUN, Shanghai, China) with ethylene diamine tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was estimated by using an enhanced bicinchoninic acid protein assay kit (Thermo Fisher, Cleveland, OH, USA). Equal amounts of total protein (25 μg) were fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) overnight. After being blocked with 5% nonfat dry milk (P0216, Beyotime, Shanghai, China) for 1 h at room temperature, the membranes were probed at 4 °C overnight with the primary antibodies (ROCK1 [1:2000; Abcam, Cambridge, UK], ROCK2 [1:1000; Abcam, UK], DLC-1 [1:1000; Abcam, UK], β-catenin [1:5000; Abcam, UK], cyclin D1 [1:10,000; Abcam, UK], and GAPDH [1:2000; Cell Signaling Technology, Beverly, MA, USA]), and then the secondary anti-mouse IgG antibody (1:1000; Beyotime, Shanghai, China) for 1 h at 37 °C. An enhanced chemiluminescence system (Tanon, Guangzhou, China) was used to detect the protein expression value. The protein levels were normalized to GAPDH.

2.6. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay (SAB, Pearland, Texas, USA) was used to measure the cell proliferation profile according to the manufacturer's protocol. Briefly, cells transfected as indicated were seeded in 96-well plates and cultured for 0 h, 24 h, 48 h, and 72 h. CCK-8 solution (10 μL in 100 μL Dulbecco's modified Eagle's medium) was added to each well and incubated for 1 h. Optical density values at a wavelength of 450 nm were measured by a microplate reader (Pulangxin, Beijing, China). Triplicates measurements were performed at each time point.

2.7. Cell cycle assay

Propidium iodide staining was used to determine the DNA content. Cells with or without treatment were harvested and resuspended in phosphate buffer saline (PBS, Solarbio, Beijing, China). Then the cells were fixed with 70% ethanol, −20 °C for 2 h. Each group was treated with RNase A (Solarbio, Beijing, China) at 37 °C for 15 min. Then, propidium iodide (7Sea Biotech, Shanghai, China) was added to the cells. The cells were incubated at room temperature in darkness for 30 min. A flow cytometer (Becton Dickinson & Company, San Diego, CA, USA) was used to analyze the DNA content. The FlowJo cell cycle analysis program (Tree Star, San Carlos, CA, USA) was used to analyze the percentage of cells at Gap phase 0 (G0)/G1, synthesis phase, and G2/mitotic phases.

2.8. Xenograft model

The assay was carried out according to the institute's guidelines for animal experiments and was approved by the independent ethics committee of Shanghai East Hospital (NO. 2016-016). An equal number of PCa cells transfected with siNC and si*DLC1* ($n=2\times10^6$) were subcutaneously injected into the right flank of 4−6-week-old BALB/c nude mice (Shanghai Laboratory Animal Company, Shanghai, China). Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. The length and width of the tumors were examined every 3 days for 33 days at Day 12 after the injection.

2.9. Statistical analysis

GraphPad Prism software version 7.0 (GRAPH PAD software Inc, CA, USA) was used for statistical analyses. Data were displayed as the mean ± standard deviation (SD) of at least three samples. Statistical significance was determined by analysis of variance (ANOVA) for multiple comparisons, and Pearson's correlation analysis was used to examine the relationship between DLC1 and ROCK1 (ROCK2). The $p<0.05$ was accepted to indicate statistical significance.

3. Results

3.1. *DLC1* was downregulated in PCa tissue and cells

To assess the expression of *DLC1* in PCa tissue, we collected its expression profile from two publicly available data sets, including the PCa data set from the Cancer Genome Atlas (TCGA) and GSE55945 data set from the Gene Expression Omnibus database. *DLC1* was clearly downregulated in PCa tissue compared with normal tissue in these two datasets (Supplementary Fig. 1A,B). Moreover, DLC1 was indicated as a suppressor of the cell cycle and Wnt pathway in PCa tissue (Supplementary Fig. 1C,D).

