

# Silencing of CDC20 suppresses metastatic castration-resistant prostate cancer growth and enhances chemosensitivity to docetaxel

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**Abstract.** The role of cell division cycle 20 (CDC20) was investigated in chemoresistance to docetaxel and the underlying mechanisms in metastatic castration-resistant prostate cancer (mCRPC). MTT assays were performed to determine effects of siRNA-mediated CDC20 knockdown on cell proliferation and anticancer activity of docetaxel. Western blot analyses were conducted to detect changes of Akt and Wnt signaling. Furthermore, *in vivo* growth of PCa was examined in nude mice treated with siCDC20 or docetaxel alone or in combination. CDC20 was overexpressed in mCRPC cells. Knockdown of CDC20 suppressed cell proliferation and enhanced anticancer effect of docetaxel with IC<sub>50</sub> reducing from 0.358 to 0.188  $\mu$ g/ml in PC3 cells and 0.307 to 0.162  $\mu$ g/ml in DU145 cells ( $P < 0.01$ ). While no change of Akt signaling was observed, inhibition of Wnt/ $\beta$ -catenin signaling was detected upon CDC20 silencing. Xenograft tumor growth was significantly reduced in nude mice by CDC20 inhibition. The additional treatment of siCDC20 achieved better anticancer effects than that of docetaxel alone. Silencing of CDC20 may be a new strategy to improve chemosensitization to docetaxel in mCRPC.

## Introduction

Prostate cancer (PCa) remains the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among men in Europe and the United States (1,2).

Despite recent therapeutic advances, most PCa cases inevitably progress to the advanced stage, castration-resistant prostate cancer (CRPC). In this setting, docetaxel-based chemotherapy is the mainstay of treatment. The majority of patients achieve an overall survival benefit, but the duration of benefit is limited and many patients eventually develop resistance (3,4). Thus, the identification of biomolecules involving in chemoresistance may improve treatment strategies in the clinical practice.

The mechanism of antimitotic drugs including docetaxel is to perturb mitotic spindle assembly, which activates the spindle assembly checkpoint (SAC). SAC induces mitotic arrest and subsequently tumor cells either die in mitosis or exit mitosis by slippage into the next phase. Cells that resist killing by premature exit show decreased sensitivity to spindle-perturbing drugs (5-8), indicating premature mitotic exit might render chemoresistance to cancer cells. CDC20 (cell division cycle 20) was first discovered in budding yeast in 1973 (9). As a critical gene for cell cycle progression, it activates the anaphase-promoting complex/cyclosome (APC/C), thus promoting mitotic exit through ubiquitin-dependent proteasomal degradation of proteins such as securin and cyclin B1 (10,11). Overexpression of CDC20 have been reported to be associated with unfavorable pathologic features and poor prognosis in various types of cancers (12). Elegant studies also revealed that knockdown of CDC20 efficiently killed slippage-prone cancer cells, suggesting an inhibitory role of CDC20 knockdown in the survival of cancer cells (13,14). However, its potential role and possible mechanisms in chemoresistance of PCa remains largely elusive.

Previously, we demonstrated that CDC20 overexpression in PCa was correlated with higher risk of biochemical recurrence (15). In this study, we found CDC20 was increased in mCRPC cell lines and siRNA-mediated knockdown of CDC20 remarkably inhibited proliferation of mCRPC cells. Interestingly, silencing of CDC20 enhanced chemosensitivity to docetaxel in mCRPC cells through inhibition of Wnt/ $\beta$ -catenin signaling. In the xenograft models, we observed intratumor injection of siRNA against CDC20 significantly reduced tumor growth and augmented chemotherapeutic effect of docetaxel.

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## Materials and methods

**Cell lines and culture conditions.** Human immortalized normal prostate epithelial cell line RWPE-1 and prostate cancer cell lines 22RV1, PC3, DU145, LNCaP were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), and PC3M was obtained from Medical Research Center of the Third Affiliated Hospital. PCa cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS). RWPE-1 cells were cultured in keratinocyte serum-free medium (Gibco). All cell lines were maintained in a humidified incubator at 5% CO<sub>2</sub> and 37°C.

