Original Article
Cullin-1 promotes cell proliferation via cell cycle regulation and is a novel in prostate cancer

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Abstract: Background: There is no reliable marker available for early detection, diagnostic confirmation, or disease prognosis available of prostate cancer (PCa). We aimed to evaluate the function of Cullin-1 and unravel its underlying molecular mechanism to develop novel treatment options equivalent to PCa. Method: We used immunohistochemistry to analyze the correlation between Cullin-1 expression and clinicopathologic variables and patient survival. The Cullin-1 level was tested in PCa cells. The role of regulation of Cullin-1 in PCa was applied in vitro and vivo. In addition, we further investigated the signaling pathway of Cullin-1 in prostate cancer cell proliferation. Result: We first discovered that Cullin-1 expression was upregulated in human PCa tissues and inversely related with PCa differentiation. We then found that high expression of Cullin-1 protein suggested a poor prognosis in PCa patients. Also, Cullin-1 promotes PCa cell proliferation in vitro and tumor growth in vivo. We then found that the mechanism of Cullin-1 regulation on cell-cycle progression is due to increased expression of p21 and p27, and decreased expression of cyclin D1 and cyclin E after Cullin-1 knockdown. Conclusion: Cullin-1 exerts multiple biological effects in the PCa cell line. Through promoting proliferation and by countering cisplatin-induced apoptosis, Cullin-1 has been deeply implicated in the pathogenesis and development of PCa.

Keywords: Cullin-1, prostate cancer, proliferation, diagnosis

Introduction

Prostate cancer (PCa) still ranks high as the leading cause of death among urological malignancies, which is the most common type of cancer and the leading cause of cancer-related morbidity among men in the United States of America. According to the assessment of the American Society of Clinical Oncology, there were 238,590 new cases of prostate cancer and 29,720 deaths in 2013 [1]. When diagnosed at its early stages, PCa can be effectively treated by surgery or radiation. However, up to one-third of patients with organ-confined PCa eventually fail local therapy and ultimately progress to advanced-staged or metastatic disease within 10 years [2]. Although early diagnosis of PCa has improved clinical outcome, metastatic PCa and hormone refractory prostate cancer (HRPC) remains one of the most challenging clinical problems，which leads to a late-stage event with a poor prognosis. PCa have a striking tendency to metastasize to bone. App-

roximately 4% of all newly diagnosed patients present with metastatic disease, and up to 85% of patients diagnosed with HRPC have metastases [3].

Traditional therapeutic strategies (chemotherapy and radiotherapy) are often associated with unsatisfying outcomes. So molecular-targeted therapy has emerged as an alternative treatment for patients with metastatic PCa or HRPC. Thus, the development of more effective therapeutic interventions based on the molecular mechanisms by which tumors develop resistance to therapeutic drugs is an urgent need. Recent work has been aimed at identifying key molecules involved in metastasis as therapeutic targets. Therefore, the potential targets for PCa therapy include tumor cell antigens that bind to molecules found at these principal sites of metastasis [4].

The cullin family of proteins was first identified in 1996 as being required for cell cycle exit in C.

Table 1. Patients' information

Characteristics	Prostate cancer (n=142)
Gender	
Male	142
Age at diagnosis	
Mean ± SD	63 ± 9
Median (range)	57 (31-82)
Tumor proportion	
< 5%	35
5-10%	62
> 10%	45
T stage	
T1	31
T2	108
T3	34
Lymph node metastasis	
N0	79
N1	41
N2	22
Gleason score	
< 7	48
= 7	62
> 7	32
PSA (ng/ml)	
Mean ± SD	17.6 ± 4
Median (range)	18.2 (7.1-32.7)
< 20	73
> 20	69
Margin status	
Positive	28
Negative	114
Neoadjuvant therapy	
Yes	88
No	63

elegans [5] and for the G1-to-S-phase transition in budding yeast [6]. Members of the cullin family (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7, PARC and APC2) provide the scaffold of CRL complexes responsible for a large portion of UPS-mediated proteolysis. The family members are characterized by an evolutionarily conserved cullin-homology domain, and serve as molecular scaffolds to facilitate the assembly of multimeric CRLs with SCF/CRL-1 as the founding member. Cullin-1 is the most extensively characterized member of the cullin family and a key component of SCF E3s, which has an indispensable role in both cell cycle progression and early embryogenesis.

