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Original Article

Cell division cycle associated 8 promotes the growth and inhibits the apoptosis of endometrial cancer cells by regulating cell cycle and P53/Rb signaling pathway

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Abstract: Objective: Cell division cycle associated 8 (CDCA8) is over-expressed in a variety of tumors and associated with tumor progression. Nevertheless, the role of CDCA8 in endometrial cancer (EC) is unclear. Therefore, this study aimed to assess the role and mechanism of CDCA8 in EC. Methods: Immunohistochemical staining was used to evaluate CDCA8 expression in EC, and its relationship with clinicopathology was analyzed. CDCA8 was knocked down or over-expressed to study its effects on cell biological behaviors. Furthermore, the feasible mechanisms of CDCA8 were examined by Western blot. Results: CDCA8 was significantly upregulated in EC tissue ($P<0.05$) and related to worse tumor grade, Figo stage, tumor (T) stage, and deep myometrial invasion ($P<0.05$). CDCA8 knockdown inhibited EC cell activities, promoted apoptosis and induced cell cycle arrest ($P<0.05$), which were reversed by CDCA8 over-expression ($P<0.05$). Besides, CDCA8 knockdown inhibited the growth of xenograft tumors in nude mice ($P<0.05$). Furthermore, CDCA8 may affect cell cycle and P53/Rb signaling pathway in EC cells. Conclusion: CDCA8 plays a role in the pathogenesis of EC and may be a target for EC treatment.

Keywords: Cell division cycle associated 8, endometrial cancer, cell proliferation, apoptosis, cell cycle, therapeutic target

Introduction

Endometrial cancer (EC) is a malignance in female genital system [1]. In recent years, the morbidity and mortality of EC have been on the rise worldwide, and the patients tend to be younger, posing a severe threat to women's health [1, 2]. It is estimated that new EC cases per year in the United States will double to 122,000 by 2030 [3]. The 5-year survival rate is 18% in metastatic patients and 17% in patients with relapsed or locally advanced EC [4, 5]. With the development of modern medicine, early diagnosis, surgery, chemotherapy and radiotherapy can significantly improve the treatment outcomes, but there is still a lack of efficient program for EC patients with early fertility preservation and late relapse [6, 7]. Tumor stage, grade, histological subtype, deep muscular invasion and lymph-vascular space invasion are indicators for EC prognosis [8]. In recent years, The Cancer Genome Atlas (TCGA) molecular classification of EC has shown a high prognostic value [9]. At present, there is no reliable early diagnosis and therapeutic target for EC. So, it is urgent to search for new biomarker for the early diagnosis of EC and identify potential therapeutic targets.

Cell division is essential for development, and its abnormality is involved in various diseases [10]. Cell division cycle associated 8 (CDCA8) modulates mitosis and cell division through correction of kinetochore binding error and stabilization of bipolar spindle [11-13]. It is highly expressed in tumors and lowly in normal cells [14]. In breast cancer cells, CDCA8 knockdown could inhibit cell growth, which was accompanied by an increase in apoptosis-related molecules [15]. CDCA8 is also involved in tumor progression in bladder cancer and breast cancer [16, 17]. It has been reported that CDCA8 is over-expressed in colorectal cancer and cutaneous melanoma, and CDCA8 is associated

with the tumor growth, progression and poor prognosis in both tumors [18, 19]. However, its function and mechanism in EC remain obscure.

To investigate the role of CDCA8 in EC, we analyzed its expression in EC and correlation with clinicopathological features. In addition, CDCA8 level was manipulated in EC cells through gene knockdown and overexpression to assess the mechanism.

Materials and methods

Immunohistochemical staining (IHC)

Retrospectively, 28 normal non-cancerous endometrial tissue samples and 54 EC tissue samples (from 2017 to 2020) were collected from Qilu Hospital of Shandong University. The inclusion criterion was tissue from patients with EC undergoing primary surgery, and the exclusion criterion was tissue from patients with recurrence or undergoing chemoradiotherapy. All sample collections were approved by the Experimental Ethics Committee of Qilu Hospital, Shandong University (approval No. KYLL-202011-052). The tissue blocks were fixed in 4% paraformaldehyde for 24 h to prepare paraffin sections. Paraffin-embedded tissue sections with a thickness of 4 μm were dewaxed using xylene and dehydrated with graded ethanol. Retrieval of antigen was pursued upon incubation with TE buffer at 95°C for 15 min. After interdicting the endogenous peroxidase activity with 0.3% H_2O_2, goat serum (Reagent A, Zhongshan Jinqiao SP-9000 general SP kit, Beijing, China) was added to block non-specific antigens for 20 min. Thereafter, sections were incubated with a rabbit polyclonal antibody against human CDCA8 (proteintech, 12465-1-AP, 1:200) at 4°C overnight. After hatching with biotin-labeled goat anti-rabbit IgG polymer (Reagent B, Zhongshan Jinqiao SP-9000 general SP kit, Beijing, China) and horseradish-labeled streptomycin (Reagent C, Zhongshan Jinqiao SP-9000 general SP kit, Beijing, China) at 37°C for 30 min, DAB reagent (Zhongshan Jinqiao, Beijing, China) was used for positive staining of respective slides. A counterstain with hematoxylin (Solarbio, Beijing, China) was also performed. CDCA8 levels, detected in the different sections, were quantified by Image-Pro Plus software 6.0, according to the ratio of integrated optical density (IOD) and area, also known as mean optical density (IOD/area). Each sample was tested 3 times.

Cell lines and culture

For EC cell lines, Ishikawa cells were maintained in RPMI 1640 medium (Gibco, Waltham, MA USA), while KLE cells in DMEM/F12 medium (Gibco).

SiRNAs and plasmid transfection

CDCA8 small interfering (si)-RNAs si-CDCA8-82, si-CDCA8-269 and si-CDCA8-623 (GenePharma, Shanghai, China) were applied to knockdown CDCA8. A non-targeting siRNA (si-NC) (GenePharma, Shanghai, China) was used as control. The CDCA8 siRNAs sequences: si-CDCA8-82, 5'-CGACCGUGAAGUGGAAAUATT-3'; si-CDCA8-269, 5'-CUGGAUAUCACCGAAAUAATT-3'; si-CDCA8-623, 5'-GAGCGGAUUUACAACAUCUTT-3' and negative control (si-NC) 5'-UUCUCCGAACGUGUCACGUTT-3'. The CDCA8 over-expressed plasmid (pCMV-CDCA8) and empty vector plasmid CON468 (pCMV-NC) were from Jikai Company (Shanghai, China). CDCA8 siRNAs and plasmid were transfected into Ishikawa and KLE cells using Lipo8000 (Beyotime, Beijing, China) according to the manufacture's protocol at room temperature. During siRNA transfection, 100 pmol siRNA and 4 μL Lipo8000 were seeded in each well of a six-well plate. During plasmid transfection, 2.5 μg plasmid and 4 μL Lipo8000 were seeded in each well of a six-well plate. Cells were collected 48 h after transfection, and the cell transfection efficiency was assessed by RT-qPCR and Western blotting.

Reverse transcription quantitative PCR (RT-qPCR)

RNA was extracted and transcribed into cDNA, followed by PCR at 37°C for 60 min, then 85°C for 10 min, and finally kept at 4°C. The primers were CDCA8 (F: 5'-CGGAGAGAGCCTGCGATTAT-3', R: 5'-AGATTTGGGCGAGACGGTTG-3') and GAPDH (F: 5'-GCACCGTCAAGGCTGAGAAC-3', R: 5'-TGGTGAAGACGCCAGTGGA-3'; BioSune, Shanghai, China). The cycle conditions were set at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 62°C for 30 sec and 72°C for 40 sec. The last step was 95°C for 15 sec and 60°C for 1 min. $2^{-\Delta\Delta CT}$ method was applied to assess the relative level of

CDCA8 [20]. Each experiment was repeated in triplicate.

Western blotting

Cells were collected and lysed in RIPA (radio immunoprecipitation assay) lysis buffer (Beyotime, P0013B, Beijing, China) to isolate proteins, which were quantified using a BCA protein kit (Beyotime, Beijing, China). The proteins were then separated using SDS-PAGE and subjected to western blot analysis using antibodies against CDCA8 (proteintech, 12465-1-AP, 1:1000 dilution), cyclinA (Santa Cruz, sc-271682, 1:200 dilution), cyclinB1 (Santa Cruz, sc-245, 1:200 dilution), cyclinD1 (Santa Cruz, sc-8396, 1:200 dilution), CDK6 (Santa Cruz, sc-7961, 1:200 dilution), CDC2 (Santa Cruz, sc-54, 1:200 dilution), Rb (Santa Cruz, sc-102, 1:200 dilution), P53 (Santa Cruz, sc-126, 1:200 dilution) and HRP-labelled secondary antibody (Thermo Fisher Scientific, 31430, 1:5000 dilution).