**Quantitative reverse transcription-polymerase chain reaction.** Total RNA isolation was performed using TRIzol (Invitrogen, NY, USA) according to the manufacturer's instructions. RNA concentration and purity ratios (OD260/280, OD260/230) were measured with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Total RNA (1.0 µg) was reverse transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis were carried out in triplicate using SYBR Green I Master kit (Roche) on LightCycler® 480 System (Roche). A human GAPDH gene was used as an endogenous control for sample normalization. PCR primers for CDC20 were as previously reported (16). Results are presented as the ratio relative to GAPDH.

**Western blot analysis.** Cells were washed twice with precold phosphate-buffered saline (PBS) and proteins were extracted using RIPA (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitor and phosphatase inhibitor. Protein concentration was measured by Bradford method. Equal amounts of protein were resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% milk for 1 h and incubated overnight at 4°C with indicated primary antibodies: CDC20 (1:2,000, ABClone), total Akt (1:1,000, Cell Signaling Technology), phospho-Akt (Ser473) (1:1,000, Cell Signaling Technology), active β-catenin (1:1,000, Cell Signaling Technology), phospho-β-catenin (Ser33/37/Thr41) (1:1,000, Cell Signaling Technology) and GAPDH (1:2,000, Cell Signaling Technology). Secondary antibodies were used to detect the primary antibodies. Protein bands were visualized using FluorChem M (ProteinSimple, CA, USA).

**SiRNA transfection.** Human siRNA oligos were purchased from Invitrogen. Human siRNA were transfected into subconfluent cells with Lipofectamine™ RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions.

**Cell proliferation/viability assay.** Cells were seeded into 96-well plates at a density of 5×10<sup>3</sup> per well, MTT assay was used to read the absorbance of 490 nm at selected time-points. In addition, treatment effects of docetaxel on cell proliferation was also examined; that is, after the adhesion culture media were replaced with that containing indicated concentration of docetaxel and MTT assays were performed at 48 h post-

seeding. Each experiment was performed in triplicate and cell proliferation is expressed as fold change ± SEM compared to values obtained on the first day of culturing. The average half maximal inhibitory concentrations (IC<sub>50</sub>) were calculated using Graphpad Prism 5.0 software.

**In vivo xenograft model of human CRPC.** The animal experiments were approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University and performed according to its guidelines. PC3 and DU145 cells (3×10<sup>6</sup> cells per mouse) were subcutaneously injected into the flank region of 5-week-old male BALB/c nude mice. Tumor size was measured every 3 days by handheld caliper and tumor volume was calculated as follows: tumor volume (mm<sup>3</sup>) = length × width<sup>2</sup> × 0.52. When the tumor volume reached 100 mm<sup>3</sup>, the mice were divided randomly into 4 groups (n=6 for each group) and tumor volumes between groups were not statistically significant. siRNA (10 µM × 50 µl) was injected into the tumor once a week. Docetaxel (10 mg/kg body weight) was administered by intraperitoneal injection every week. Thereafter, tumor volumes were measured every 7 days. After 5 weeks of treatment, mice were sacrificed and primary tumors were excised, weighed, and captured.

**Statistical analysis.** Statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA). Results were presented as means ± SEM of three independent experiments conducted at least in triplicate. Two-tailed Student's t-test was used to compare results between groups. P<0.05 was considered statistically significant.

## Results

**CDC20 expression is increased in mCRPC cell lines.** To investigate the potential role of CDC20 in PCa cells, we performed RT-PCR and western blotting to determine the levels of CDC20 mRNA and protein in normal epithelial cell line of prostate RWPE-1 and several PCa cell lines, including primary PCa cell line (22RV1), mCRPC cell lines (PC3, PC3M, DU145) and metastatic hormone-sensitive cell line (LNCaP). As shown in Fig. 1, although CDC20 was modestly expressed in RWPE-1, it was remarkably increased in mCRPC cell lines both at mRNA and protein levels. PC3 and DU145 were chosen for further investigation.

