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Downregulation of CDC20 suppressed cell proliferation, induced apoptosis, triggered cell cycle arrest in osteosarcoma cells, and enhanced chemosensitivity to cisplatin

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Osteosarcoma (OS) is a common malignant bone tumor that occurs in adolescents or children under the age of 20, which is extremely difficult to cure and has a high recurrence rate. Recent studies showed that cell division cycle 20 (CDC20) overexpression is associated with poor prognosis in patients with osteosarcoma. However, the function of CDC20 in osteosarcoma has not been investigated clearly. In this study, we aim to explore the role of CDC20 in two independent human OS cell lines' biological phenotype and chemotherapy sensitivity. We applied multiple approaches to measure cell growth, cell cycle, and apoptosis with or without deregulation or overexpression of CDC20. We found that the downregulation of CDC20 by siRNA or apcin suppressed cell proliferation, induced apoptosis, and triggered cell cycle arrest. Consistently, overexpression of CDC20 in normal cells promoted cell growth, inhibited apoptosis. What's more, the additional treatment with siCDC20 or Apcin achieved better anticancer effects than that of cisplatin alone. Furthermore, Bim and p21 were upregulated in OS cells following Apcin treatment. Altogether, the results of the present study demonstrated that targeting CDC20 could be useful for the treatment of OS, and might be a promising solution for the treatment of the OS with cisplatin insensitivity.

Key words: osteosarcoma, CDC20, cell growth, apoptosis, chemosensitivity

Osteosarcoma (OS) is the most common primary bone malignant tumor worldwide, predominantly affecting the population of children, teenagers, and young adults. The peak incidence of OS is occurring in the second decade of life during the adolescent growth spurt [1, 2]. At present, treatments for OS mainly are local control surgery and intensive multiagent chemotherapy, including cisplatin, doxorubicin, ifosfamide, methotrexate, etc [3]. However, the 5-year survival rate for OS patients with metastases, especially pulmonary metastases, is only 20%. If untreated, OS may run an ongoing course with local and systemic progression and causes of death within a few months [4]. The current treatment strategies for metastatic OS are limited and typically result in poor prognosis and relapse. So it is imperative and beneficial to develop novel therapeutic agents to improve survival rates in patients with OS.

The anaphase promoting complex/cyclosome (APC/C) is a complex of large multimeric cullin-RING ubiquitin ligases that plays an important role in the cell cycle. RING finger E3 ubiquitin ligase cell division cycle 20 homolog (CDC20) serves as a function of the activator of APC/C during the metaphase-anaphase transition [5, 6]. It has been reported that the CDC20 expression is high in a number of malignancies and is associated with tumorigenesis and progression [7]. Furthermore, CDC20 upregulation has also been reported to indicate poor prognosis in the urothelial bladder, uterine cervix, colorectal, pancreatic ductal, oral squamous cell, gastric, and lung cancers. These findings revealed that CDC20 may be a promising novel therapeutic target for cancer treatment [8–11]. Furthermore, Apcin, as the inhibitor of CDC20, has been reported to induce metaphase arrest and apoptotic cell death in multiple myeloma, indicating that APC/C-CDC20 inhibitors may be effective therapeutic agents targeting cohesion defective cancers [12]. However, the antitumor properties and mechanism of Apcin in OS have not been previously investigated. The aim of the present study was to expound on the effect and mechanism of antitumor properties by inhibiting the expression of CDC20 in a human OS cell line.

Materials and methods

Cell culture. Human osteosarcoma cell lines MG63 and U2OS were purchased from the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientifc, Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientifc, Inc.) and 1% penicillin/streptomycin (HyClone™; GE Healthcare Life Sciences, Logan, UT, USA) at 37 °C in a 95% humidified and 5% CO_2 incubator.

CDC20 silencing by siRNA. Cells with 80% confluence were transfected using Lipofectamine RNAi MAX (Invitrogen) in Opti-MEM medium (Invitrogen). We transiently transfected cells with CDC20 siRNA using Lipofectamine RNAi MAX (Invitrogen, USA) in Opti-MEM medium (Invitrogen, USA) according to the manufacturer's instructions. 48 h after the transfection, cells were harvested and the efficiency of transfection was measured by western blot (WB). For the knockdown of CDC20 *in vivo,* the lentivirus vector of plko.1-shCDC20 or negative control plko.1-shNC were used, and the infected cells were screened by puromycin (puro) at the concentration of 2 μg/ml 48 h after infection for at least one week.

Cell viability assay. Cells were seeded in 96-well plates at a density of 1×10^3 cells/well. Cell counting Kit-8 (CCK-8, Dojindo, Japan) was used to measure cell viability following treating cells with different treatments. After treatments, the culture medium was removed, and cells were washed with PBS. A total of 100 μl fresh medium with 10 μl CCK-8 solution was added to each well for 2 h at 37 °C. Optical density (OD) was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The OD readings of the treatment groups were divided by their corresponding control readings to obtain the ratio of cell viability.