MTT assay

Ishikawa and KLE cells were inoculated into five replicate wells of 96-well plates (2×10^3 cells/well), and cultured for 5-8 days. Cell viability was detected every 24 h (at 24, 48, 72, 96, 120, 144, 168, and 192 h). Next, 10 μL of MTT solution (5 mg/mL, Thermo Fisher Scientific, M6494) was added to each well, followed by incubation for 4 h. The MTT solution was then discarded, and DMSO was added to each well for 10 min. The absorbance of each well was measured at 490 nm using a microplate reader (Tecan Austria GmbH, 30050303, Austria).

Colony formation assay

Cell suspensions were seeded into 6-well plates and cultured for 10 days. The cell colonies were rinsed 3 times, fixed with methanol for 15 min, and dyed with 1% crystal violet (Solarbio, Beijing, China) for 30 min at room temperature. After washing, cell colonies in each group were counted.

Wound healing assay

The Ishikawa and KLE cells in 6-well plates (5×10^5 cells/well) were cultured, and a scraping device (pipette) was used to form a scratch followed by washing and observation at 0 h and 24 h under a fluorescent inverted microscope (Nikon, Ti-E, JPN). The scratch width was calculated as the mobility ratio = (0 h scratch width - 24 h scratch width)/0 h scratch width.

Transwell assay

Cells were harvested without FBS and inoculated in the upper chamber of transwell (8 μm pore size; Corning Costar, MA, USA) with or without Matrigel (BD Biosciences, CA, USA) at a density of 1.5×10^5 cells per well. The medium was placed in lower chamber, and cells were transferred to lower chamber. After incubation, the cells were fixed with methanol for 15 min and dyed with crystal violet (Solarbio, Beijing, China) for 30 min at room temperature. Five distinct optical fields were randomly assessed with the fluorescent inverted microscope (Nikon, Ti-E, JPN) at 100× magnification. All tests were performed in triplicate.

Cell cycle assay

Cells were rinsed with PBS, resuspended with 1 mL of DNA staining solution and 10 μL permeabilization solution (Multi Sciences, Hangzhou, China) after centrifugation (800 rpm, 5 min, at room temperature), and reacted for 30 min before detection. The cell cycle was examined by FACSCalibur flowcytometry (Beckman Coulter Inc., A00-1-1102, USA). The results were analyzed using the FlowJo X 10.0.7 (BD Biosciences).

Cell apoptosis assay

Cells were digested, washed and added with 100 μL of 1× binding buffer, 5 μL of Annexin V-APC and 5 μL of 7-AAD (Becton, Dickinson and Company, USA) to measure the apoptosis using FACS Calibur flow cytometer (Beckman Coulter Inc., A00-1-1102, USA).

Xenograft experiments

To establish stable cell lines, the cells were infected with lentivirus carrying shRNA targeting CDCA8 (sh-CDCA8; Jikai Company, 96461-1, Shanghai, China) or negative control precursor (sh-NC; Jikai Company, LVCON313, Shanghai, China). The target sequence of CDCA8 (sh-CDCA8) was 5'-GAGCGGATTTACAACATCT-3'. The negative control (sh-NC) sequence was 5'-TTCTCCGAACGTGTCACGT-3'. The amount of lentivirus in the sh-NC group was 0.8 μL and the lentivirus titre was 2.5×10^9 TU/

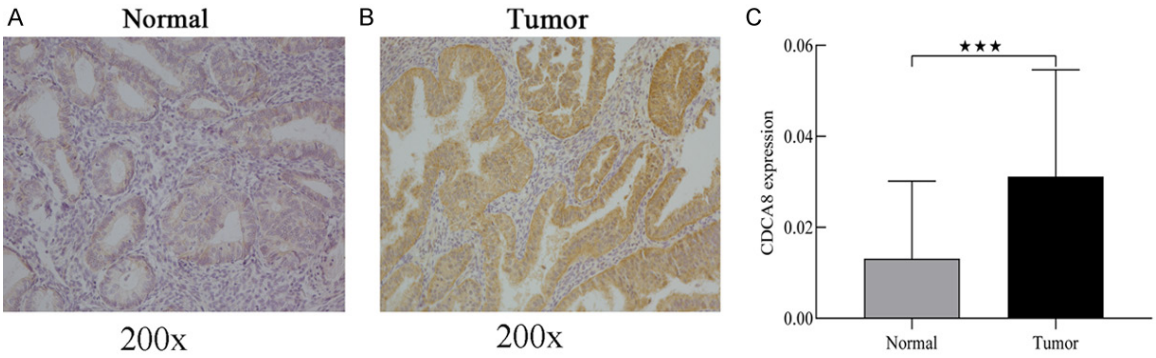


Figure 1. Immunohistochemical examination of CDCA8 expression in normal endometrial tissue and EC tissue. A: Representative images of CDCA8 protein expression in normal endometrial tissue (200×); B: Representative images of CDCA8 protein expression in EC tissue (200×); C: Differential expression of CDCA8 protein in normal endometrial tissue and EC tissue. Compared with the normal group, ***P<0.001. CDCA8: cell division cycle associated 8; EC: endometrial cancer. Scale bar: 50 μm.

mL. At 72 h after transfection, the cells were treated with purinomycin (Sigma-Aldrich) (0, 1, 2.5, 5, 7.5, 10 μg/mL), and the minimum effective concentration of purinomycin was determined to be 5 μg/mL. So, the cells were cultured with 5 μg/mL purinamycin for seven days to obtain stable cell line.

The animal experiments were performed according to the Animal Experimental Ethics Committee guidelines of Qilu Hospital, Shandong University (approval No. 21095). A total of 10 female BALB/c nude mice (4 weeks old, 18-20 g) were purchased from GemPharmatech (Jiangsu, China). Ishikawa cells stably expressing sh-NC and sh-CDCA8 were digested, and 200 μL Ishikawa cell resuspension solution was subcutaneously injected into the axilla area of the mice at a density of 5×10^7 cells/mouse. Tumor volume = 0.5× $(\text{width})^2$ × length. All mice were euthanized by cervical dislocation 32 days after injection.

Statistical methods

GraphPad Prism Version 8.0.1 (GraphPad Software, USA) was used for data processing. The data were shown as mean ± SD and analyzed by Student's t-test or two-way ANOVA, followed by Sidak's multiple comparisons post-test. P<0.05 refers to a significant difference.

Results

CDCA8 was over-expressed in EC and associated with worse clinicopathological characteristics

An increased expression of CDCA8 protein was revealed in EC tissue samples (**Figure 1B**) compared with non-cancerous endometrial samples (**Figure 1A**; P<0.05, **Figure 1C**). According to the median value, patients with EC were divided into a high CDCA8 group and a low CDCA8 group. Then, we analyzed the relationship between CDCA8 protein expression and clinicopathological features of EC. As shown in **Table 1**, up-regulated expression of CDCA8 protein was correlated with advanced Figo stage, T stage and tumor grade (P<0.05). CDCA8 was significantly upregulated in serous carcinoma relative to that in endometrioid carcinoma (P<0.05). CDCA8 was higher in patients with myometrial invasion greater than 1/2 than in those with less than 1/2 (P<0.05). The age of EC patients and the presence of lymphatic metastasis and distant metastasis were not associated with CDCA8 expression. This may be related to small patients number with lymphatic and distant metastasis.

CDCA8 knockdown and over-expression in Ishikawa and KLE cells

CDCA8 was knocked down by siRNA transfection and over-expressed by plasmid transfection. Significantly reduced CDCA8 was observed in Ishikawa and KLE cells after si-RNA-623 transfection (P<0.05). Follow-up experiments were performed with si-CDCA8-623 (si-CDCA8). The CDCA8 expression was significantly promoted in Ishikawa and KLE cells after plasmid transfection (P<0.05). At a mRNA level, CDCA8 knockdown efficiency in Ishikawa and KLE cells was 68.72% and 97.12%, respectively, and the over-expression efficiency of CDCA8 in Ishikawa and KLE cells was over 700% and 500%, respectively (**Figure 2A**). At a protein level, the

Table 1. Association between CDCA8 and clinicopathological characteristics of EC

Clinicopathological characteristics	Total (N)	CDCA8 expression (N)		*P*-value
		High	Low	
Age	54			
>60		14	11	0.413
≤60		13	16	
Figo stage				
IA	38	8	16	0.023
IB		10	4	
IA	54	8	16	0.028
IB-IV		19	11	
I	54	18	20	0.551
II-IV		9	7	
Tstage				
T1a	38	8	16	0.023
T1b		10	4	
T1a	54	8	16	0.028
T1b-T4		19	11	
T1	54	18	20	0.551
T2-T4		99	7	
Grade				
G1	37	3	10	0.011
G3		16	8	
G1	54	3	10	0.038
G2+G3		23	18	
M stage	54			
M0		25	27	0.957
M1		1	1	
N stage	54			
N0		20	26	0.100
N1+N2		6	2	
Histology	54			
endometroid		16	24	0.043
non-endometroid		10	4	
Myometrialinvasion	54			
>1/2		12	5	0.040
<1/2		15	22	

Note: CDCA8: cell division cycle associated gene 8; EC: endometrial cancer; N: number.

knockdown efficiency of CDCA8 in Ishikawa and KLE cells was 71.39% and 76.93%, respectively, and the over-expression efficiency of CDCA8 in Ishikawa and KLE cells was over 90% and 150%, respectively (**Figure 2B**).