Next, qRT-PCR was used to examine the relative mRNA levels of *DLC1*, *ROCK1*, and *ROCK2* in 25 pairs of human PCa

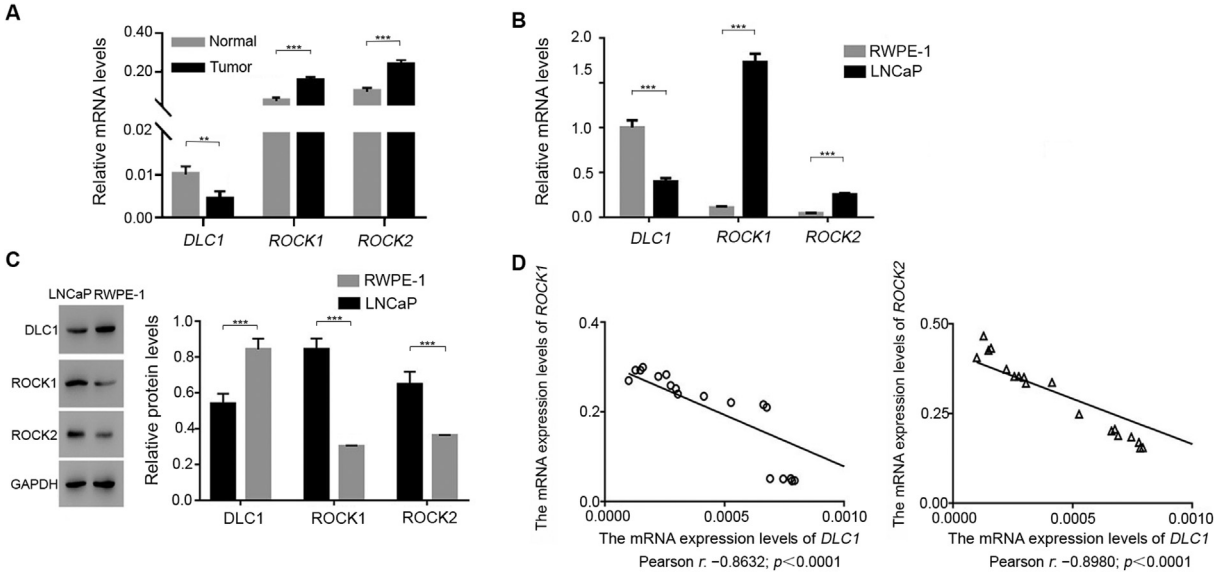

Figure 1 DLC1 was downregulated in PCa tissue and PCa cells. (A) The mRNA levels of *DLC1*, *ROCK1*, and *ROCK2* were examined in PCa tissues ($n=25$ in each group). (B) The relative mRNA levels of *DLC1*, *ROCK1*, and *ROCK2* in LNCaP and RWPE-1 cells were presented, respectively. (C) The relative protein levels of DLC1, ROCK1, and ROCK2 in LNCaP and RWPE-1 cells were presented, respectively. (D) *DLC1* mRNA was negatively correlated with *ROCK1* mRNA or *ROCK2* mRNA in PCa tissue, $n=25$ for each group. DLC1, deleted in liver cancer 1; PCa, prostate cancer; ROCK, Rho-associated protein kinase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. ** $p<0.01$, *** $p<0.001$.

and matched paracancerous tissue. As shown in Fig. 1A, the relative mRNA level of *DLC1* was much lower in PCa tissue than in paracancerous tissue, which was opposite to that of *ROCK1* and *ROCK2*.

Then, we compared the relative mRNA levels of *DLC1*, *ROCK1*, and *ROCK2* between PCa cell LNCaP and normal prostate epithelial cell RWPE-1. The relative mRNA and protein levels of DLC1 were significantly decreased in LNCaP cells compared with that in RWPE-1 cells. However, the relative mRNA and protein levels of ROCK1 and ROCK2 were much higher in LNCaP cells than in RWPE-1 cells (Fig. 1B,C). Furthermore, correlation analysis indicated that *DLC1* was negatively correlated with *ROCK1*and *ROCK2* in LNCaP cells (Fig. 1D).