To evaluate the function of Cullin-1 in prostate cancer, we used immunohistochemistry to analyze the correlation between Cullin-1 expression and clinicopathologic variables and patient survival. In addition, we further investigated the role of Cullin-1 in prostate cancer cell proliferation.

Materials and methods

Human tissues and cell lines

A total of 142 pairs of human primary PCa tissues and their adjacent NCTs were collected between 2006 and 2009 at Huashan Hospital, Fudan University. These tissue samples were immediately snap-frozen in liquid nitrogen. All of the human materials were obtained with informed consent. The clinical information for the PCa patients is included in the **Table 1**. Two human PCa cell lines (PC-3, DU-145) and HEK-293T cell line were purchased from the American Type Culture Collection (ATCC). DU-145 and PC-3 cells were cultured in Ham's F-12 media (Invitrogen, USA) with L-glutamine (300 mg/L, NaHCO3 1.5 g/L) and 10% FBS. HEK-293T cells were cultured in DMEM. Cells were incubated with 5% CO_2 at 37°C.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentrations were determined using a NanoDrop ND-1000 (NanoDrop, USA). cDNA was synthesised with the PrimeScript RT reagent kit (TaKaRa, Japan) using 500 ng total RNA as template. qPCR analyses were conducted to quantitate mRNA relative expression using SYBR Premix Ex Taq (TaKaRa, Japan) with beta-actin as an internal control (44-46). The results of qPCR were defined from the threshold cycle (Ct), and relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method [7]. PCR was performed using an ABI 7900HT instrument (Applied Biosystems, USA). The primers used for PCR analysis were listed in the Supplementary Data (Table S1).