CDCA8 promoted EC cells proliferation

As seen in **Figure 3A** and **3B**, CDCA8 knockdown suppressed the proliferation of Ishikawa and KLE cells and reduced the clonogenic ability (P<0.05). However, CDCA8 over-expression increased the proliferation rate and clonogenic ability of Ishikawa and KLE cells (P<0.05, **Figure 3C** and **3D**). Furthermore, to investigate the effect of CDCA8 on tumor growth in vivo, Ishikawa and KLE cell lines with stable knockdown of CDCA8 were obtained by lentivirus transfection (**Figure 4A**). Transfection of sh-CDCA8 significantly restrained the expression of CDCA8 in Ishikawa and KLE cells, and the CDCA8 knockdown efficiency was higher in Ishikawa cells than in KLE cells (P<0.05, **Figure 4B** and **4C**). Ishikawa cells with sh-CDCA8 and the corresponding control were subcutaneously injected into nude mice, and IHC staining confirmed that the CDCA8 expression was inhibited (P<0.05, **Figure 4F**). CDCA8 knockdown significantly inhibited xenografted tumor growth (**Figure 4D**) and resulted in reduced tumor mass and tumor weight (P<0.05, **Figure 4E**). Collectively, our data revealed that CDCA8 promoted EC cells proliferation, and knockdown of CDCA8 inhibited EC cells growth.

CDCA8 knockdown inhibited EC cell migration and invasion abilities

As shown in **Figure 5A** and **5D**, si-CDCA8 significantly inhibited the cell migration ability (P<0.05) with lower number of migrated and invaded cells (P<0.05, **Figure 5B**, **5C**, **5E** and **5F**). While CDCA8 over-expression promoted the cell migration and invasion ability. Wound healing assay showed that cell migration ability in pCMV-CDCA8 group was significantly higher than that in pCMV-NC group (**Figure 6A** and **6D**). Transwell assay showed that the number of migration and invasive cells in pCMV-CDCA8 group was higher than that in pCMV-NC group (P<0.05, **Figure 6B**, **6C**, **6E** and **6F**).

CDCA8 knockdown promoted the apoptosis of EC cells while CDCA8 over-expression inhibited EC cell apoptosis

As shown in **Figure 7A-C**, si-CDCA8 increased the cell apoptosis (P<0.05), with lower apopto-

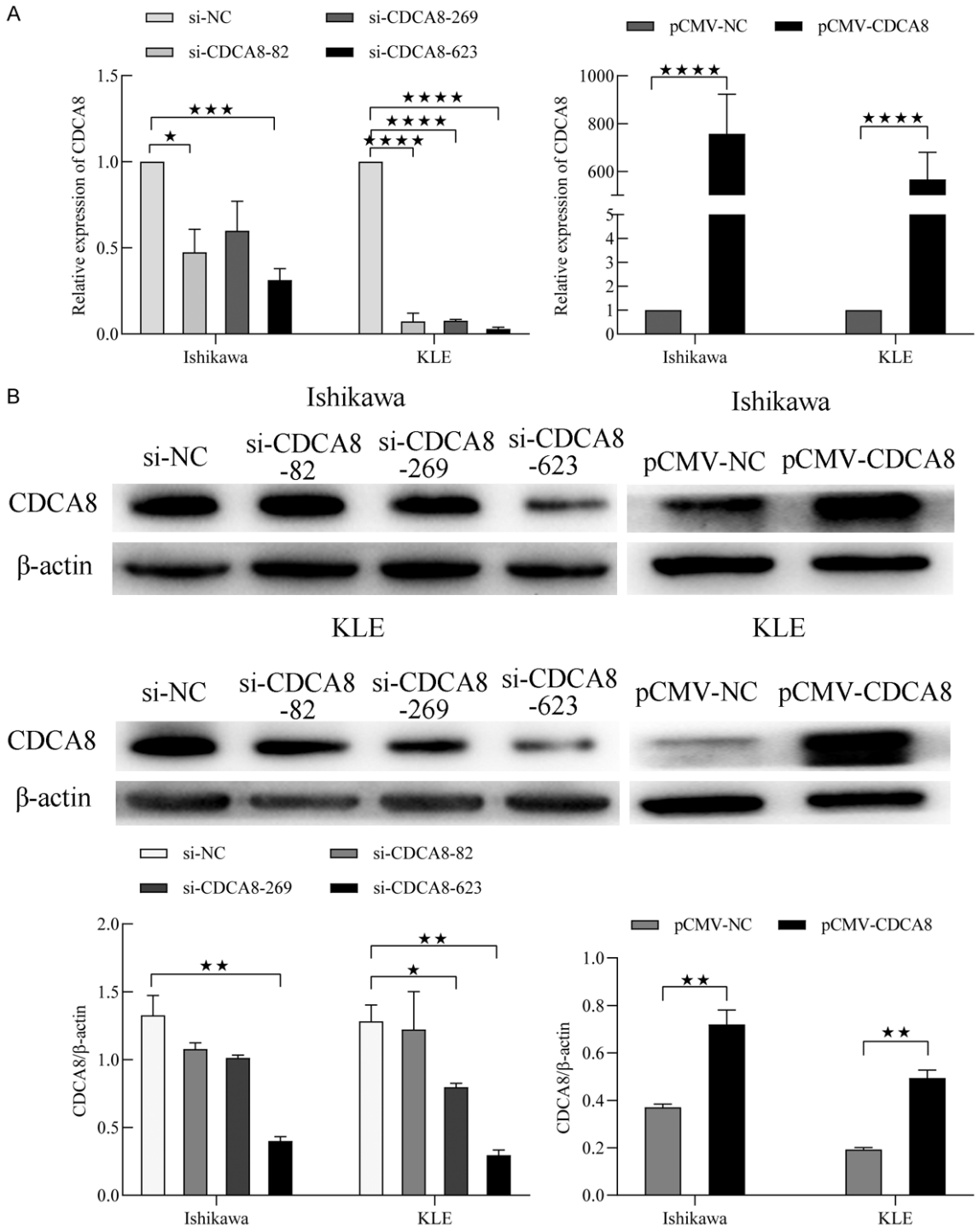


Figure 2. CDCA8 knockdown and over-expression in endometrial cancer Ishikawa and KLE cells. A: Three siRNAs transfection inhibited CDCA8 mRNA expression, while plasmid transfection promoted CDCA8 mRNA expression in Ishikawa and KLE cells; B: Three siRNAs transfection inhibited CDCA8 protein expression while plasmid transfection promoted CDCA8 protein expression in Ishikawa and KLE cells. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; NC: negative control; si: small interfering (RNA); pCMV: plasmid.

sis in pCMV-CDCA8 group than that in pCMV-NC group (P<0.05, **Figure 7D-F**), indicating that down-regulation of CDCA8 could promote cell apoptosis.