Cell cycle analysis. The OS cells were seeded in a 6-well plate overnight. After 48 hours of treatments, cells were harvested, washed, and resuspended in 70% cold alcohol and kept at 4 °C overnight. Then, cells were suspended in 1×10^6 cells/ml in PBS and incubated with 0.1 mg/ml RNaseI and 50 mg/ml PI at 37 °C for 30 min. The cell cycle was further detected with a FACS flow cytometer (BD, USA).

Cell apoptosis assay. Cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well. Then cells were treated with different treatment, as indicated. Cells were harvested, washed twice with cold PBS, and centrifuged. The supernatants were discarded and the cells were resuspended in 1× Annexin-binding buffer. A total of 5 μl Annexin V-APC (BD Biosciences, Franklin Lakes, NJ, USA) was added to the cells at room temperature for 15 min, then 5 μl propidium iodide (PI) solution (BD Biosciences) was added. The ratio of apoptotic cells was measured by flow cytometry (BD FACS, BD Biosciences).

Western blot analysis. Cells were washed twice with cold PBS, and total protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was quantified by a bicinchoninic acid protein assay kit (Thermo Fisher Scientifc, Inc., Grand Island, NY, USA). A total of 20 μg protein (for each sample) was loaded and separated by 10% or 12.5% SDS-PAGE, then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C (anti-CDC20, anti-GAPDH, anti-Bcl-2, anti-Bax, anti-caspase-3, anti-cleaved-caspase-3, anti-p21, and anti-p53 for 1:1,000 dilution; Cell Signaling Technology, Inc., Danver, MA, USA). Following three washes with TBS/0.1% Tween-20 (TBST), the membranes were incubated with anti-rabbit or anti-mouse immunoglobulin G for 1 h at room temperature (for 1:5,000 dilution; Cell Signaling Technology, Inc.). The membranes were incubated in enhanced chemiluminescence (ECL) solution (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were densitometrically quantified and normalized to GAPDH.

Mice U2OS tumor model. The xenograft model of human MG63 cells was established and maintained in accordance with the guidelines of NIH. MG63 cells were infected with lentivirus plko.1-shCDC20, or negative control, 5×10^6 treated cells were subcutaneously into the right flank of the mice (5/group). When the right flank tumors were about 150 mm^3, mice were intraperitoneally injected with cisplatin (5 mg/kg in 0.9% isotonic saline solution). All mice were sacrificed 4 weeks after treatment. The tumor sizes were measured 7 days apart and the tumor volumes were calculated: V (cm3) = width^2 (cm^2) × length (cm)/2.

Statistical analysis. The results are analyzed by using SPSS 16.0 statistical software and GraphPad Prism 7.0 software. It was considered to be a significant difference when $p<0.05$. Unpaired t-tests or Mann-Whitney U tests were used to compare the two groups, and multiple group comparisons were analyzed with one-way ANOVA. All experiments were performed at least three times.

Results

CDC20 is highly expressed in sarcoma tissues and its overexpression may predict poor prognosis of sarcoma patients. To investigate the possible role of CDC20 in OS, we analyzed messenger RNA (mRNA) expression levels of CDC20 in tumor tissues and the matched normal tissues of sarcoma patients using data from the TCGA database. We found CDC20 was significantly upregulated in sarcoma (Figure 1A), according to the GEO database through UALCAN and GEPIA. The relationship between CDC20 expression and prognosis was analyzed by the *KMPLOT* online analysis tool, which indicated that patients with higher CDC20 expression were correlated with poor survival probability. Collectively, these results indicated that CDC20

was highly expressed in sarcoma, and its high expression is associated with poorer prognosis in OS (Figure 1B).

CDC20 siRNA transfection decreases CDC20 expression, suppresses cell growth, induces cell apoptosis, and cell cycle arrest. To explore the role of CDC20 in OS cells, CDC20 siRNA and siNC were transfected into MG63 and U2OS cells, respectively. Then we measured the expression of CDC20 at protein levels in OS cells by western blotting (WB) analysis. Our WB results showed that the protein level of CDC20 was obviously knocked down in both MG63 and U2OS cells transfected with CDC20 siRNA (Figure 2A). To dissect the function of CDC20 in OS cells, we measured cell viability in OS cells after CDC20 siRNA transfection. The CCK-8 assays were carried out to detect the ability of cell growth in OS cells with CDC20 siRNA infection for 0, 24, 48, and 72 hours, respectively. Our data demonstrated that the CDC20 knockdown suppressed cell proliferation (Figures 2B, 2E). The Annexin V+- PI- assays were performed to explore whether the inhibition of CDC20 could induce cell apoptosis. We observed that apoptosis of OS cells was accelerated in both MG63 and U2OS cells transfected with CDC20 siRNA (Figures 2D, 2G). Moreover, we found that the depletion of CDC20 induced cell cycle arrest at the G2/M phase in OS cells (Figures 2C, 2F). Our work implied that CDC20 inhibition suppressed cell proliferation, enhanced cell apoptosis in OS cells, and induced cell cycle arrest.