Vector constructs and lentivirus transduction

The open reading frame (ORF) of Cullin-1 was amplified by nested PCR and cloned into the pLVX-IRES-Neo vector (Clontech, USA). The
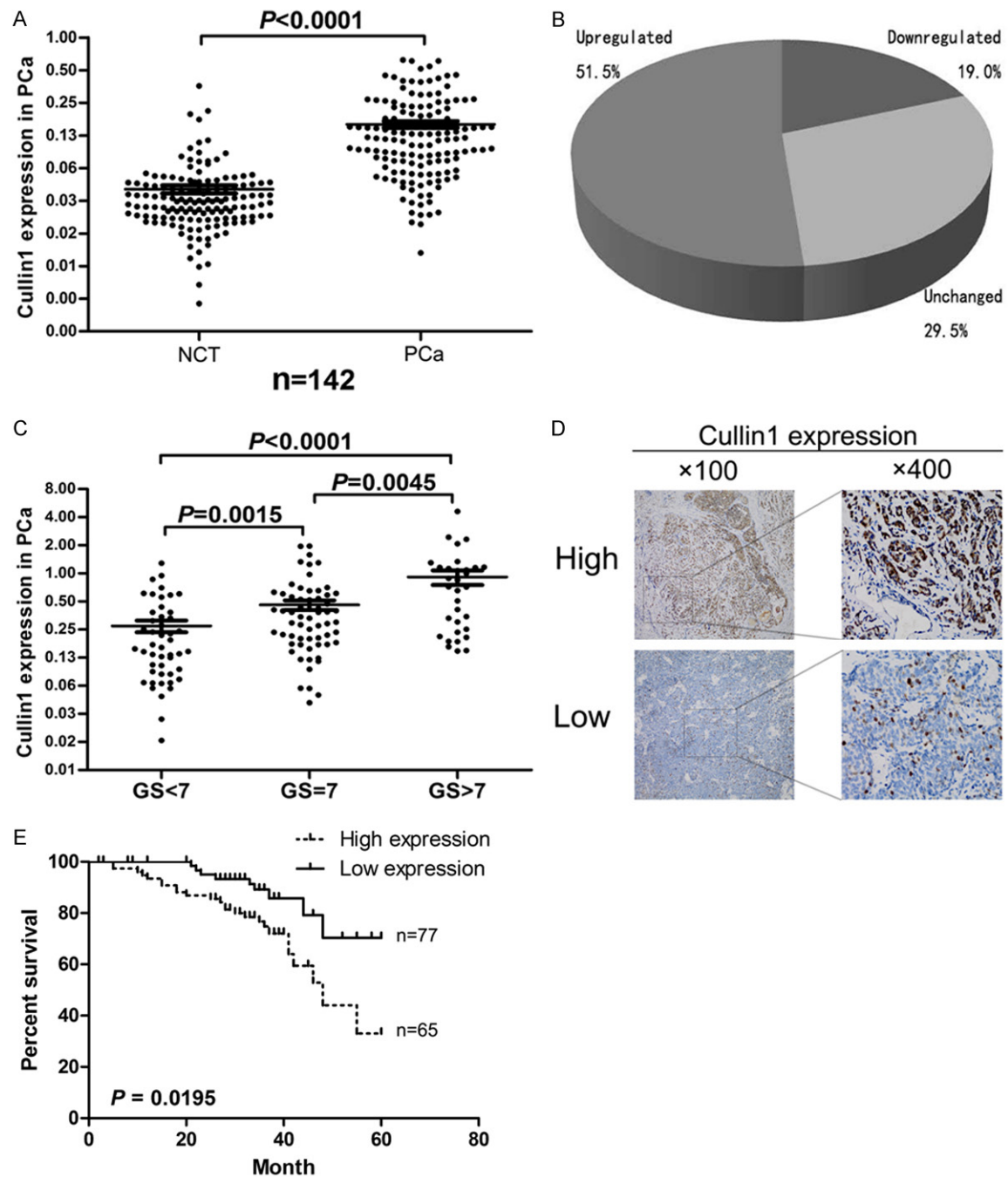
Figure 1. Cullin 1 expression was frequently increased in PCa patients. A. Comparison of Cullin 1 expression levels in 142 matched pairs of PCa tissues and noncancerous tissues (NCTs). The qRT-PCR results showed that Cullin 1 expression was significantly upregulated in tumor tissues (GAPDH used as an internal control). B. Cullin 1 expression was upregulated in 51.5%, downregulated in 19.0% and unchanged in 29.5% of the PCa samples. C. Box plots of expression levels of Cullin 1 in 3 groups (48 with GS < 7, 62 with GS = 7, 32 with GS > 7). Cullin 1 expression was upregulated in GS > 7 patients and downregulated in GS < 7 compared with GS = 7 patients. D. Cullin 1 protein showed a typical nucleus staining in Pca tissues and more than 50% cells with nucleus staining was determined as high expression (original magnification, × 100 or × 400). E. The percentage of OS in the Cullin 1 low-expression group was significantly higher than that of patients in the ARL5A high-expression group.

primers and endonuclease sites used for the vector constructs are shown in the Supplementary Data (Table S1). Virus particles were

harvested 48 hours after cotransfecting pLVX-Cullin-1 with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G into HEK-

293T cells using Lipofectamine 2000 reagent (Invitrogen, USA). PC-3 and DU-145 cells were infected with recombinant lentivirus-transducing units plus 6 µg/mL polybrene (Sigma, USA).

Oligonucleotide transfection

Small interfering RNA (siRNA) of Cullin-1 were synthesised (Ribobio, China). Oligonucleotide transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. The final concentration siRNA in the transfection mixture was 50 nM.

Cell proliferation assay and colony formation assay

Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) according to the manufacturer's instructions. For the colony formation assays, 1,000 cells per well of PC-3 and DU-145 were incubated in medium containing 10% FBS for 2 weeks. The colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 20 minutes. The number of colonies containing more than 30 cells was counted using an inverted microscope.

Xenograft and intravenous tumor model

Male BALB/c athymic nude mice at 5 weeks of age (Vital River, Beijing, China) were bred in licensed SPF (special pathogen-free) grade laboratory. A total of 2×10^6 cells (DU145 and PC-3, stably expressing Cullin-1 or the vector control) in 200 µl of PBS were injected subcutaneously at right flanks of each mouse. After transplantation, the growth of the subcutaneous tumors was assessed twice a week. Tumor size was monitored by measuring the length and width with callipers, and volumes were calculated with the formula: $(L \times W^2) \times 0.5$, where L is length and W is width of each tumor. The mice were sacrificed after a period of 5-7 weeks, and the weight of subcutaneous tumors were recorded. All protocols were approved by Fudan University animal ethics committee.