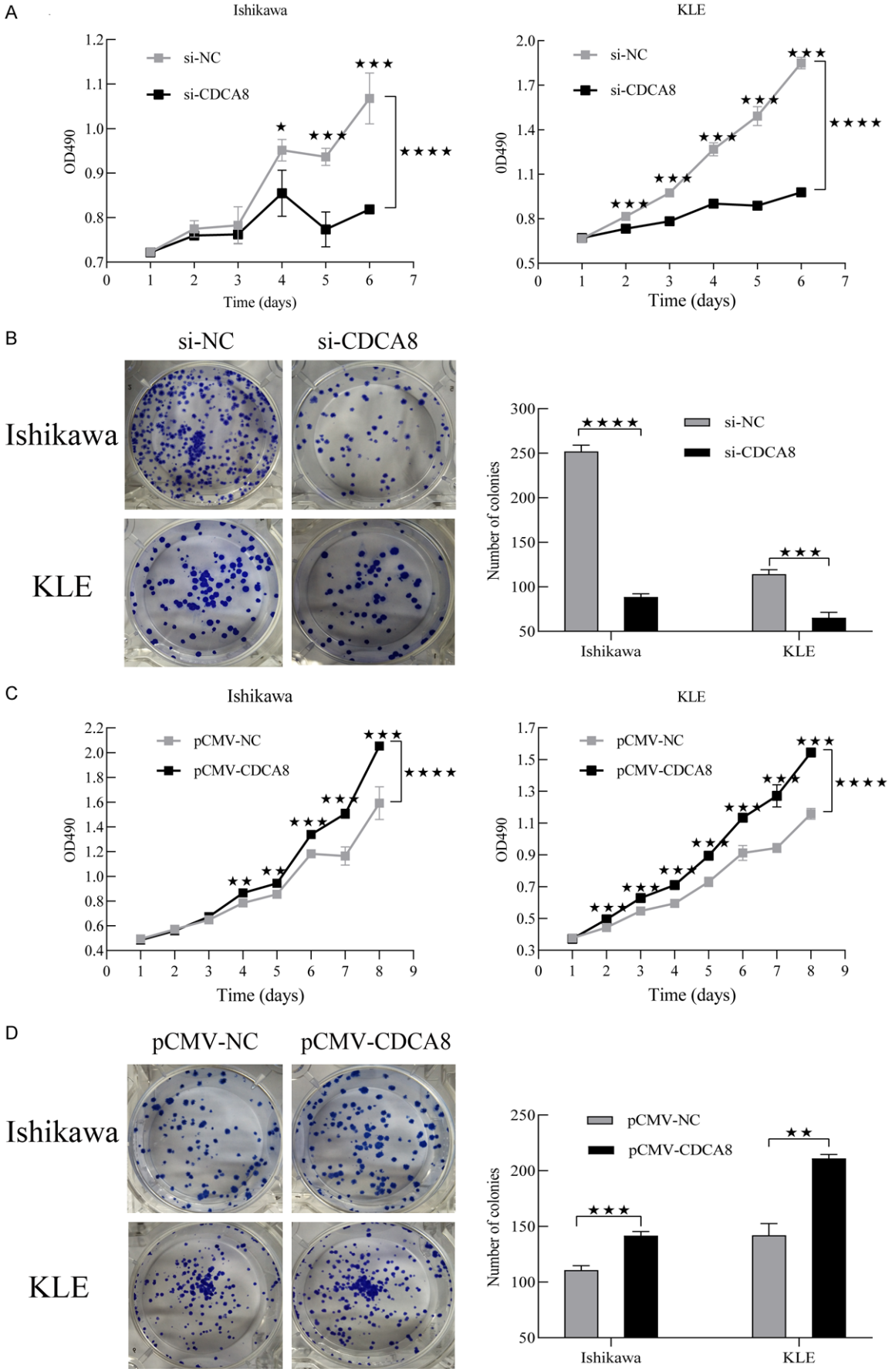
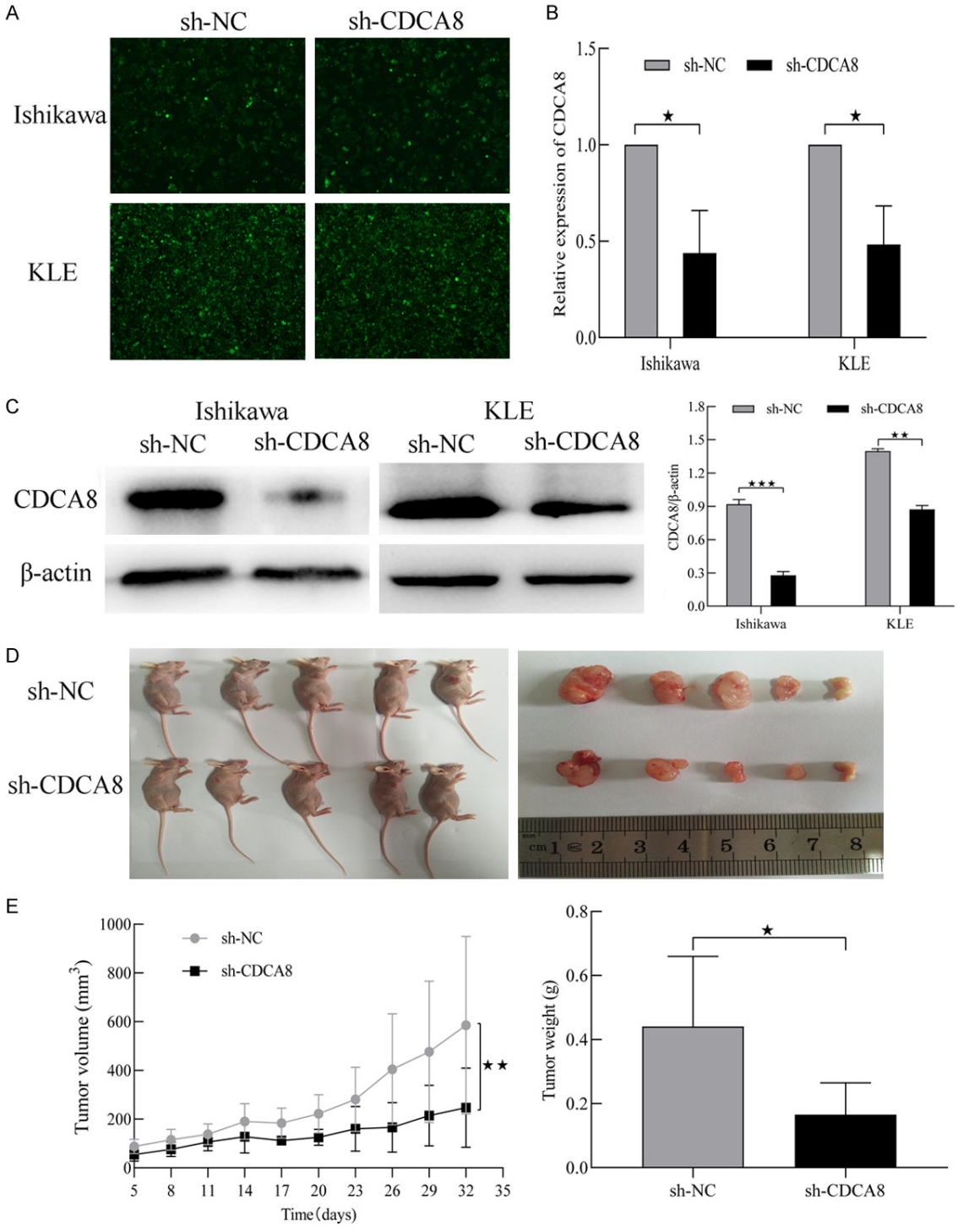

A
Ishikawa
KLE
si-NC
si-CDCA8
OD490
0D490
Time (days)
B
si-NC
si-CDCA8
Ishikawa
KLE
Number of colonies
C
Ishikawa
KLE
pCMV-NC
pCMV-CDCA8
OD490
Time (days)
D
pCMV-NC
pCMV-CDCA8
Ishikawa
KLE
Number of colonies

Figure 3. Effect of CDCA8 on the proliferation of endometrial cancer Ishikawa and KLE cells. A: CDCA8 knockdown reduced the proliferative capacity of Ishikawa and KLE cells; B: CDCA8 knockdown reduced the clonogenic ability of Ishikawa and KLE cells; C: CDCA8 over-expression promoted the proliferative capacity of Ishikawa and KLE cells; D: CDCA8 over-expression promoted the clonogenic ability of Ishikawa and KLE cells. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; NC: negative control; si: small interfering (RNA); pCMV: plasmid.



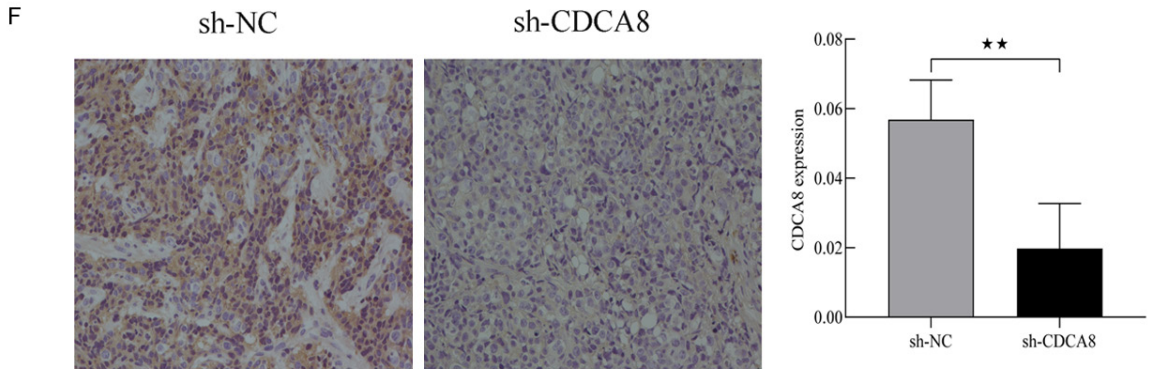


Figure 4. CDCA8 gene stable knockdown inhibits EC cell tumor growth in nude mice xenografts. A: GFP expression in Ishikawa and KLE cells under fluorescence microscope after lentivirus transfection (100×); B: Lentivirus transfection inhibited the expression of CDCA8 mRNA in Ishikawa and KLE cells; C: Lentivirus transfection inhibited the expression of CDCA8 protein in Ishikawa and KLE cells; D: Xenograft tumor model in nude mice subcutaneously injected with Ishikawa cells with stable knockdown of CDCA8 or the control; E: Volume and weight of tumors from mice subcutaneously injected with Ishikawa cells with stable knockdown of CDCA8 or the control; F: CDCA8 immunohistochemical staining of xenograft tumors (200×) and statistical analysis. *P<0.05, **P<0.01. CDCA8: cell division cycle associated 8; NC: negative control; sh: short hairpin (RNA); GFP: green fluorescent protein.