3.2. Knockdown and overexpression of *DLC1* in LNCaP cells

To further assess the function of *DLC1* in LNCaP cells, we induced *DLC1* knockdown and overexpression in LNCaP cells. For overexpression, the full-length *DLC1* cDNA was inserted into a lentiviral vector. Then, the recombinant vector (*oeDLC1*) and mock plasmid (oeNC) were transfected into LNCaP cells. The untreated cells acted as a blank control (Blank). Marked overexpression of DLC1 was clearly identified in oe*DLC1*-transfected cells (Fig. 2A,B).

For the silencing assay, three siRNAs targeting human *DLC1* (*siDLC1-1*, *siDLC1-2* and *siDLC1-3*) and nonspecific scramble siRNA (*siNC*) were synthesized and transfected into LNCaP cells. *DLC1* siRNAs strongly reduced the endogenous expression of *DLC1*. Moreover, *siDLC1-1* showed a stronger effect than *siDLC1-2* and *siDLC1-3* (Fig. 2C,D). Therefore, oe*DLC1* transfected and *siDLC1-1*-transfected cells were chosen for the following analyses.

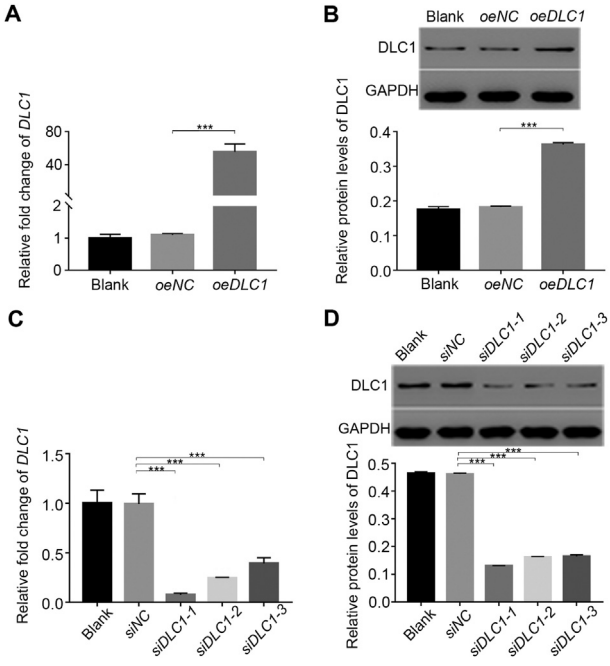

Figure 2 Overexpression and knockdown of DLC1 in LNCaP cells. (A) The mRNA levels of *DLC1* in *oeDLC1*-transfected cells were examined using qRT-PCR. (B) The protein levels of DLC1 in *oeDLC1*-transfected cells were examined using Western blot. (C) The relative mRNA levels of *DLC1* in *siDLC1*-transfected cells were examined using qRT-PCR. (D) The relative protein levels of DLC1 in *siDLC1*-transfected cells were examined using Western blot. DLC1, deleted in liver cancer 1; PCa, prostate cancer; siNC, negative control siRNA; oeNC, negative control of overexpression; oeDLC1, overexpression of DLC1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time real-time polymerase chain reaction. *** $p<0.001$.