Apcin suppresses cell growth, triggers cell apoptosis, and cell cycle arrest by decreasing CDC20 expression. We then wondered whether the CDC20 inhibitor Apcin can affect the human OS cell biological phenotype. WB analysis was performed for MG63 and U2OS cells following treatment with the designated concentrations of Apcin for 48 h. Our WB results showed that the protein level of CDC20 was suppressed in both MG63 and U2OS cells treated with Apcin (Figure 3A). The CCK8 results revealed that OS cell proliferation was significantly suppressed by Apcin in a time- and dose-dependent manner (Figure 3B). Treatment with 40 or 80 μM Apcin caused slight OS cell growth inhibition at day one and day two, and an obvious inhibition at day three both in MG63 and U2OS cells. These results demonstrated that Apcin exhibited its anti-tumor characteristics in human OS cells. Moreover, we found that Apcin induced cell cycle arrest at the G2/M phase in OS cells (Figure 3C). It was further investigated whether Apcin affected apoptosis in human OS cells. MG63 and U2OS cells were treated with the designated concentrations of Apcin for 48 h and cell apoptosis was assessed using Annexin V- PI assays. We revealed that Apcin treatment induced significant cell apoptosis in a dose-dependent manner (Figure 3D). Our work implied that CDC20 inhibition restrained cell proliferation and enhanced cell apoptosis in OS cells.

CDC20 overexpression promotes cell proliferation and suppresses cell apoptosis. To further define the role of CDC20 in OS cells, CDC20 cDNA and empty vector were transfected into MG63 and U2OS cells, respectively. The CCK-8 assays were exerted to measure the cell viability in OS cells with CDC20 overexpression. We found that the CDC20 cDNA transfection promoted cell proliferation in OS cells (Figures 4A, 4D). Consistently, the numbers of cell apoptosis were decreased from 9.2% to 4.5% after CDC20 cDNA transfection in U2OS cells (Figure 4C). Similarly, the percentage of cell apoptosis in the CDC20 cDNA group was 4.7% compared with 10.7% in the control cDNA group in MG63 cells (Figure 4F). Overexpression of CDC20 didn't affect the cell cycle in U2OS and MG63 cells (Figures 4B, 4E). All the data suggested that CDC20 overexpression promoted cell growth and inhibited cell apoptosis.

Downregulation of CDC20 affects protein expression. Since we identified OS cells with downregulation of CDC20

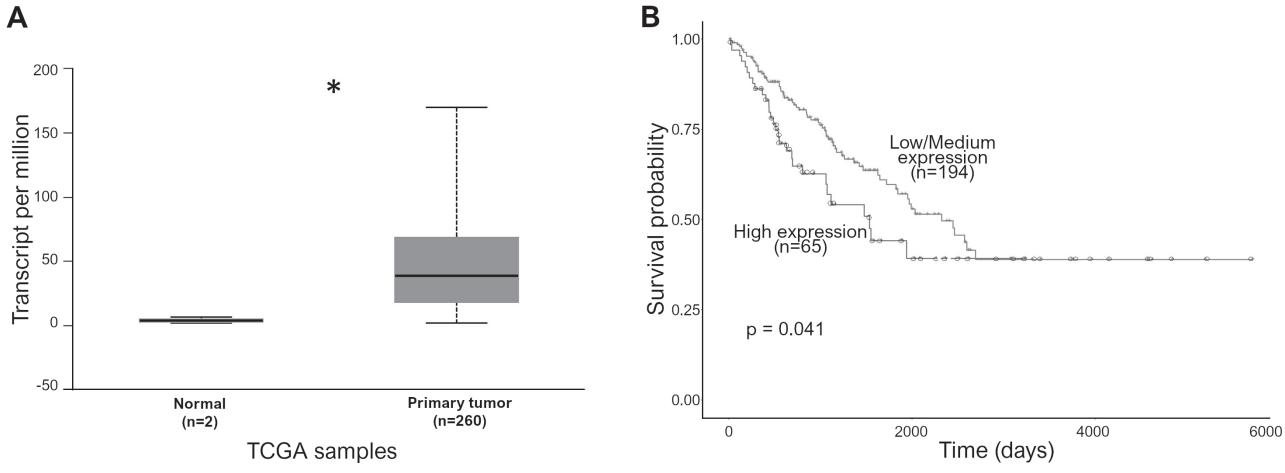

Figure 1. High expression of CDC20 in sarcoma tissues may predict the poor prognosis of sarcoma patients. A) CDC20 was significantly highly expressed in sarcoma tissues. B) The high expression of CDC20 was closely related to the poor survival probability.