CDCA8 knockdown induced cell cycle arrest of EC cells

The proportion of cells in the G0/G1 phase was reduced, while the proportion of cells in the G2/M phase was increased after CDCA8 knockdown in Ishikawa and KLE cells (P<0.05, **Figure 8A** and **8B**), without difference between the CDCA8 over-expression group and the control group (**Figure 8C** and **8D**). It is indicated that CDCA8 knockdown could induce G2/M phase arrest in Ishikawa and KLE cells.

Mechanism of CDCA8 in EC cells

After CDCA8 knockdown, the expressions of cyclin D1, cyclin A and CDK6 were reduced, and the expressions of cyclin B1 and CDC2 were increased in Ishikawa and KLE cells (P<0.05, **Figure 9A** and **9B**). However, cyclin A was increased after CDCA8 over-expression (P< 0.05, **Figure 9C** and **9D**). This indicates that CDCA8 over-expression could promote cell DNA synthesis. In addition, the expression levels of signaling pathway molecules P53 and Rb were also examined. It was observed that their expression were increased in the CDCA8 knockdown group, and decreased in the CDCA8 over-expression group (P<0.05, **Figure 10**), indicating that CDCA8 may regulate P53/Rb signaling pathway.

Discussion

CDCA8 participates in the modulation of cell division [11]. It has been observed that CDCA8 is over expressed in several malignant tumors, including EC, and is closely associated with poor pathological stage, grade, distant, lymphatic metastasis and prognosis in patients [21]. However, the biological function of CDCA8 in EC has not been established. Initially, we found higher CDCA8 level in EC tissue, which was related to worse tumor grade, Figo stage, tumor (T) stage, deep myometrial infiltration. CDCA8 is involved in cell growth [14]. The expression of CDCA8 was up-regulated in hepatocellular carcinoma, and CDCA8 knockdown inhibited hepatocellular carcinoma cell growth and monoclonal formation, affected cell cycle and promoted cell apoptosis. Therefore, CDCA8 may be an effective therapeutic target for hepatocellular carcinoma [22]. CDCA8 was also essential for lung cancer cell growth, and over-expression of CDCA8 could promote lung cancer cell growth [23]. In addition, CDCA8 knockdown could reduce the proliferation of human embryonic stem cells [14]. In pancreatic cancer, knockdown of KIF23 inhibited pancreatic cancer cell growth by affecting CDCA8 levels [24]. In ovarian cancer and glioma, knockdown of CDCA8 could inhibit tumor cell growth [25, 26]. Our results exhibited that knockdown of CDCA8 significantly inhibited EC cell growth and colony formation, which were reversed by CDCA8 over-expression. Similarly, animal experiments confirmed that knockdown of CDCA8 inhibited the growth of xenografts in nude mice. These findings indicated that CDCA8 may be a target for the treatment of EC.

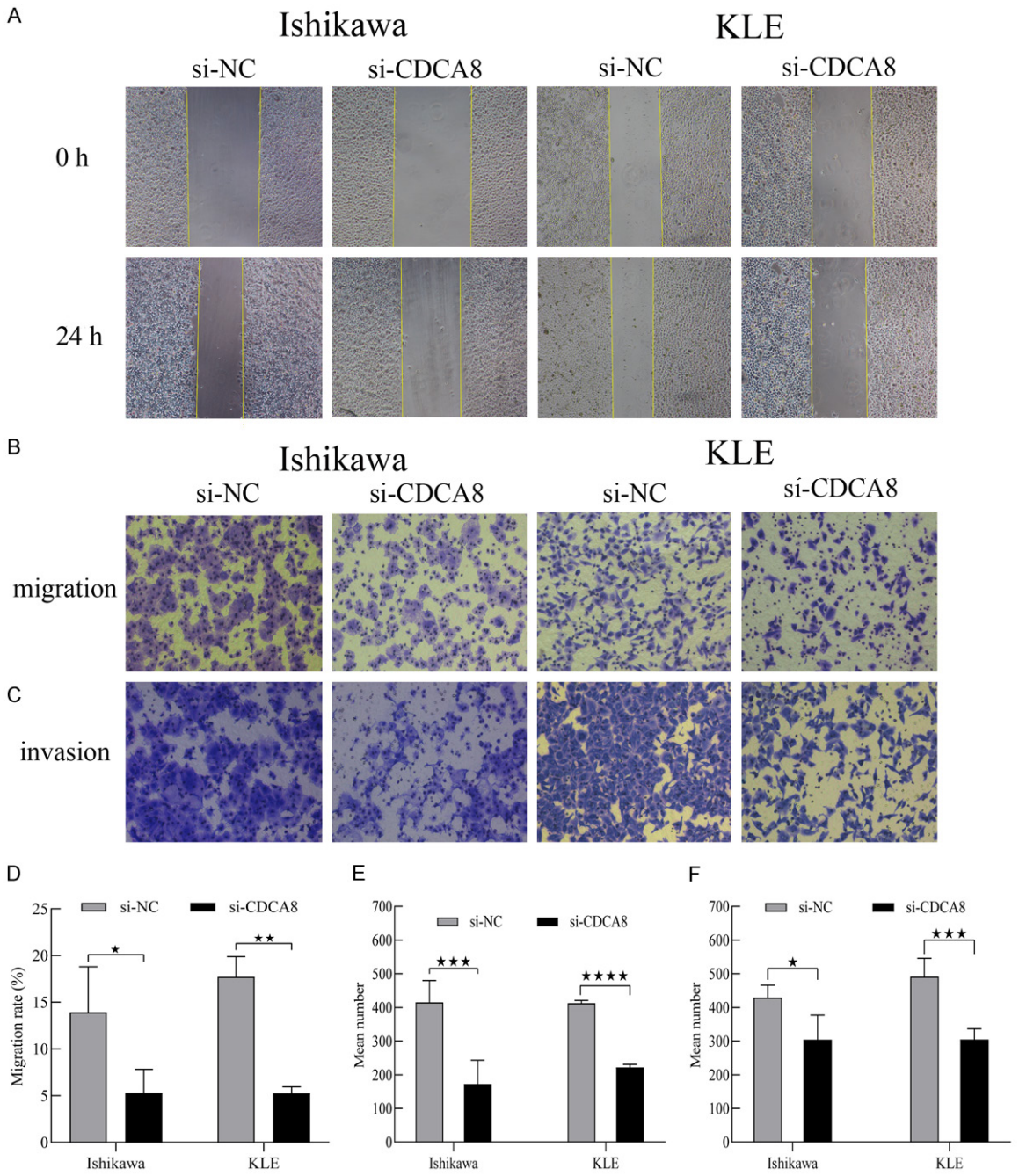


Figure 5. CDCA8 knockdown inhibits the migration and invasion of EC cell lines. A, D: Representative micrographs of wound healing assays in Ishikawa and KLE cells (100×) and their migration rates; B, E: Representative micrographs of Transwell cell migration experiments in Ishikawa and KLE cells (100×) and their quantitative statistical results; C, F: Representative micrographs of Transwell cell invasion experiments in Ishikawa and KLE cells (100×) and their quantitative statistical results. *P<0.05, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; EC: endometrial cancer; NC: negative control; si: small interfering (RNA); pCMV: plasmid. Scale bar: 100 μm.

CDCA8 is not only an oncogene that promotes tumor genesis and development, but also essential for tumor invasion and metastasis [27]. In this study, knockdown of CDCA8 significantly inhibited EC cell abilities, which was reversed by CDCA8 over-expression. CDCA8 over-expression could promote cell abilities in lung cancer [23] and glioma [26]. Previous