**Silencing of CDC20 suppresses cell proliferation of mCRPC cells.** In order to determine the effect of CDC20 on proliferation of CRPC cells, siRNA against CDC20 was transfected into PC3 and DU145 cells. As determined by western blot analysis, endogenous expression of CDC20 was significantly reduced by the two siRNA, especially the siRNA-1 (Fig. 2A and B). The MTT assays showed that knockdown of CDC20 remarkably suppressed proliferation of mCRPC cells in comparison with the control group (Fig. 2C and D), indicating CDC20 promotes proliferation of mCRPC.

**Inhibition of CDC20 augments chemosensitivity to docetaxel in mCRPC cells through Wnt/β-catenin signaling.** To further investigate the possible role of CDC20 in regulating chemosensitivity of CRPC cells, cells were transfected with siRNA

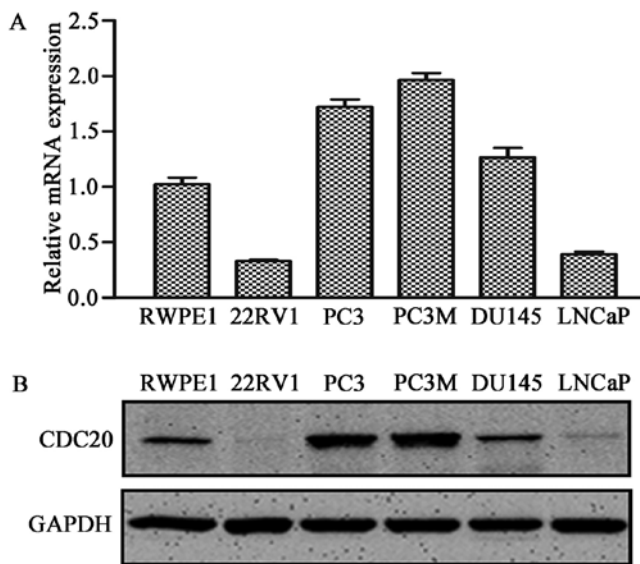


Figure 1. CDC20 expression levels in immortalized epithelial cell line and prostate cancer cell lines. (A) Relative mRNA expression of CDC20 determined by qRT-PCR. Data are expressed as the mean  $\pm$  SEM. (B) Protein expression of CDC20 determined by western blotting.

and then treated with a mock agent or docetaxel of indicated concentration for another 48 h. After silencing of CDC20, the  $IC_{50}$  of docetaxel was significantly reduced from 0.358 to 0.188  $\mu$ g/ml ( $P < 0.01$ ) in PC3 cells and 0.307 to 0.162  $\mu$ g/ml ( $P < 0.01$ ) in DU145 cells (Fig. 3A and B). To clarify the mechanisms of action, we detected the effect of CDC20 silencing on Akt signaling and Wnt/ $\beta$ -catenin signaling because of their reported involvement in cancer chemoresistance. No changes were observed in the expression levels of phospho-Akt and total Akt protein. However, phospho- $\beta$ -

catenin on Ser33/37/Thr41 residues (inactivated  $\beta$ -catenin) was increased and total  $\beta$ -catenin was decreased, indicating Wnt/ $\beta$ -catenin signaling was inhibited upon CDC20 inhibition. These results indicate that inhibition of CDC20 augmented chemosensitivity to docetaxel in mCRPC cells, which is achieved partly through inhibiting Wnt/ $\beta$ -catenin signaling (Fig. 3C-F).

*Silencing of CDC20 reduces tumor growth and enhances chemotherapeutic effect of docetaxel in CRPC xenografts.* To validate the research results *in vitro*, we performed *in vivo* studies in nude mice. The tumor growth curves are shown in Fig. 4A and B. Consistent with the results of *in vitro* studies, silencing of CDC20 significantly reduced tumor growth at different time-points, showing better anticancer effect than docetaxel. In addition, the average weights of harvested tumors in groups treated with control saline, siCDC20, docetaxel and a combination of siCDC20 and docetaxel were 1148.5 $\pm$ 168.0, 801.3 $\pm$ 50.8, 579.2 $\pm$ 98.4, 258.6 $\pm$ 103.7 mg for PC3 tumors and 972.3 $\pm$ 100.1, 767.5 $\pm$ 79.4, 541.8 $\pm$ 118.1, 171.8 $\pm$ 80.1 mg for DU145 tumors respectively ( $P < 0.001$  compared with the control) (Fig. 4C and D).