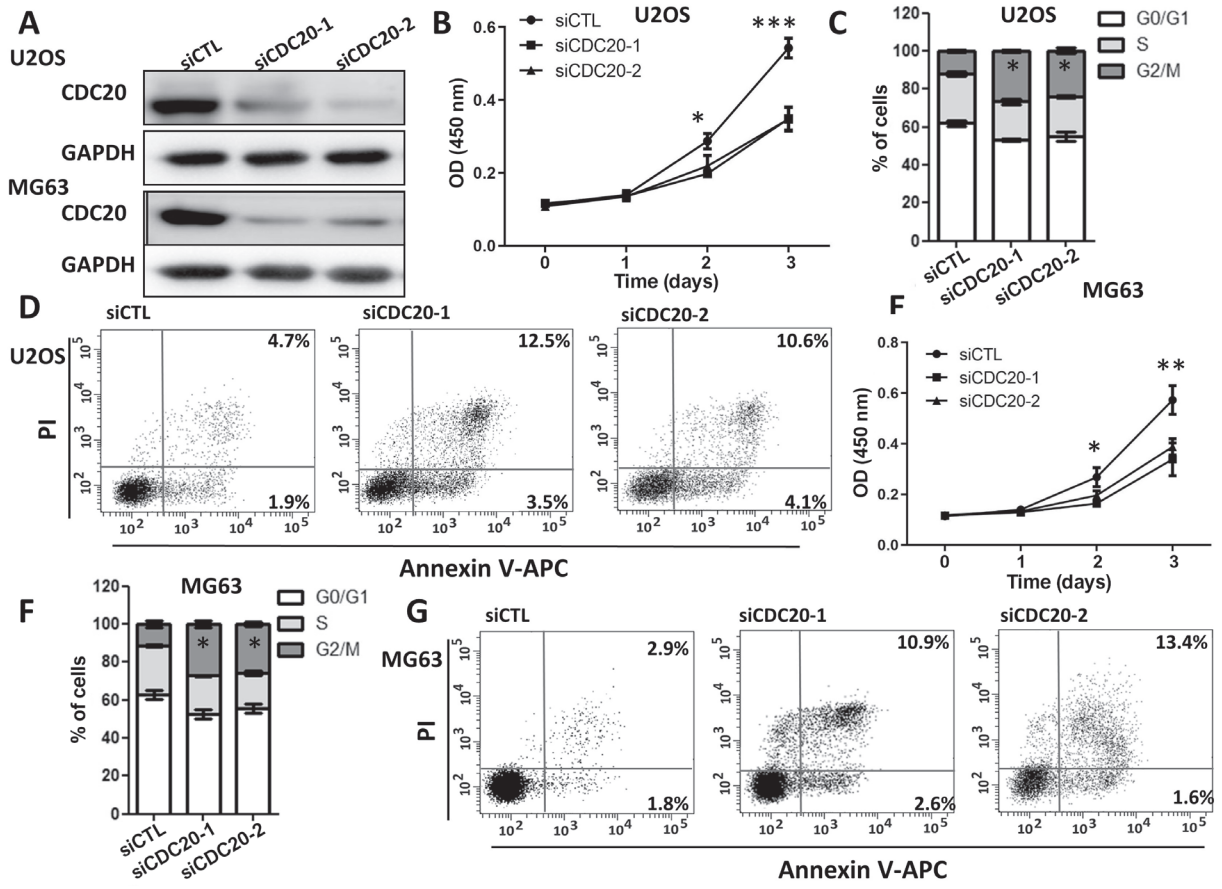

Figure 2. CDC20 siRNA transfection decreases CDC20 expression, suppresses cell growth, induces cell apoptosis, and cell cycle arrest. A) *CDC20* **was significantly inhibited by siRNA in U2OS and MG63 cells. B, E) CCK-8 assays showed that the knockdown of CDC20 remarkably suppressed the proliferation of U2OS and MG63 cells. Data are expressed as fold change ± SEM compared to the absorbance at OD490 nm on the first day. C, F) Knockdown of CDC20 arrested cell cycle at the G2/M period D, G) The apoptosis rate of MG63 and U2OS cell lines was significantly increased after CDC20 siRNA transfection. *p<0.05, **p<0.01, data represents the mean ± S.D.**