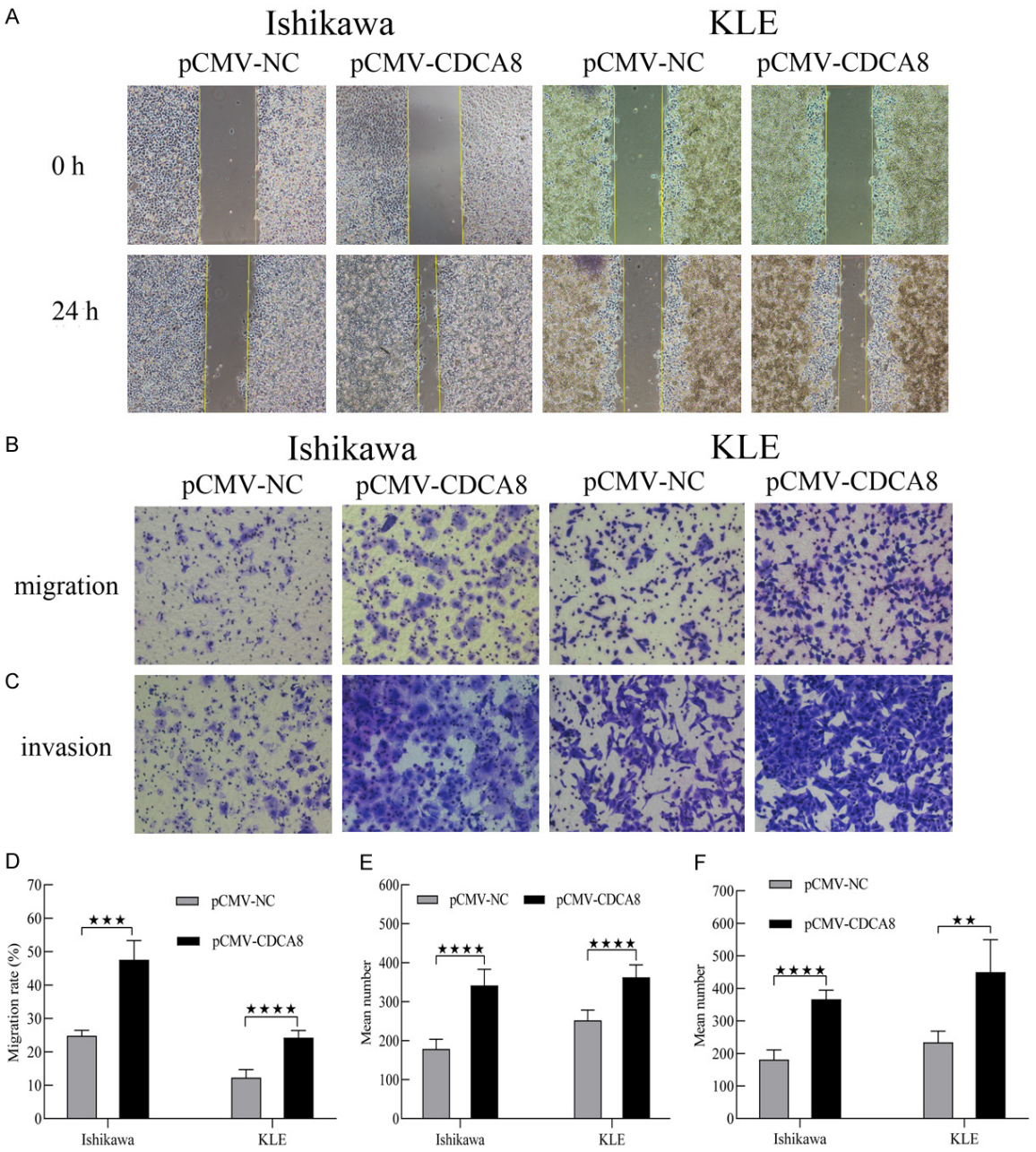


Figure 6. CDCA8 over-expression promotes the migration and invasion of EC cell lines. A, D: Representative micrographs of wound healing assays in Ishikawa and KLE cells (100×) and their migration rate; B, E: Representative micrographs of Transwell cell migration experiments in Ishikawa and KLE cells (100×) and their quantitative statistical results; C, F: Representative micrographs of Transwell cell invasion experiments in Ishikawa and KLE cells (100×) and their quantitative statistical results. **P<0.01, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; EC: endometrial cancer; NC: negative control; pCMV: plasmid. Scale bar: 100 μm.

studies have found association of high CDCA8 expression with lymph node metastasis in cutaneous melanoma [19]. Li et al. found an association of high CDCA8 expression with lymphatic and distant metastasis in EC [21]. Liu et al. found relation of CDCA8 gene with EC progression and prognosis [28]. These findings are consistent with the findings in our study, suggesting that CDCA8 up-regulation could promote EC cell migration and invasion and lead to poor prognosis.

In bladder cancer, knockdown of CDCA8 could inhibit cell growth and induce cell apoptosis

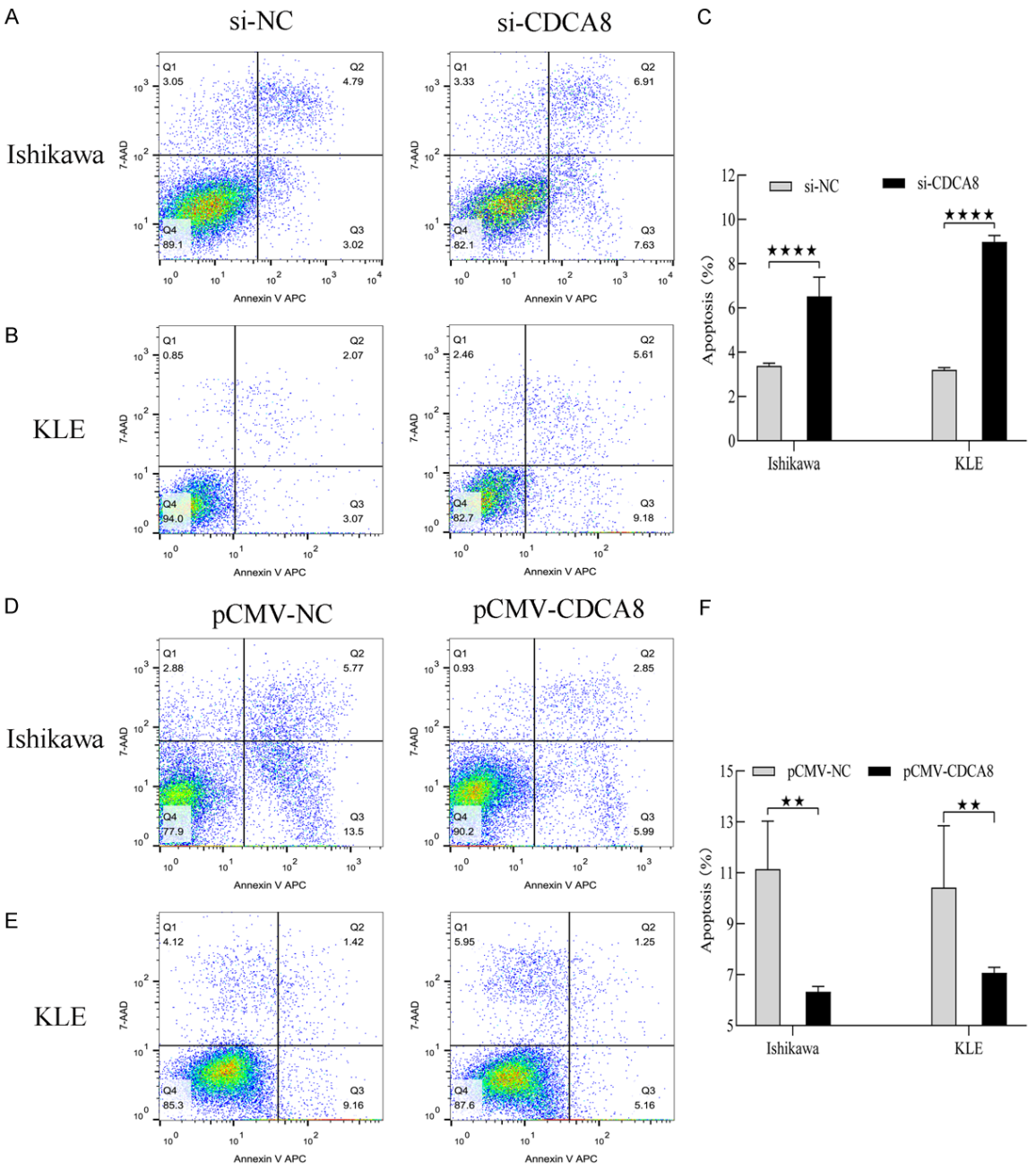


Figure 7. Effect of CDCA8 on the apoptosis of endometrial cancer Ishikawa and KLE cells. A: CDCA8 knockdown promoted the apoptosis of Ishikawa cells; B: CDCA8 knockdown promoted the apoptosis of KLE cells; C: Apoptosis in Ishikawa and KLE cells after CDCA8 knockdown; D: CDCA8 over-expression reduced the apoptosis of Ishikawa cells; E: CDCA8 over-expression reduced the apoptosis of IKLE cells; F: Apoptosis in Ishikawa and KLE cells after CDCA8 over-expression. **P<0.01, ****P<0.0001. CDCA8: cell division cycle associated 8; NC: negative control; si: small interfering (RNA); pCMV: plasmid.