Cell-cycle analysis

Thirty-six hours after transfection, the cells were treated with 1 µg/ml aphidicolin. After 12 h, the cells were incubated in fresh medium containing 50 ng/ml nocodazole for 0, 3, 6 h or 0, 4, 6 h. Then, the cells were fixed with 70% cold ethanol at 4°C overnight, and stained with 40 µg/ml propidium iodide in hypotonic fluorochrome buffer for 30 min. Samples were then analyzed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA).

Western blot

Harvested proteins were first separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, USA). The membranes were blocked with 5% nonfat milk and incubated with a mouse anti-Cullin-1 polyclonal antibody at a dilution of 1:500 (Abcam, USA) or a mouse anti-beta-actin monoclonal antibody at a dilution of 1:1,000 (Sigma, USA). The membranes were subsequently incubated with a goat anti-mouse horseradish peroxidase secondary antibody (Sigma, USA). The protein complex was detected using enhanced chemiluminescence reagents (Pierce, France). Endogenous beta-actin was used as the internal control.

Immunohistochemical staining

Tissue arrays were constructed using the 142 paired PCA tissues and NCTs. Immunohistochemical staining was performed on 4 µm sections of paraffin-embedded tissues to determine the expression level of CULLIN-1 protein. In brief, the slides were incubated in CULLIN-1 antibody (Abgent, USA) diluted 1:200 at 4°C overnight. The subsequent steps were performed using the EnVision™ FLEX High pH visualisation system according to the manufacturer's instructions (DAKO, Demark).