## Discussion

Prostate cancer (PCa) is initially responsive to androgen deprivation therapy. Unfortunately, many patients eventually become resistant and progress to metastatic castration-resistant prostate cancer (mCRPC), which is an incurable and aggressive phenotype. For several years, docetaxel chemotherapy has been the primary standard of care for patients with mCRPC, though it prolongs survival only a limited time (17). PCa is a highly heterogeneous disease (18). It is likely that some cancer cells are intrinsically resistant to docetaxel and some

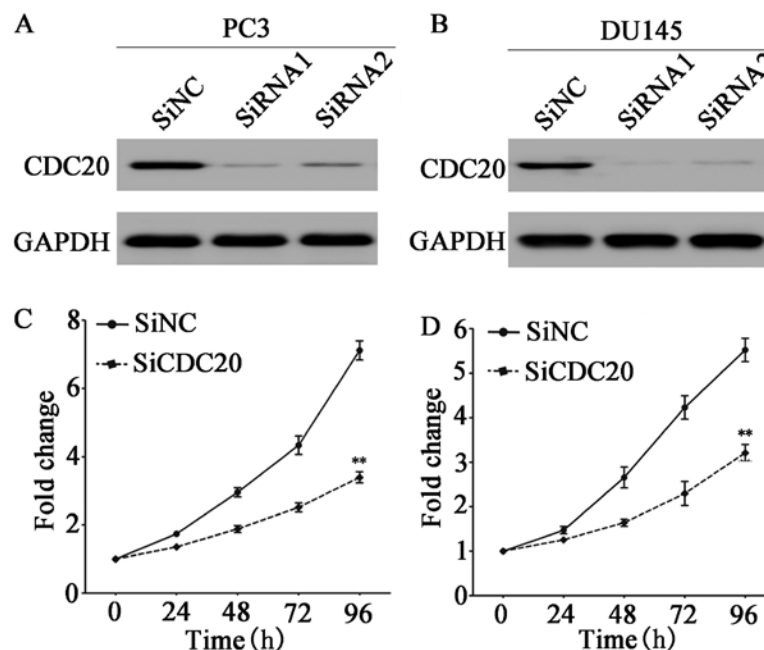


Figure 2. Knockdown of CDC20 suppressed proliferation of mCRPC cells. CDC20 protein expression in PC3 cells (A) and DU145 cells (B) was significantly inhibited by siRNA. MTT assays showed knockdown of CDC20 remarkably suppressed proliferation of PC3 (C) and DU145 cells (D). Data are expressed as fold change  $\pm$  SEM compared to the absorbance at OD490 nm on the first day, \*\* $P < 0.001$  using Student's t-test.