[29]. In osteosarcoma, knockdown of CDCA8 significantly controlled cell growth by regulating cell cycle [30]. CDCA8 is an independent prognostic factor for hepatocellular carcinoma. CDCA8 gene silencing significantly inhibited cell growth and resulted in G2/M phase arrest [31, 32]. It was also found that CDCA8 was over-expressed in ovarian cancer and involved in the occurrence, invasion and chemotherapy resistance of ovarian cancer [25]. The over-expression of CDCA8 was found to promote cell proliferation, while silencing of CDCA8 reversed this

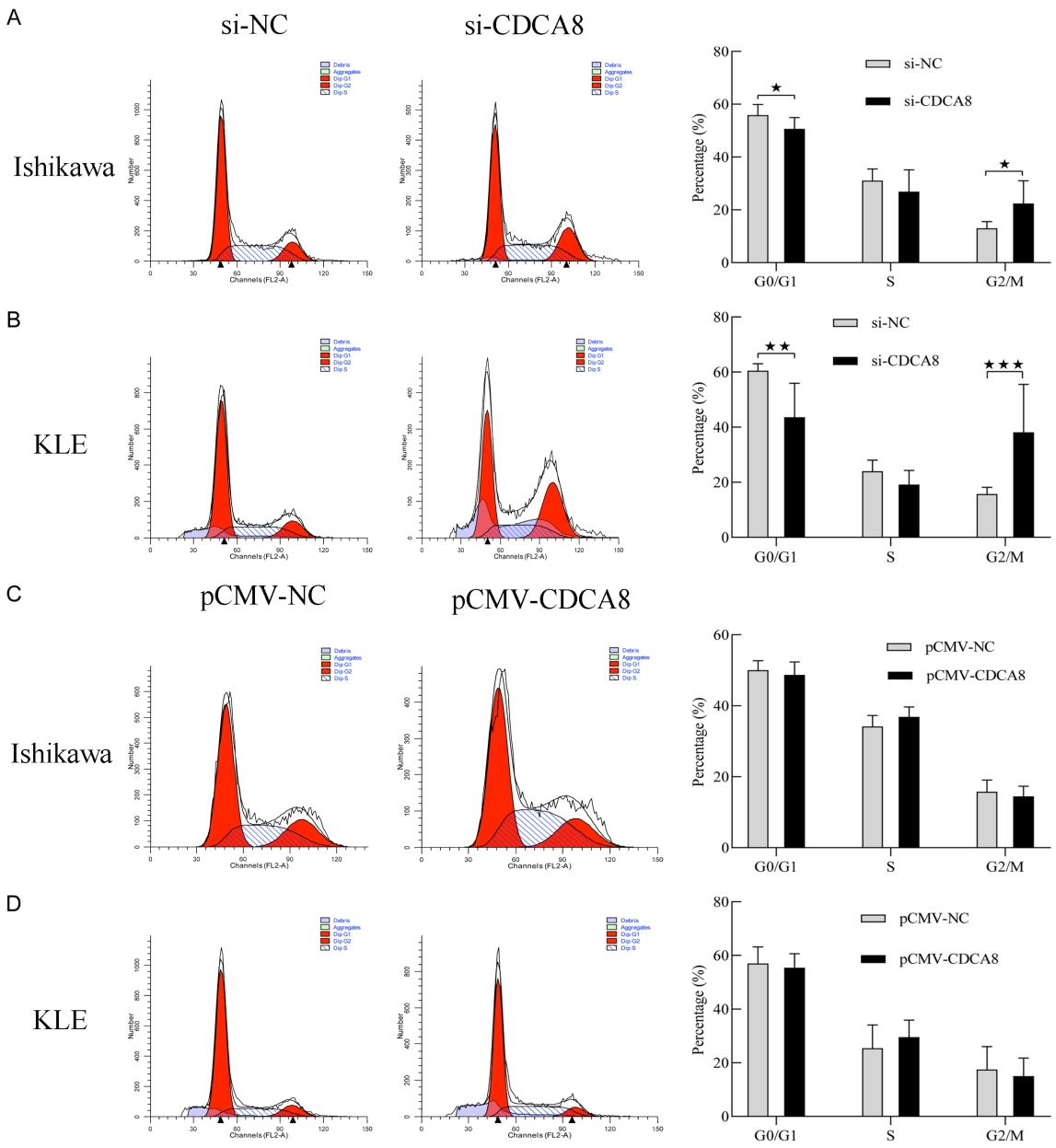


Figure 8. Effects of CDCA8 on the cell cycle of EC cell lines. A: Cell cycle in Ishikawa cells with CDCA8 knockdown; B: Cell cycle in KLE cells with CDCA8 knockdown; C: Cell cycle in Ishikawa cells with CDCA8 over-expression; D: Cell cycle in KLE cells with CDCA8 over-expression. *P<0.05, **P<0.01, ***P<0.001. CDCA8: cell division cycle associated 8; EC: endometrial cancer; NC: negative control; si: small interfering (RNA); pCMV: plasmid.

effect. So, CDCA8 silencing combined with olaparil may lead to some progress in the targeted therapy of ovarian cancer [25]. CDCA8 could also promote the cell behaviors of esophageal cancer [33]. This is consistent with the findings of flow cytometry in this study, which exhibited that CDCA8 over-expression inhibited EC cell apoptosis, while CDCA8 knockdown promoted EC cells apoptosis and resulted in G2/M phase arrest.

Through GSEA analysis of EC data in TCGA, Li et al. found that CDCA8 could play a role by affecting many biological processes related to cell activities [21]. The G1/S phase and G2/M phase are two key checkpoints in the cell cycle that regulate the transition of cells from one phase to another [34]. In general, cyclin, cyclin dependent kinase (CDK) and CDK inhibitor (CDKI) work together to regulate the cell cycle [35]. Various causes, such as increased expres-

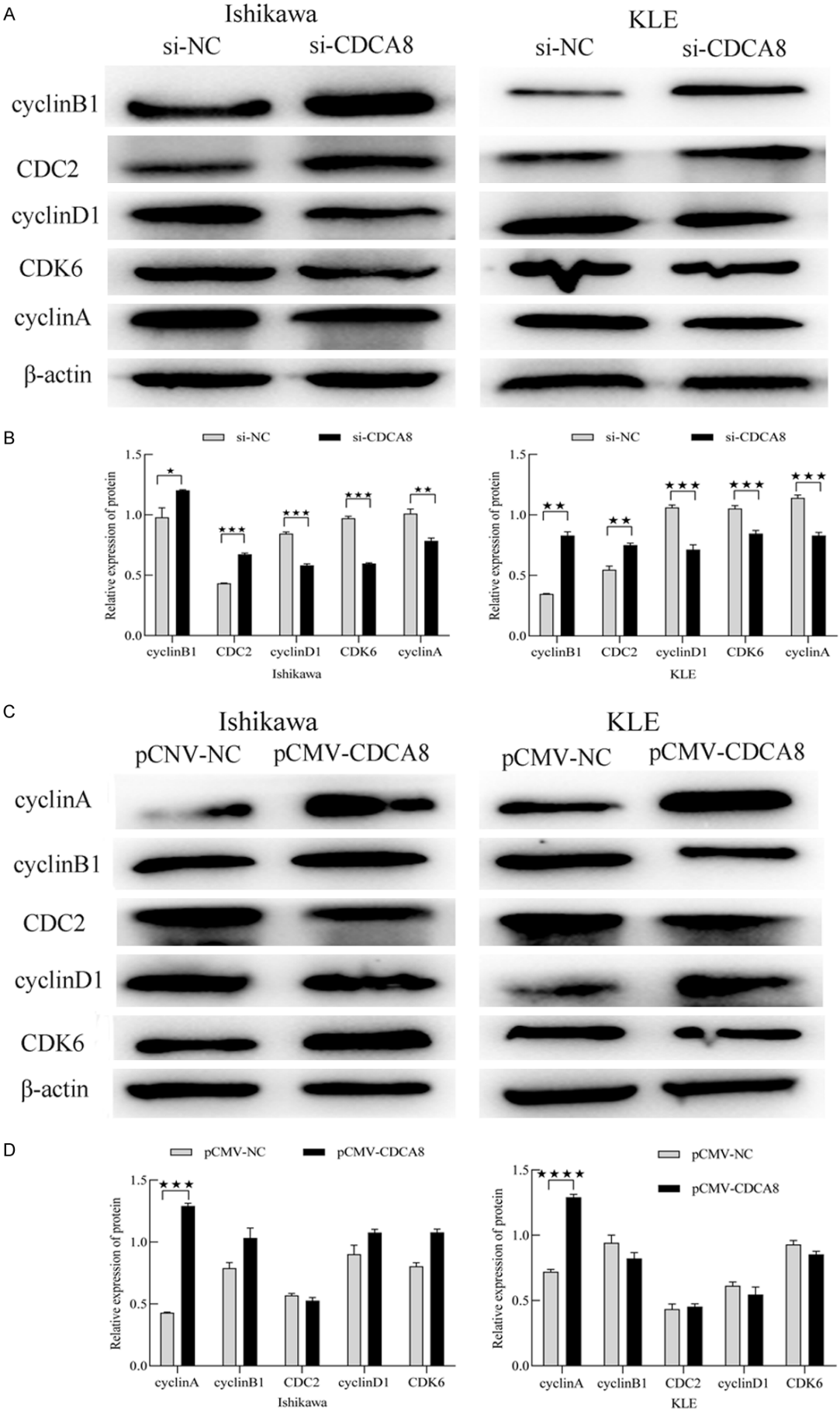

A
Ishikawa
si-NC
si-CDCA8
KLE
si-NC
si-CDCA8
cyclinB1
CDC2
cyclinD1
CDK6
cyclinA
β-actin
B
si-NC
si-CDCA8
Relative expression of protein
cyclinB1
CDC2
cyclinD1
CDK6
cyclinA
Ishikawa
KLE
C
Ishikawa
pCNV-NC
pCMV-CDCA8
KLE
pCMV-NC
pCMV-CDCA8
cyclinA
cyclinB1
CDC2
cyclinD1
CDK6
β-actin
D
pCMV-NC
pCMV-CDCA8
Relative expression of protein
cyclinA
cyclinB1
CDC2
cyclinD1
CDK6
Ishikawa
KLE

Figure 9. Effects of CDCA8 on the cell cycle-related proteins of EC cell lines. A and B: Western blot analysis of cell cycle-related protein expression in Ishikawa and KLE cells with CDCA8 knockdown; C and D: Western blot analysis of cell cycle-related protein expression in Ishikawa and KLE cells with CDCA8 over-expression. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; EC: endometrial cancer; NC: negative control; si: small interfering (RNA); pCMV: plasmid.