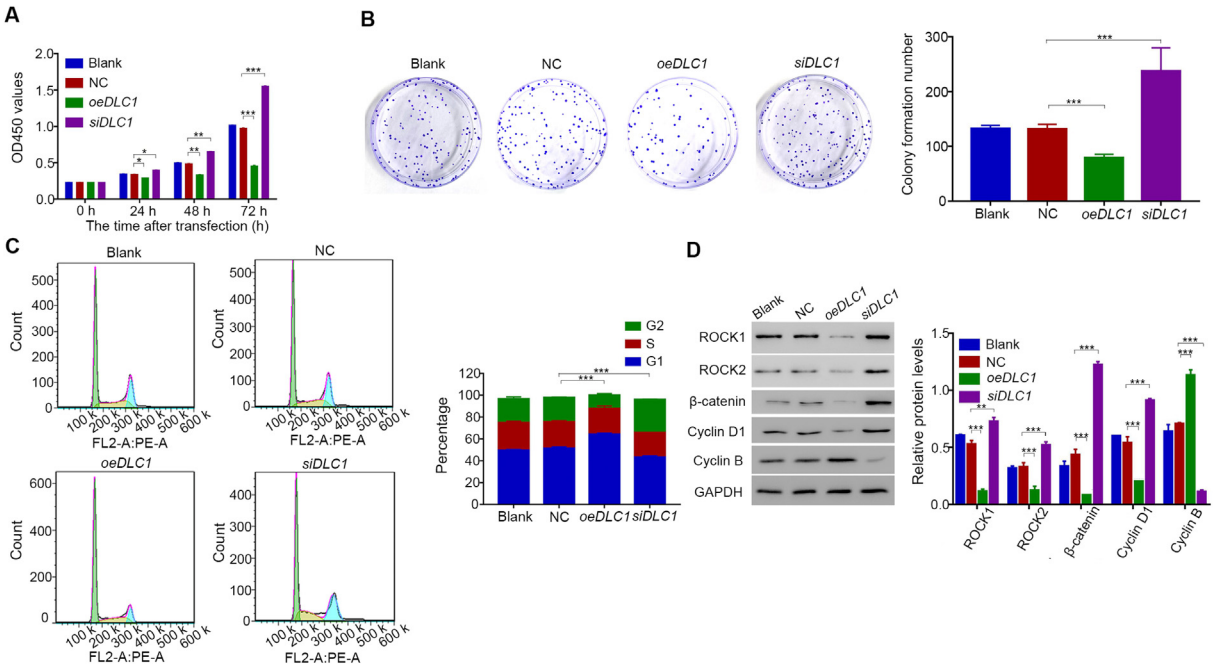

Figure 3 DLC1 overexpression suppressed the proliferation and cell cycle of LNCaP cells. (A) Cell proliferation was detected 12 h, 24 h, 48 h, and 72 h after transfection with NC (both *siNC* and *oeNC*), *oeDLC1*, and *siDLC1*.(B) Colony formation assay was performed in cells as indicated above. (C) Cell cycle profiles of cells transfected with NC, *oeDLC1*, or *siDLC1* were examined by using flow cytometry. (D) The protein levels of ROCK1, ROCK2, β-catenin, cyclin D1, and cyclin B in different transfected cells. DLC1, deleted in liver cancer 1; PCa, prostate cancer; ROCK, Rho-associated protein kinase; OD450, optical density 450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; oeDLC1, overexpression of DLC1; *siDLC1*, DLC1 siRNA; S, synthesis phase; G1, gap phase 1; G2, gap phase 2. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.3. *DLC1* overexpression inhibited the proliferation and cell cycle of LNCaP cells

CCK-8 assay was performed to examine cell proliferation. As shown in Fig. 3A, the cell proliferation of the Blank and NC groups showed no significant difference. However, the proliferation rate markedly decreased in *oeDLC1*-transfected cells, whereas it was significantly upregulated in *siDLC1*-transfected cells. These results demonstrated that DLC1 was an anti-proliferative factor in PCa cells. Moreover, overexpression of *DLC1* deeply decreased the colony formation of human LNCaP cells, while the opposite results was obtained in *siDLC1* transfected cells (Fig. 3B).

Next, flow cytometry was utilized to assess the cell cycle in different cells as indicated. As shown in Fig. 3C, the G0/G1 phase cell population was significantly decreased in *siDLC1*-transfected cells compared with that in NC-transfected cells. Moreover, *DLC1* siRNA prolonged the G2/M phase in LNCaP cells, whereas the opposite results were obtained in *oeDLC1* transfected cells.