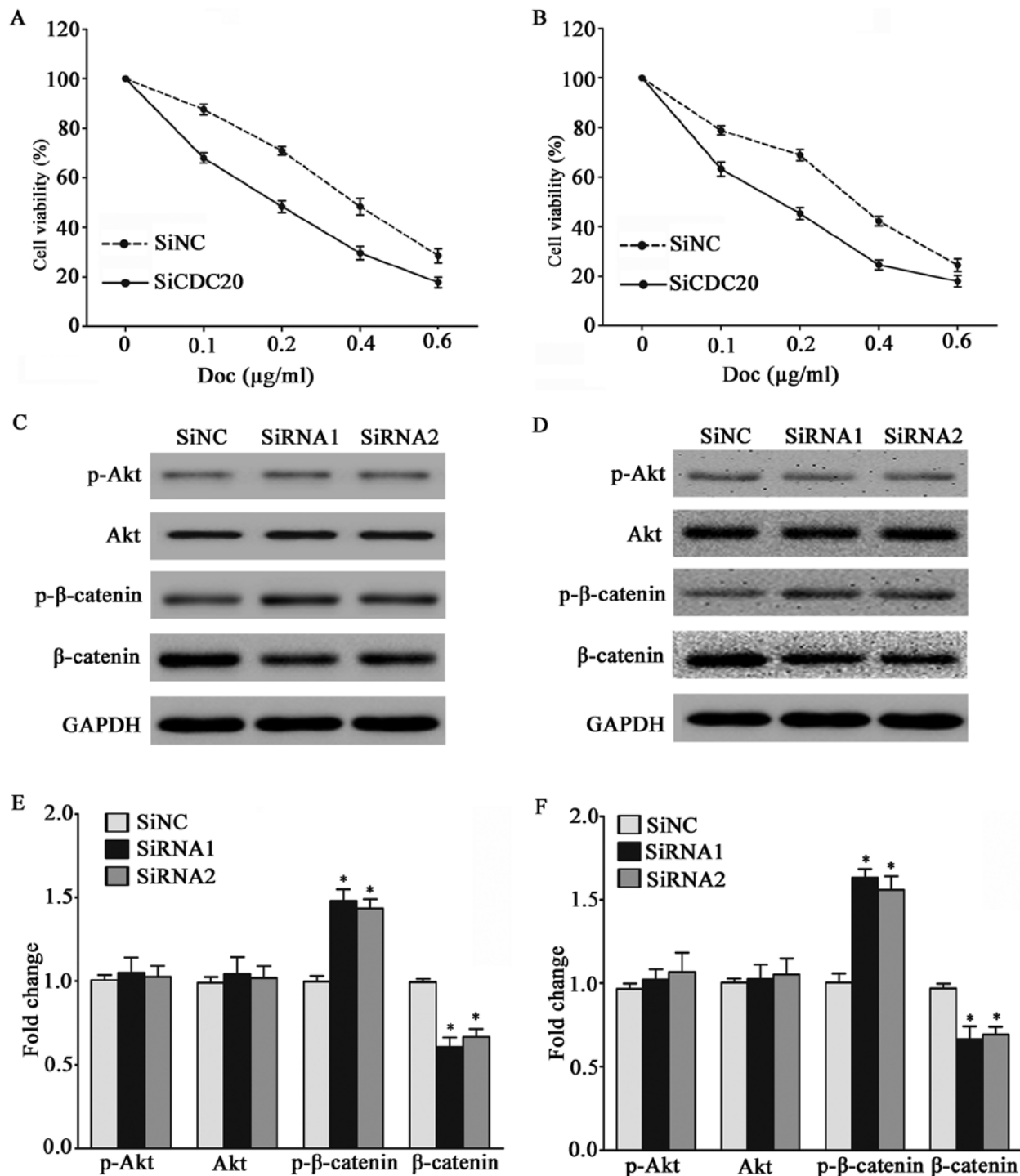


Figure 3. Inhibition of CDC20 augments chemosensitivity to docetaxel in mCRPC cells through inhibiting Wnt/β-catenin signaling. Inhibition of CDC20 enhanced anticancer effect of docetaxel in PC3 (A) and DU145 cells (B). Knockdown of CDC20 inhibited Wnt/β-catenin signaling whereas it did not change the Akt pathway. Western blot analysis of expression changes of Akt and Wnt/β-catenin signaling in PC3 (C) and DU145 (D) cells. Protein expression levels in PC3 (E) and DU145 (F) cells were standardized by GAPDH and presented as fold change  $\pm$  SEM compared to the control group. Cells were treated with non-targeted siRNA (SiNC), siRNAs targeting CDC20 (SiRNA1 and SiRNA2). All experiments were performed three times independently, \* $P < 0.05$  using Student's t-test.

other cancer cells acquire resistance during the chemotherapy. Therefore, resensitizing PCa to docetaxel has been a great challenge and is of critical significance for improving treatment strategies.