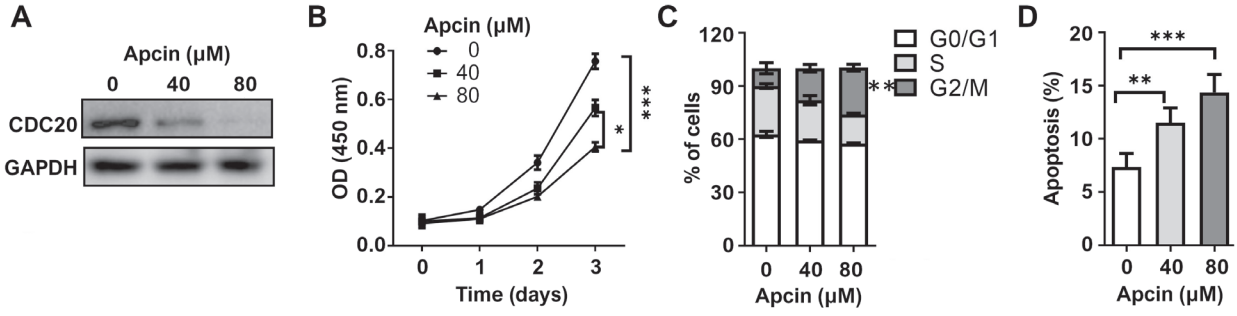

Figure 3. Apcin inhibits CDC20 expression, slows down cell growth, triggers cell apoptosis, and cell cycle arrest. A) The protein expression of CDC20 in U2OS cells and MG63 cells was significantly inhibited by Apcin. B) CCK-assays showed inhibition S.D. of CDC20 remarkably suppressed proliferation of U2OS cells. C) Apcin treatment arrested cell cycle at the G2/M period. D) The apoptosis rate of U2OS cell lines was significantly increased after Apcin treated.*p<0.05, **p<0.01, ***p<0.001, data represents the mean ± S.D.

were much more sensitive to cisplatin. Next, we further explore whether the related proteins involved in CDC20-mediated tumorigenesis were affected in MG63 cells by WB. We found that the protein level of CDC20 was decreased by siRNA transfection and Apcin treatment. The expression of Bcl was decreased, while Bax was increased by siRNA and Apcin. But caspase 3 showed no distinct difference. As a possible target of tumor therapy, the pro-apoptotic protein molecule Bim was investigated in this study. The increased expression of Bim was also observed in the MG63 cells