Moreover, Western blot analysis was used to quantify the protein levels of ROCK1, ROCK2, β-catenin, cyclin D1, and cyclin B (the key protein of G2/M phase) in cells as indicated. Importantly, the protein levels of ROCK1 and ROCK2 markedly decreased in *oeDLC1*-transfected cells but increased in *siDLC1*-transfected cells (Fig. 3D). Interestingly, DLC1 overexpression also significantly inhibited the expression of β-catenin and cyclin D1, while promoted the expression of cyclin B in PCa cells.

3.4. The ROCK inhibitor Y27632 rescued the function of DLC1 in LNCaP cells

To further analyze the connection between DLC1 and ROCK, a specific ROCK inhibitor Y27632 (10 mmol/L; ACS-3030; ATCC, VA, USA) was used to treat *siNC*- and *siDLC1*-transfected cells. As shown in Fig. 4A, Y27632 significantly inhibited the cell proliferation rate of *siNC*- and *siDLC1*-transfected cells and deeply reduced the colony formation in *siDLC1*-transfected cells as well (Fig. 4B). Moreover, the function of *siDLC1* in the cell cycle was abolished by Y27632 (Fig. 4C). In addition, we also examined the protein levels of β-catenin and cyclin D1 in *siDLC1*-transfected cells as indicated. As shown in Fig. 4D, Y27632 markedly suppressed the expression of β-catenin and cyclin D1, but promoted cyclin B expression in *siDLC1*-transfected cells. Taken together, these results demonstrated that the ROCK inhibitor Y27632 played a key role in rescuing the function of DLC1 in PCa cells.

3.5. Knockdown of *DLC1* promoted the tumorigenicity of LNCaP cells *in vivo*

To further examine the effect of DLC1 on tumorigenicity *in vivo*, a PCa model was established in nude mice. An equal number of LNCaP cells that were transfected with *siNC* and *siDLC1* were hypodermically injected into nude mice. Then, the mice were treated with Y27632 once the tumor formed. Subsequently, the tumor was determined every 3 days for 33 days (starting at Day 12). All