Statistical analyses

The results are presented as the mean values ± SEM. Overall survival rates were calculated actuarially according to the Kaplan-Meier method with log-rank test and were measured from the day of surgery. Differences between groups were estimated using the χ^2 test, Student's t test, the Mann-Whitney U test, repeated-measures ANOVA test. Relationships were explored by Spearman's correlation. A value of $P < 0.05$ was considered statistically significant. SPSS 16.0 package (IBM, USA) and Graphpad prism 5.0 software (GraphPad Software, USA) were used for statistical analyses and scientific graphing, respectively.
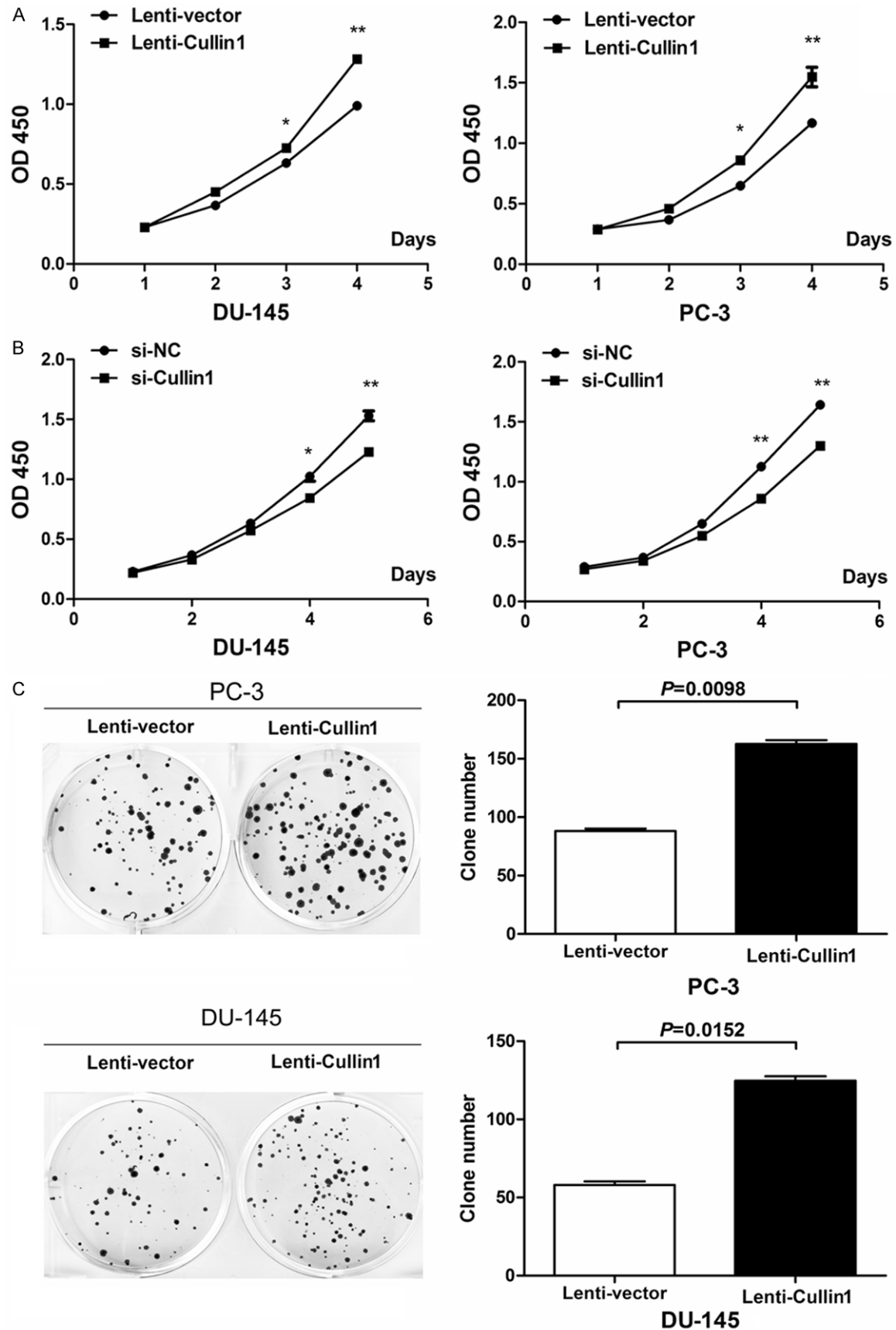
Figure 2. Cullin 1 promotes PCa cell proliferation in vitro. A, B. Cullin 1 overexpression promotes the proliferation of PC-3 and DU145 cells. Cullin 1 knockdown represses the growth rate of PC-3 and DU145 cells. The number of cells was determined using the Cell Counting Kit-8 assay (*: P < 0.05, **: P < 0.01, ***: P < 0.001). C. The effect of Cullin 1 on colony formation was evaluated by crystal violet staining of Cullin 1 lentiviral vector-infected PC-3 and DU145 cells. Cullin 1 overexpression enhanced the colony formation rates (P = 0.0098 and P = 0.0152).
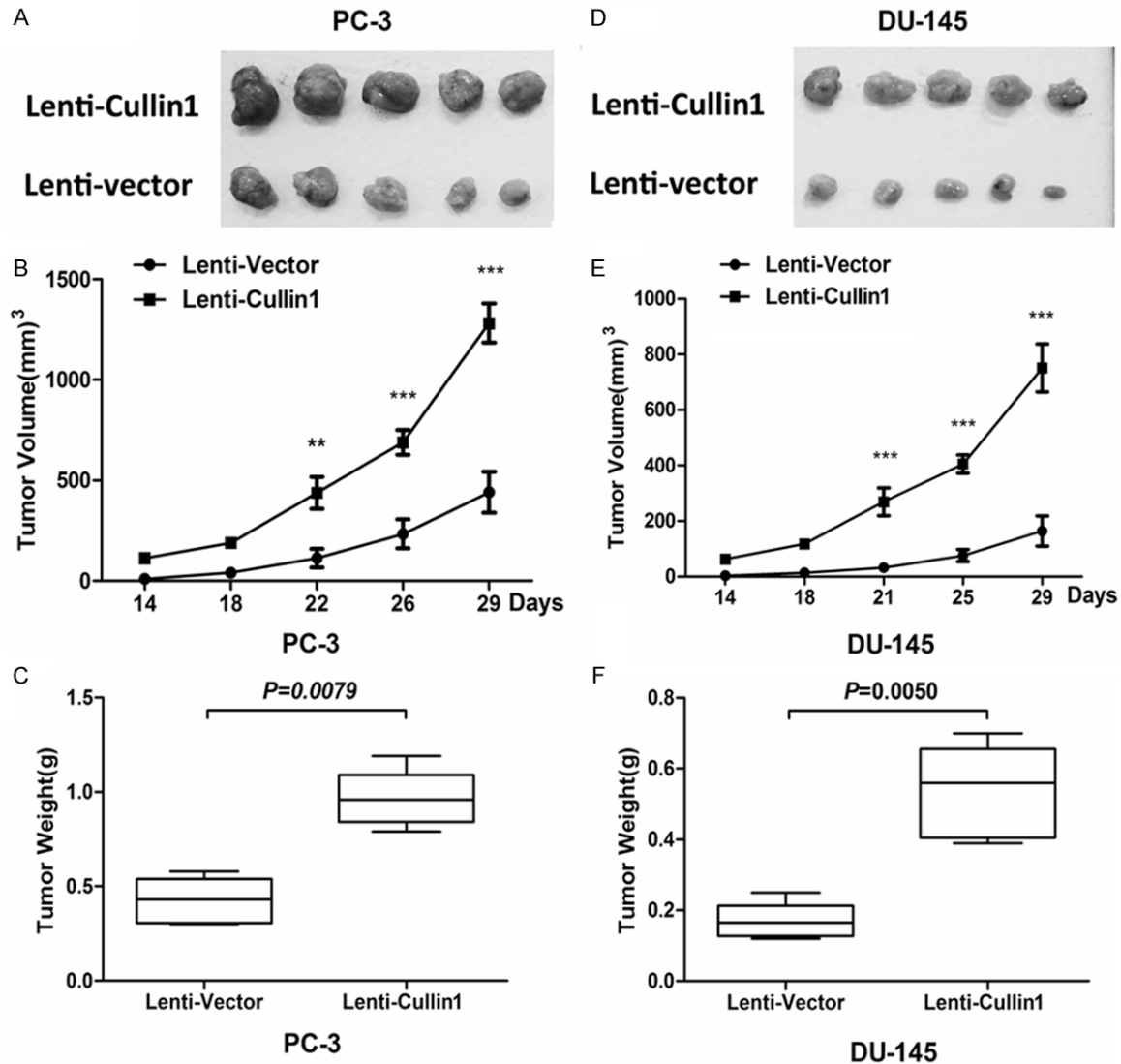