Cell division cycle 20 (CDC20), is a WD40 repeat domain-containing E3 ligase that associates with and activates the

anaphase-promoting complex (APC). APC<sup>CDC20</sup> recognizes the D-box or KEN box of substrates to promote proteasomal degradation of securin and cyclin B, triggering transition from metaphase to anaphase and mitotic exit (19). It has been reported that multiple cancer types showed increased CDC20 expression and its expression level correlated with clinico-

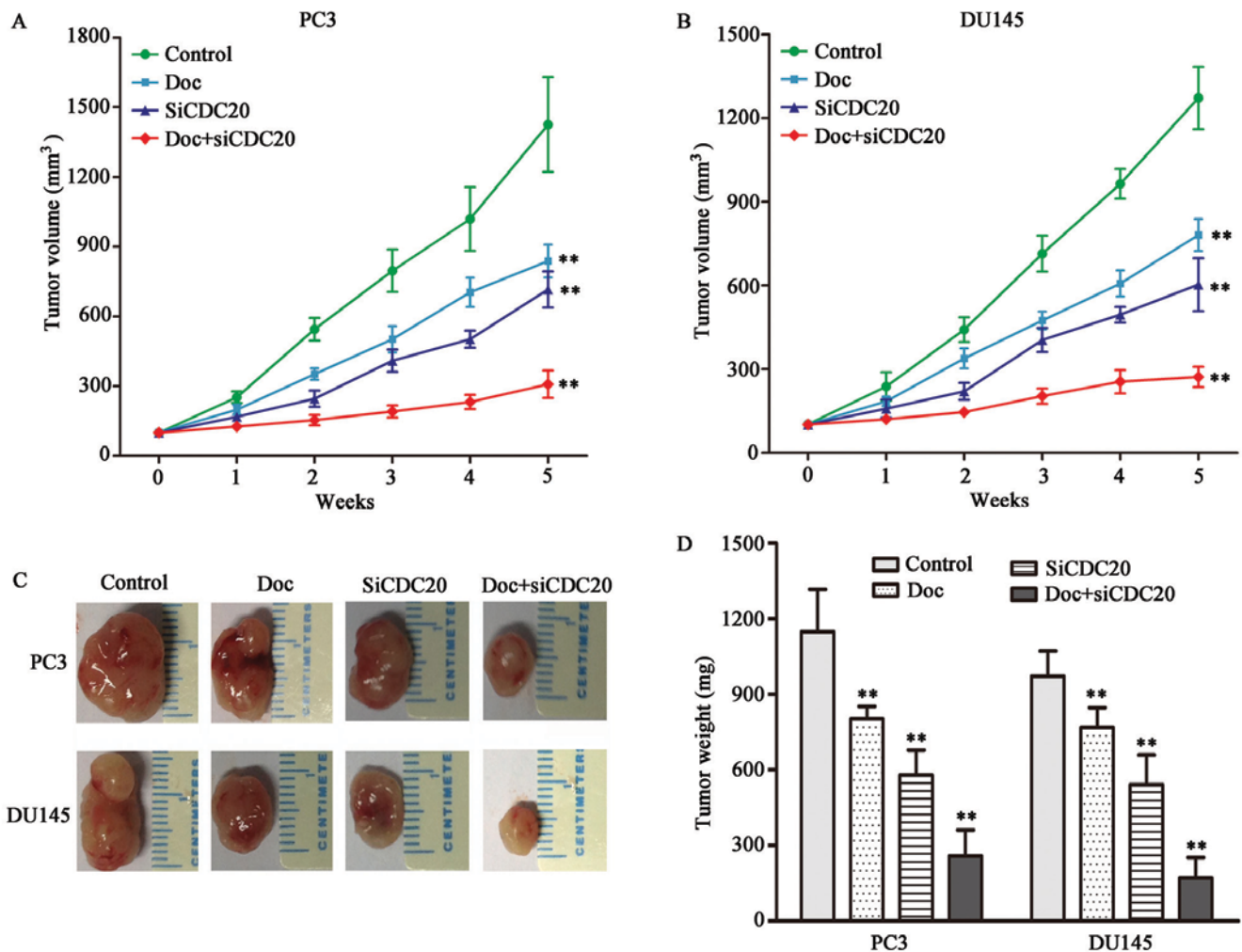


Figure 4. Effects of siRNA against CDC20 and docetaxel on xenograft tumor growth. Tumor growth curves of PC3 (A) and DU145 (B) cells in mice treated with indicated treatments (n=6 for each group). (C) Representative images of harvested tumors after 5 weeks of treatments. (D) Average weight of harvested tumors in each group. Control, intraperitoneal injection of saline. Doc, intraperitoneal injection of docetaxel (10 mg/kg body weight). siCDC20, intratumoral injection of siRNA against CDC20 (10  $\mu$ M x 50  $\mu$ l). Data are presented as mean  $\pm$  SEM. \*\*P<0.001 compared with control group using Student's t-test.

pathological characteristics as well as prognosis (20,21). In PCa, we previously demonstrated high expression level of CDC20 associated with higher Gleason scores and predicted biochemical recurrence (15). Yet, there is no direct evidence on the role of CDC20 in chemotherapy of PCa.

In this study, we observed modest expression of CDC20 in normal epithelial cell line of prostate RWPE-1, which might be explained by the essential role of CDC20 for cell cycle progression in both normal and cancerous cells (13,19). We also revealed that CDC20 was upregulated in mCRPC cell lines, representing an oncogenic role of CDC20 in PCa. Further, we demonstrated that inhibition of CDC20 achieved better antitumor effect than docetaxel, in agreement with the previous proposal that blocking mitotic exit was a better cancer therapeutic strategy than other cell cycle processes (14,22). Silencing of CDC20 enhanced chemosensitivity to docetaxel both *in vitro* and *in vivo*, indicating CDC20 is a potential therapeutic target. In concert with our finding, depleting endogenous CDC20 induced chemosensitization in head and neck cancer cells (23). Indeed, inhibitors of CDC20 have provided a therapeutic window in a variety of