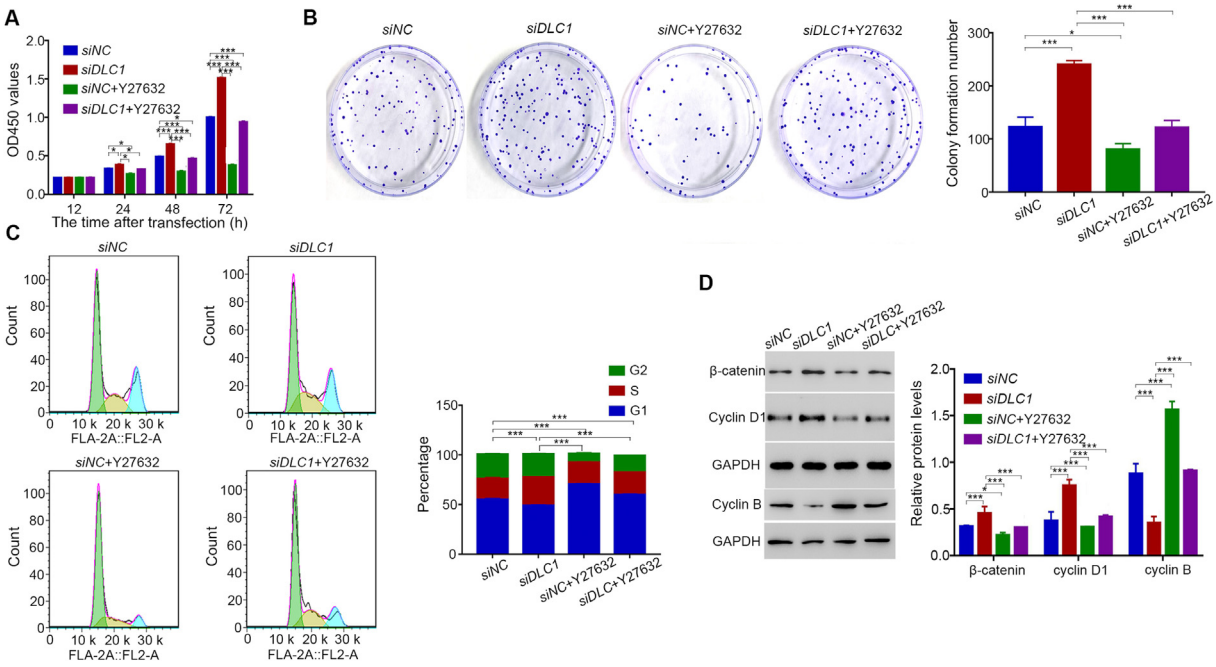

Figure 4 The ROCK inhibitor Y27632 rescued the function of DLC1 in LNCaP cells. (A) Cell proliferation was detected at 12 h, 24 h, 48 h, and 72 h in cells transfected with *siNC*, *siDLC1*, or *siNC* and treated with Y27632. (B) Colony formation assay was performed in cells as indicated above. (C) Y27632 reduced the effect of si*DLC1* in PCa cell cycle regulation. (D) Western blot was used to examine the protein levels of β-catenin, cyclin D1, and cyclin B in cells as indicate above. ROCK, Rho-associated protein kinase; PCa, prostate cancer; *siNC*, negative control siRNA; DLC1, deleted in liver cancer 1; *siDLC1*, DLC1 siRNA; G1, Gap phase 1; G2, Gap phase 2; S, synthesis phase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. * $p<0.05$; ** $p<0.01$; ** $p<0.001$.

of the injected cells were able to clearly develop tumors in nude mice. However, both the tumor volume and weight were significantly increased by injection with *siDLC1*-transfected cells compared to injection with *siNC*-transfected cells. Interestingly, Y27632 abolished the tumorigenicity of PCa cells transfected with *siDLC1* (Fig. 5A,B).

Further, the protein level of DLC1 was also increased by Y27632 *in vivo*. (Fig. 5C) Meanwhile, the levels of β-catenin and cyclin D1 were significantly decreased by Y27632. These findings were consistent with those *in vitro*. Therefore, the ROCK inhibitor Y27632 was a promising agent in the treatment of PCa.

4. Discussion

Testosterone is needed for PCa cells to maintain growth and progression during its early stages. Hence, androgen-deprivation therapy has been identified as a useful approach in the treatment of PCa at its initial phase [23]. However, this method is not effective for androgen depletion-independent cells. Therefore, a novel useful biomarker for PCa is urgently needed.

In the current study, we investigated the biological function of DLC1 in LNCaP cells. Silencing and overexpression of *DLC1* were induced by using RNAi and lentiviral vector transduction in LNCaP cells. The results obtained from these two experimental sections were consistent, which make our results more robust.

A previous study reported that DLC1 suppressed the progression of hepatocellular carcinoma cells via inhibiting ROCK [3]. Moreover, overexpression of DLC1 significantly inhibits the proliferation of cutaneous squamous cell carcinoma [24]. Moreover, a similar result was obtained in Burkitt's lymphoma cells [25]. In the current research, our results demonstrated that DLC1 was downregulated in PCa tissues and cells. Moreover, DLC1 overexpression markedly suppressed the proliferation and cell cycle progression of LNCaP cells. Therefore, our findings indicated that DLC1 was an anti-proliferation and cell cycle factor in PCa cells. Importantly, our results not only elucidated that DLC1 was a tumor suppressor in LNCaP cells, but also demonstrated its potential value as a biomarker for PCa diagnosis. Furthermore, our analysis indicated a negative correlation between DLC1 and ROCK in PCa tissue and cells. Therefore, DLC1 was involved in the ROCK pathway in PCa tissues and cells. DLC1 might suppress the growth of PCa cells by inhibiting ROCK1 and ROCK2.

A previous report stated that DLC1 suppressed the growth of colon cancer cells by inhibiting Wnt/β-catenin signaling [26]. In the present study, DLC1 overexpression markedly inhibited the expression of β-catenin in LNCaP cells. It has been confirmed that ROCK is the upstream factor of β-catenin in breast cancer and colon carcinoma cells [27, 28]. Therefore, β-catenin might be a component in the DLC1/ROCK signaling pathway in PCa cells. Our findings firstly illustrated a negative correlation between DLC1 and β-catenin in PCa cells.