Figure 3. Cullin 1 enhance PCa cell growth in vivo. The effect of Cullin 1 on tumor formation in a nude mouse xenograft model. Lentiviral vector- and Cullin 1- infected PC-3 or DU145 cells were injected s.c. into the right flank of each nude mouse. The tumor formation rate was 5/5 for both two cells. The tumor volume and weight of the Cullin 1 group was significantly increased compared with the control group. A-C. The results of mouse xenograft model for PC-3 cells. D-F. The results of mouse xenograft model of DU145 cells.

Results

Cullin-1 expression was upregulated in human PCa tissues

The expression level of Cullin-1 was examined using qRT-PCR in a cohort of 142 PCa patients. Cullin-1 expression was remarkably upregulated in 73 of the 142 (51.5%) PCa tissues when compared to the corresponding NCTs (**Figure 1A**, **1B**). To demonstrate the relationship of Cullin-1 expression and tumor differentiation, the patients were separated into 3 groups stratified by their Gleason scores. Importantly, level of Cullin-1 was highest in GS > 7 patients and lowest in GS < 7 patients. These results showed that Cullin-1 expression was inversely related with PCa differentiation (**Figure 1C**).
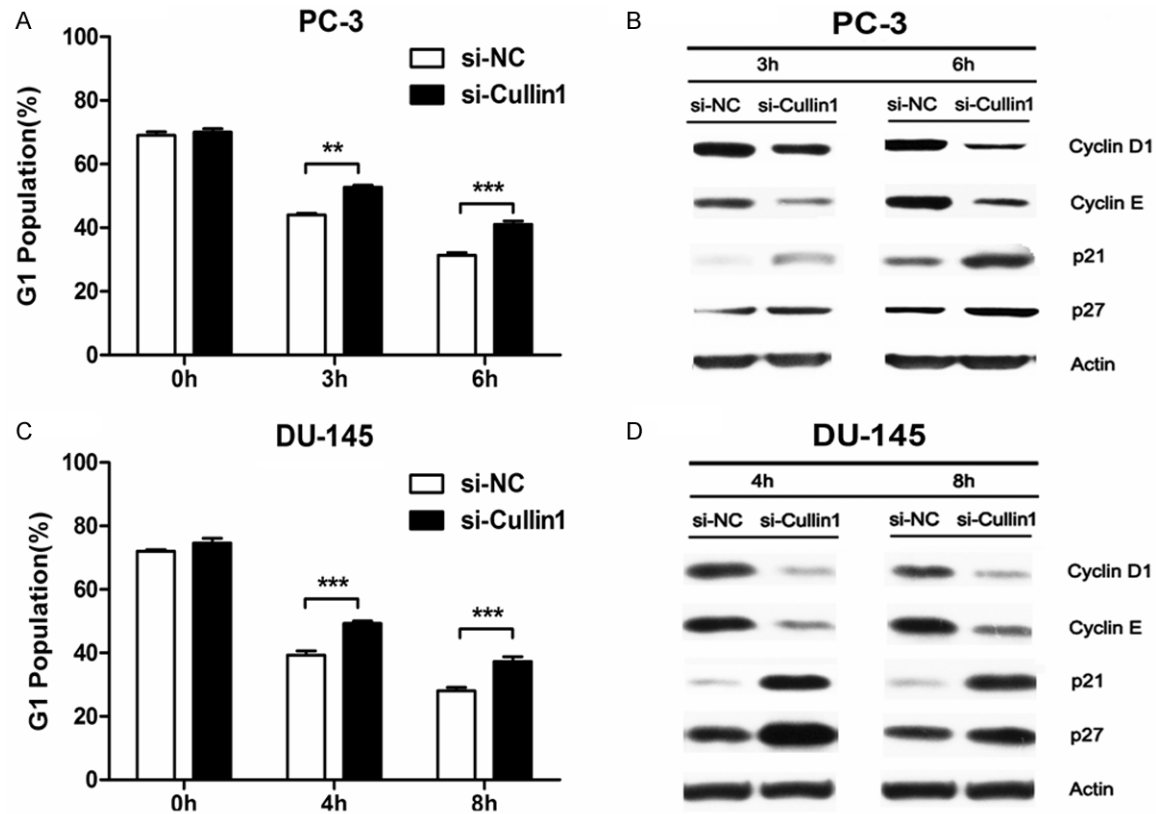
Figure 4. Cullin 1 promotes PCa cell proliferation via cell-cycle regulation. A, C. The percentage of G1 population cells was measured by flow cytometry after Cullin 1 knockdown in PC-3 or DU145 cells. Cullin 1 knockdown cells have significantly higher G1 population than control cells. B, D. Western blot analysis of the relative protein levels of cyclin D1, cyclin E, p21 and p27 in Cul1 knockdown and control cells. β-Actin was used as a whole cell protein loading control.

High expression of Cullin-1 protein suggested a poor prognosis in PCa patients

To further confirm this result, we measured the protein expression levels of Cullin-1 using immunohistochemistry (IHC) methods. The Cullin-1 protein showed a typical nucleus staining in PCa tissues and more than 50% cells with nucleus staining was determined as high expression (**Figure 1D**). More importantly, enhanced immunoreactivity of Cullin-1 in PCA tissues was inversely correlated with overall survival (OS) and suggested a poor prognosis for the PCa patients (**Figure 1E**).

Cullin-1 promotes PCa cell proliferation in vitro

The high expression of Cullin-1 in PCa suggests it contributes to tumorigenesis. To confirm this inference, a cell proliferation assay revealed that overexpression of Cullin-1 significantly enhanced the growth rate of PC-3 and DU145 cells (**Figure 2A**). In contrast, silencing of Cullin-1 expression with siRNA significantly repressed the growth of PC-3 and DU145 cells (**Figure 2B**). Besides that, a colony formation assay confirmed that Cullin-1 overexpression promotes the proliferation of PCa cells (**Figure 2C**).

Cullin-1 promotes PCa tumor growth in vivo

To assess the function of Cullin-1 in *vivo*, Cullin-1 overexpressed stable cell lines (PC-3 and DU145) was constructed and tumor formation assays in a nude mouse model was performed. Compared with the empty vector control, overexpression of Cullin-1 promotes tumor growth with remarkable tumor volume and weight in nude mice (**Figure 3A-E**).