cancers. Zeng *et al* reported that TAME (tosyl-L-arginine methyl ester), a small molecule, reduced CDC20 association with APC and resultantly suppressed APC activation (24,25). Another small molecule, apcin, has recently been validated to bind CDC20 to prevent substrate recognition, thus competitively inhibiting the ubiquitination of CDC20 substrates (26). Other biocompounds such as Withaferin A, NAHA, ganodermanontriol (GDNT) and BCHHD leads to decreased CDC20 expression to exert anticancer activity (27-30). Based on our study, we propose that a combination of microtubule-targeting agents with inhibitors of mitotic exit could be much more effective than treatment using a single anticancer agent.

Mechanistically, we explored the changes of Akt and Wnt pathways upon CDC20 knockdown, because Akt and Wnt pathway have been reported to be implicated in protecting tumor cells against chemotherapeutic drugs (31,32). Surprisingly, we identified depression of Wnt/ $\beta$ -catenin signaling and no change of Akt pathway, suggesting CDC20 silencing achieved antitumor effect in PCa via inhibition of Wnt/ $\beta$ -catenin signaling rather than Akt signaling. Consistently, an elegant study proposed that knockdown of

CDC20 blocked Wnt signaling (33). Several studies have provided explanations for this finding. Firstly, Wnt/ $\beta$ -catenin signaling has been discovered to be highly activated in prostate cancer stem cells (CSCs) (34). Secondly, CSCs have been proven to be key players associated with chemoresistance in PCa (35). Thirdly, it was documented that  $\beta$ -catenin directly interacted with the activation function 2 region (AF2) of the ligand binding domain of androgen receptor (36,37), which was considered the critical role in PCa development, progression and chemoresistance. Furthermore, activation of  $\beta$ -catenin increased the membrane located efflux pump P-glycoprotein(P-gp) expression, resulting in an active efflux of anticancer agents from cells and thus inducing multidrug resistance (38-40).

In conclusion, this study presents the preliminary evidence that CDC20 is significantly involved in mediating chemoresistance to docetaxel partly through inhibiting Wnt/ $\beta$ -catenin signaling. Therefore, treatment strategies directed toward silencing CDC20 may improve chemosensitization to docetaxel in mCRPC.

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