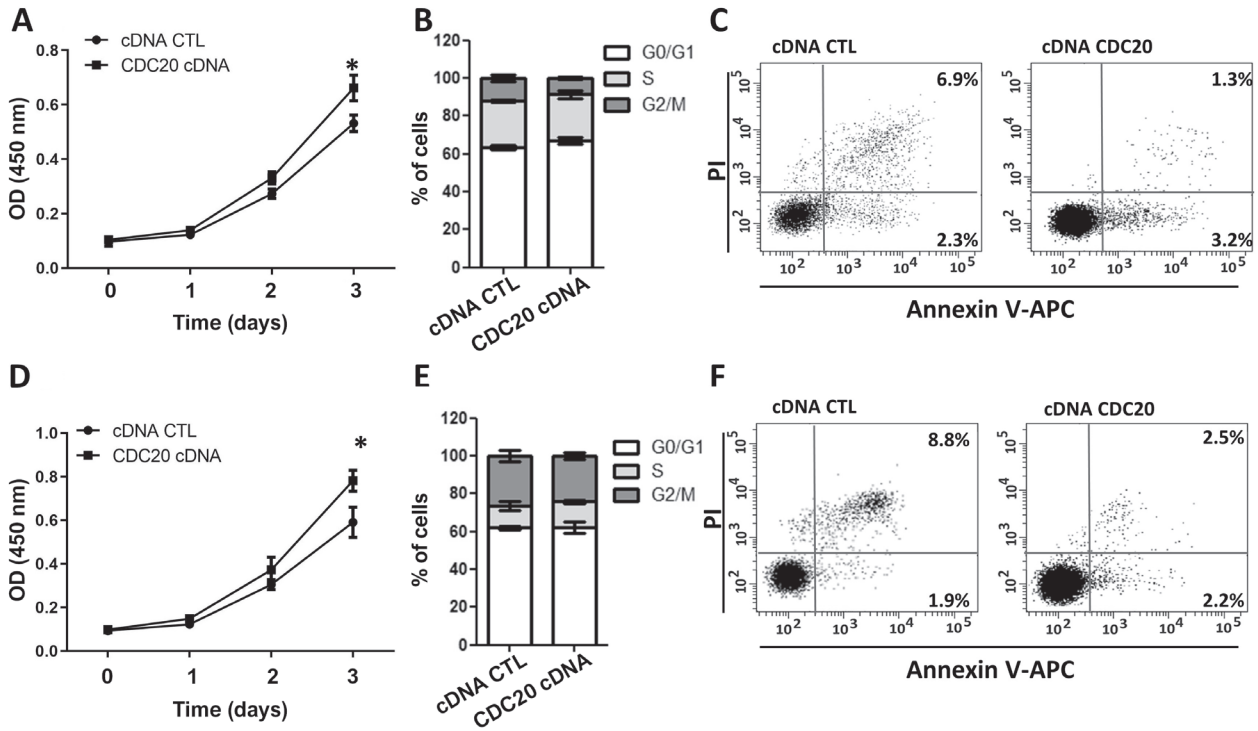

Figure 4. CDC20 cDNA transfection increases cell growth and has no effect on cell cycle and apoptosis rate. A, D) CCK-assays showed overexpression of CDC20 remarkably increased proliferation of U2OS and MG63 cells. Data are expressed as fold-change ± SEM compared to the absorbance at OD490 nm on the first day; B, E) Cell cycle and C, F) apoptosis rate showed no difference between control and CDC20 cDNA transfection groups in U2OS and MG63 cell lines. *p<0.05, data represents the mean ± S.D.

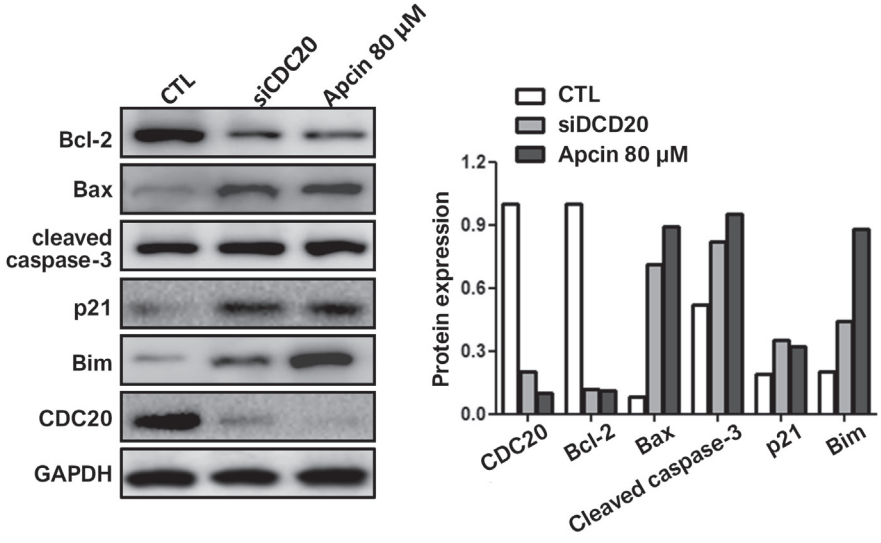

Figure 5. Inhibition of CDC20 affects apoptosis and cell cycle-associated pathway. A) The expressions of Bcl-2, Bax, caspase-3, p21, and Bim were analyzed by WB in MG63 cell lines. B) Graphs showing the densitometric intensity of immunoblots.

treated with downregulation of CDC20. Furthermore, p21, one of the CDC20 treatment (Figure 5).

Downregulation of CDC20 increases the sensitivity to chemotherapy of human OS cells. MG63 cells transfected with siRNA or treated with Apcin were exposed to *in vitro* treatment with 0, 0.01, 0.1, 1.0, 10, 25, and 50 μM cisplatin. At 48 hours after treatment, a CCK-8 assay was employed to evaluate the cell viability. The responses of CDC20 knockdown or Apcin-treating cells to cisplatin treatment were inhibited in a dose-dependent manner after 48 h; MG63 cells

with different treatments were exposed to 0, 0.1, 1.0, 10, and 25 μM cisplatin, the numbers of cell apoptosis were increased in a dose-dependent manner after 48 h. The results showed that CDC20-knockdown or Apcin-treated cells are more significantly sensitive than the control group (Figures 6).

CDC20 downregulation sensitizes OS cells to cisplatin *in vivo*. To further validate the *in vitro* experiments results, the effect of CDC20 on chemosensitivity to cisplatin was investigated in a mouse model of osteosarcoma. We calculated the volumes and weights of tumors during and after *in vivo* treatments period. As expected, tumor volumes were decreased in both cisplatin-treated groups. But in the cisplatin combined with shCDC20 treated group, the tumors almost were completely eliminated (Figures 7A, 7B). And meanwhile, the tumors were still obvious in the CTL group. Besides, we also detected the related proteins by WB. The protein level of CDC20 was decreased in the cisplatin combined with shCDC20 group. Consistent with the results of WB *in vitro*, the expression of Bcl-2 was decreased, while Bax, Bim, cleaved-caspase-3, and p21 were increased both in cisplatin and cisplatin combined with shCDC20 treated group *in vivo* (Figure 7C). These results demonstrated that knockdown of CDC20 in the U2OS cells suppressed the osteosarcoma cell proliferation and increased cell apoptosis, as well as enhanced the chemosensitivity to cisplatin.