Cullin-1 promotes PCa cell proliferation via cell-cycle regulation

We then examined whether Cullin-1 affect PCa cell growth is due to cell-cycle regulation. Our

data showed that Cullin-1 knockdown cells have significantly higher G1 population than control cells in both the cell lines using fluorescence activate cell sorting. Western blot results showed that the mechanism of Cullin-1 regulation on cell-cycle progression is due to increased expression of p21 and p27, and decreased expression of cyclin D1 and cyclin E after Cullin-1 knockdown (**Figure 4**).

Discussion

In China, the morbidity associated with prostate cancer is significantly lower, although it has increased over the last few years. Due to the limitations in health awareness and financial resources, the majority of patients in China have middle- and advanced-stage disease at diagnosis. Currently there is no reliable marker available for early detection, diagnostic confirmation, or disease prognosis available [8].

Although among the 8 cullins, only Cullin-1 is a component of the SCF E3 ligases, which serves as a rigid scaffold in SCF complex assembly, and aberrant expression of Cullin-1 results in the dysfunction of SCF E3 ligases [9]. Another study showed that Cullin-1 might function as a tumor suppressor by regulating PLK4 protein levels [10]. However, less is known about differential Cullin-1 expression in tumors, although Bai et al. recently described the relationship between Cullin-1 expression and poor prognosis in gastric cancer [11]. Similarly, we also found that Cullin-1 expression was upregulated in human PCa tissues and inversely related with PCa differentiation. Besides, we found that high expression of Cullin-1 protein suggested a poor prognosis in PCa patients.

This study suggested that Cullin-1 promoted PCa cell proliferation in vitro. Cullin-1 promotes PCa cell proliferation via cell-cycle regulation, which due to increased expression of p21 and p27, and decreased expression of cyclin D1 and cyclin E after Cullin-1 knockdown. Pyo et al. also found that Cullin-1 expression and cyclin D1 expression are associated with malignant progression and aggressive behavior of thyroid papillary carcinoma [12].

Our data suggested that Cullin-1 promotes PCa tumor growth in vivo. As we known that rictor associates with Cullin-1 to form a functional E3 ubiquitin ligase. Rictor, but not Raptor or mTOR alone promotes SGK1 ubiquitination. Akt and SGK1 through complexing with mTOR, Rictor also regulates cell signaling pathways by controlling the stability of key signaling components like SGK1 [13]. SGK1 activity has been demonstrated previously to be activated by the PI3K kinase pathway [14]. SGK1 is very unstable in normal conditions, and upon serum or growth factor induction, acute activation of the PI3K kinase pathway, which subsequently activates the Akt and S6K kinases, might also contribute to the induction of SGK1 stability by disrupting the Rictor/Cullin-1 complex. The PI3K/Akt/mTOR signaling pathway is well-documented to be frequently deregulated and serves as an oncogenic pathway in several kinds of carcinomas [15]. It has been well documented that the mTOR signaling pathway regulates protein synthesis in response to various growth factors and consequently affects both cell survival and cell proliferation [16]. In our previous research, we found that the inhibition of PI3K/Akt/mTOR signaling, by wortmannin and rapamycin, abolished HIF-1α-induced Snail and Slug expression levels, suggesting that this pathway is critical for their expression [17]. So the Cullin-1 might also involve in this signaling pathway in PCa.

In summary, Cullin-1 exerts multiple biological effects in the PCa cell line. Through promoting proliferation and by countering cisplatin-induced apoptosis, Cullin-1 has been deeply implicated in the pathogenesis and development of PCa. More explorations on specific details of the molecular mechanisms are warranted in the future.

Acknowledgements

Disclosure of conflict of interest

None.

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Table S1. Primers sequences

Primer name	Sequence (5' to 3')	Product size (bp)
Primers for the detecting of CUL1 expression		
CUL1-q-F1	CAGCTCCACCATCAACATCAAC	200
CUL1-q-R1	AGTGCTGCTGGCATACACATT	
Primers for the cloning of CUL1 ORF		
CUL1-OUT-F	CCCTGTTGCGTGGGAGTGAC	1816
CUL1-OUT-R	AGCTGCTGATTTTGGCTGTCA	
CUL1-IN-F	GGCCGAATTC*ATGGAGGAAAGCATGGAAGAGG	1130
CUL1-IN-R	AATAGGATCC*CTATCTGTACCCCCTGGAACTTGCACATG	

*Sequences underlined are sites for restriction enzyme.