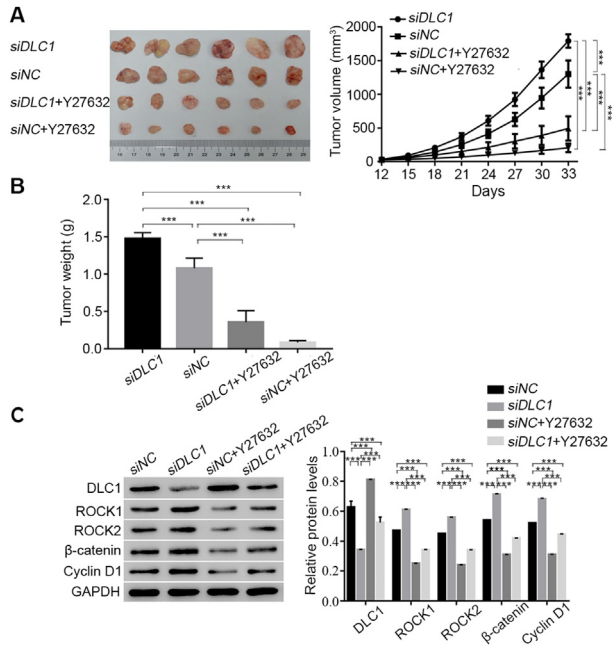

Figure 5 The ROCK inhibitor Y27632 inhibited the tumorigenicity of LNCaP cells that transfecting with *siDLC1 in vivo*. (A and B) The tumor volume and weight in nude mice injected with PCa cells transfected with *siNC* or *siDLC1*, with or without Y27632 treatment ($n=6$ in each group). (C) The relative protein levels of DLC1, ROCK1, ROCK2, β-catenin, and cyclin D1 in different tumors as indicated. ROCK, Rho-associated protein kinase; PCa, prostate cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DLC1, deleted in liver cancer 1; *siDLC1*, DLC1 siRNA; *siNC*, negative control siRNA. *** $p<0.001$.

Cyclin D1 belongs to the G1 cyclin family and is identified as a positive regulator of cell cycle progression [21]. Moreover, a previous report demonstrated that DLC1 suppressed the growth of renal carcinoma cells by regulating cyclin D1 [29]. Importantly, our studies also obtained similar results. Therefore, DLC1 might inhibit the cell cycle of PCa cells by suppressing cyclin D1. Moreover, *DLC1*-knockdown significantly promoted the tumorigenicity of LNCaP cells *in vivo*, which was markedly suppressed in the presence of Y27632, the ROCK inhibitor. Therefore, Y27632 is a potential agent for the treatment of PCa. Targeting the ROCK signaling pathway might provide a novel perspective for the treatment of PCa.

5. Conclusion

In the present study, our results indicated that DLC1 overexpression markedly suppressed the proliferation and cell cycle progression of PCa cells and negatively correlated with ROCK expression in PCa cells and tissue. Our findings not only deepen the understanding of DLC1 in PCa cells but also provide novel insight into PCa therapy.

Author contributions

Study concept and design: Weihua Chen.
Data acquisition: Hua Gong, Kang Chen.
Data analysis: Lan Zhou, Yongchao Jin.
Drafting of manuscript: Hua Gong.
Critical revision of the manuscript: Weihua Chen.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

This study was supported by the Key Scientific Research Project of Shanghai Municipal Commission of Health and Family Planning (No. 201640014), the project of Natural Science Foundation of Jiangxi (No. 20171BAB205019), and the Special Diseases Program of Pudong New Area Health System (No. PWZzb2017-06). All authors sincerely acknowledged the support given by Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital and Shanghai East Hospital, School of Medicine, Tongji University, Shanghai, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajur.2021.12.007.

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