Discussion

OS is the most common human primary malignant bone tumor that primarily affects children and adolescents. At present, patients are mainly treated with surgery or adjuvant chemotherapy. However, the 5-year survival outcome of a recurrent or advanced OS is still extremely poor [4]. Since the survival period of OS has not apparently prolonged over

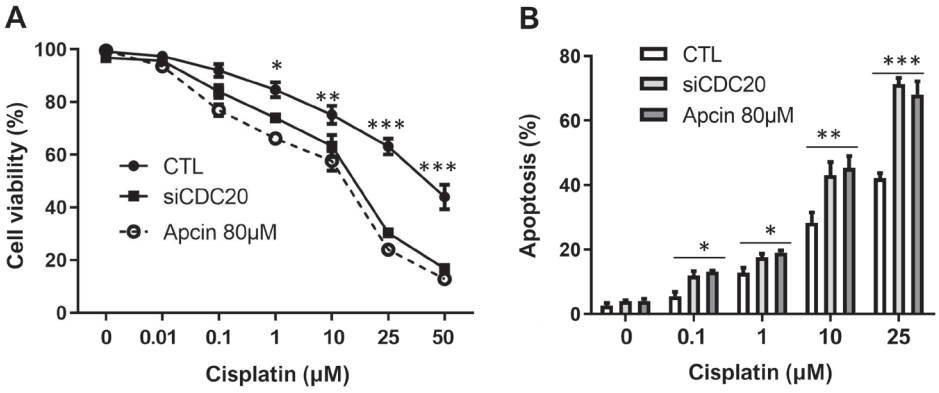

Figure 6. Inhibition of CDC20 augments the chemosensitivity to cisplatin in MG63 cells. A) CCK-8 assays showed cisplatin-treated combined with CDC20 inhibition remarkably decreased cell viability of U2OS compared with cisplatin-treated. Data are expressed as fold-change ± SEM compared to the absorbance at OD490 nm on the first day. B) Apoptosis rate of cisplatin-treated combined with CDC20 inhibition was significantly higher than that in cisplatin-treated cells. *p<0.05, **p<0.01, ***p<0.001, data represents the mean ± S.D.

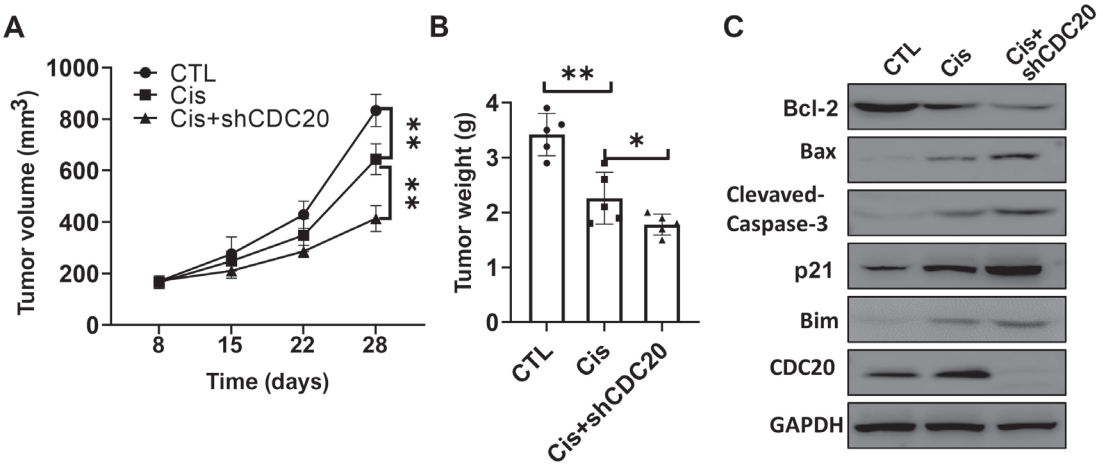

Figure 7. Knockdown of CDC20 improves the anti-tumor effect of cisplatin *in vivo*. 2×10^6 conditional MG63 cells were infected with the lentivirus vector of plko.1-shCDC20 or plko.1-shNC and screened with puro, then subcutaneously injected in the rear flank of nude mice (5 per group). A, B) The tumor size (mm^3) and the weight on day 28 were measured. C) The expressions of related proteins in tumor tissues were determined by WB. *p<0.05, **p<0.01, data represents the mean ± S.D.

the past two decades, new drugs and improved strategies for chemotherapy are urgently needed to be introduced to apply greater benefit to patients. Cisplatin is one of the most commonly drugs applied for OS due to its unique therapeutic advantages, including high efficiency and mild side effects. The main mechanism of cisplatin is causing tumor cells death by DNA damage. However, some patients with osteosarcoma are resistant to cisplatin treatment for the increased DNA repair ability of tumor cells. Hence cisplatin resistance is frequently reported, which means that the enhancement of cisplatin sensitivity is important for chemotherapy [13, 14]. Zheng et al. demonstrated that MAX dimerization protein 1(MXD1)-mediated hypoxia-induced cisplatin resistance in osteosarcoma cells by inhibiting the expression of phosphatase and tensin homolog (PTEN) [15]. And the miRNAs have also been identified as novel modulators including miR-133b, miR-21, and miR-214 in cisplatin resistance of osteosarcoma cells [16]. Therefore, understanding the mechanism of chemoresistance of cisplatin is vital to develop a more effective treatment for OS.

CDC20 is a key cofactor of the APC/C E3 ubiquitin ligase, with the function of regulating APC/C ubiquitin activity on the substrates for their subsequent degradation by the proteasome [17]. Ubiquitination is a post-translational modification of regulating a lot of key cellular proteins involved in various cellular processes including cell proliferation, differentiation, apoptosis, cell cycle, DNA damage repair (DDR), and senescence [18]. Besides, CDC20 is also a target of the spindle assembly checkpoint (SAC), which means that it plays important functions in chromosome segregation, mitotic exit as well as DDR [19]. There is increasing evidence showing that CDC20 plays an important role in tumor formation and development. A comprehensive analysis argues that the overexpression of CDC20 in malignant tumors was correlated with higher tumor grade and stage, such as squamous cell carcinoma, gastric cancer, colorectal cancer, and lung cancer. CDC20 is often overexpressed in the majority of human cancers, supporting its oncogenic role in promoting tumorigenesis, and thus CDC20 is a legitimate target of drug development for the treatment of human malignancies [20–25]. Consistent with the former findings, we also found CDC20 overexpressed in OS and its overexpression predicts adverse clinical outcomes (Figure 1B). Due to the key oncogenic role of CDC20 in tumorigenesis, CDC20 inhibitors are promising therapeutic agents targeting CDC20-high-expression cancers. Among these, Apcin prevents the substrate recognition of CDC20 and competitively inhibits APC/C-dependent ubiquitylation by binding to CDC20 [26, 27]. In line with it, inhibition of CDC20 by its siRNA and Apcin inhibited the cell growth, induced apoptosis, blocked the cell cycle to the G2/M phase of OS cells. We also observed that overexpression of CDC20 decreased apoptosis and stimulated proliferation.

Besides, what's more important is that we found combined CDC20 inhibition with cisplatin augmented chemosensitivity to cisplatin in MG63 cells both *in vitro* and *in vivo*. Chemotherapy is the main method for cancer therapy and cisplatin is the most commonly used chemotherapy drug for osteosarcoma. However, chemoresistance to cisplatin has become an intractable problem. So far, the mechanisms of chemoresistance to cisplatin are not fully understood, and several mechanisms have been reported, including mutation of drug targets, filtration of chemotherapy-resistant cells, and alterations in drug metabolism [28]. In this case, we try to increase the fitness of chemosensitive cells to outcome the chemoresistance. In the present study, OS cells with CDC20 inhibition are hypersensitive to cisplatin treatment and could therefore be specifically targeted.

The results of our present study demonstrated that targeting CDC20 could be useful for the treatment of OS, then we explored the potential mechanisms. Apoptosis is generally considered as an anti-oncogenic process for the elimination of mutant or damaged cells [29, 30]. B cell lymphoma 2 (BCL-2) family genes, including Bcl, Bax, Bim, caspase 3, and so on, have a central role in regulating programmed cell death by controlling pro-apoptotic and anti-apoptotic intracellular signals. And the tumor cells may utilize some of several molecular mechanisms to decrease apoptosis and acquire resistance to chemotherapeutics [31–34]. As might be expected, the inhibition of CDC20 reduced the expression of anti-apoptotic proteins such as Bcl-2, and downregulation of the pro-apoptotic proteins BAX. Caspase-3 is reported to facilitate tumor cell survival after the chemotherapy and radiation therapy. Accordingly, we speculated whether CDC20 regulated the biological behavior of the tumor by anti-apoptotic BCL-2 family proteins. The results of *in vivo* investigations showed that cisplatin treatment also affected the expression of Bcl family proteins, and cisplatin combined with shCDC20 amplified the effect.

However, further investigation on the mechanisms of CDC20 in OS cell chemosensitivity is needed, and much more efforts, including enlarging the sample size and confirming our results with more cell lines. And unfortunately, we cannot find the *in vivo* type of CDC20 inhibitor Apcin, which means that we can only study and observe the Apcin effect on OS cells *in vitro* but not *in vivo*. In conclusion, our *in vivo* and *in vitro* experiments revealed that the CDC20 gene has an important role in malignant tumors by serving as a useful biological marker to predict prognosis in patients with OS and that the knockdown or inhibition of CDC20 enhanced osteosarcoma cell chemosensitivity. CDC20 might affect the chemosensitivity of osteosarcoma by regulation of the apoptosis-related pathways. Therefore, CDC20 may serve as a potential therapeutic target for osteosarcoma.

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