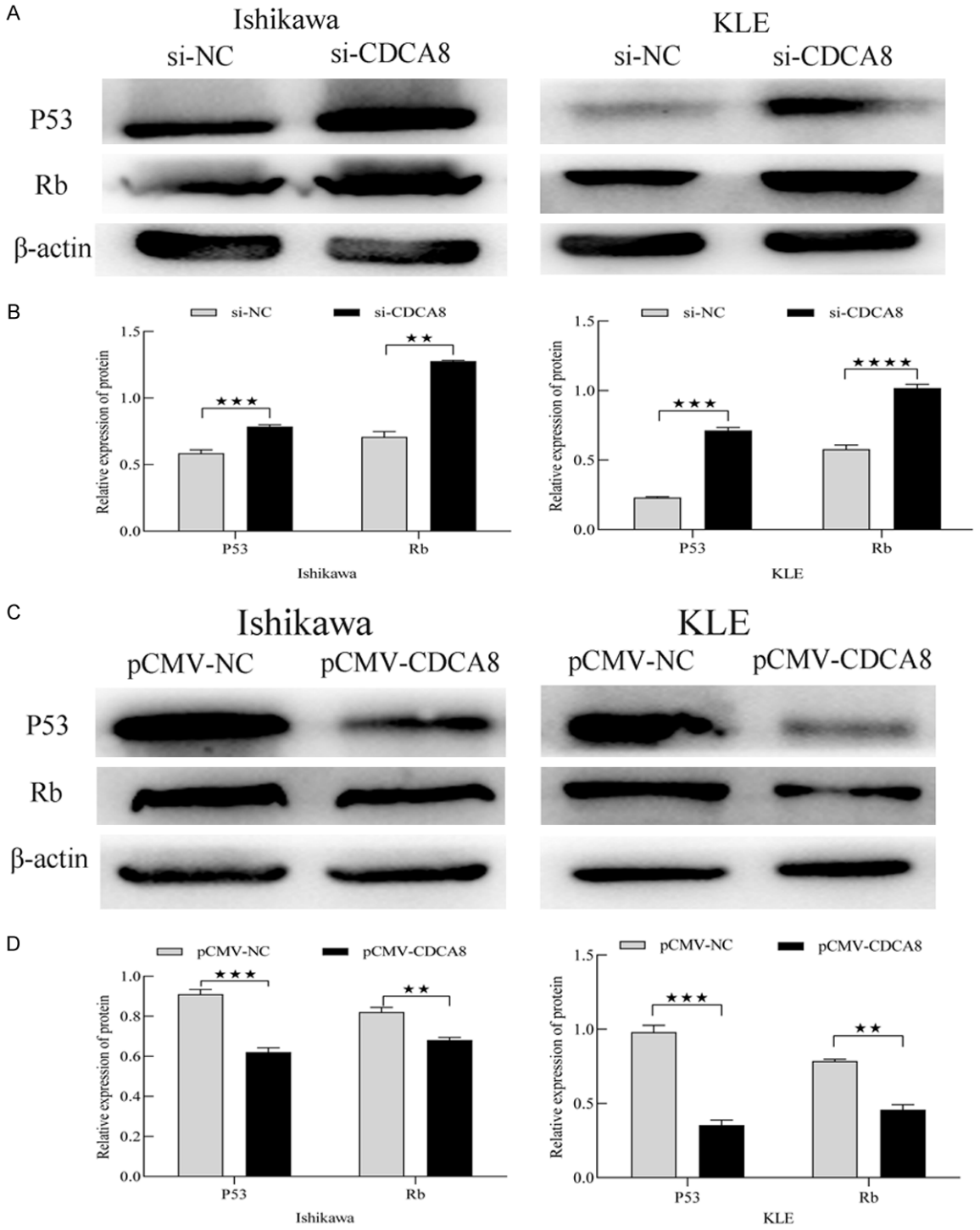


Figure 10. Effects of CDCA8 on P53/Rb signaling pathway of EC cell lines. A and B: Western blot analysis of p53 and Rb expression in Ishikawa and KLE cells with CDCA8 knockdown; C and D: Western blot analysis of P53 and Rb expression in Ishikawa and KLE cells with CDCA8 over-expression. **P<0.01, ***P<0.001, ****P<0.0001. CDCA8: cell division cycle associated 8; EC: endometrial cancer; NC: negative control; si: small interfering (RNA); pCMV: plasmid.

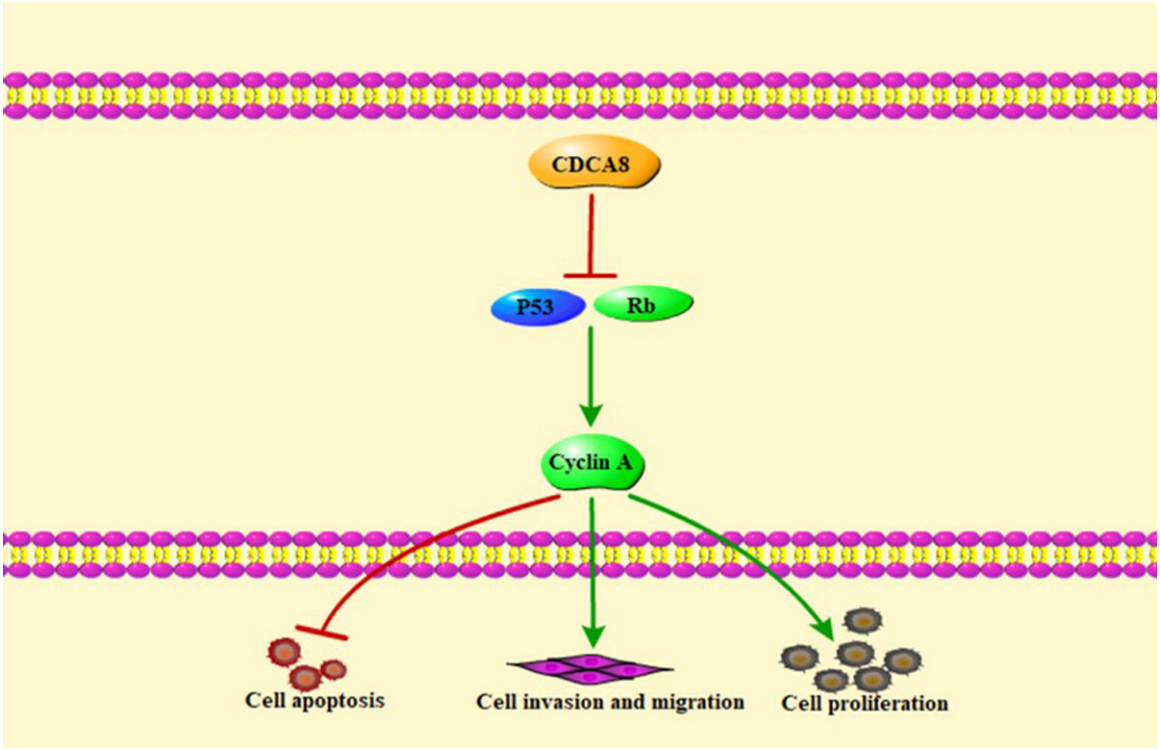


Figure 11. Schematic graph of the effect of CDCA8 on endometrial cancer cell activities.

sion of CDK or cyclin and decreased expression of CDKI, can cause cell cycle regulation disorder and excessive cell proliferation [36]. Activation of cyclin D and cyclin E promotes cell cycle from G1 phase to S phase transition. Cyclin D mainly binds to CDK4/CDK6 and mediates G1 to S phase transition. Cyclin B can promote G2 phase to M phase. Cyclin A functions in the S phase, and cyclin A acts with CDK or CDKI to regulate S phase. CDC2, also known as cyclin-dependent kinase 2 (CDK2), promotes cell from S phase to G2/M phase transition. In this study, CDCA8 knockdown resulted in increased expression of cyclin B1 and CDC2 (CDK2), and decreased expression of cyclin D1, CDK6 and cyclin A in EC samples, which are consistent with the G2/M phase arrest caused by CDCA8 knockdown in EC cells. On the contrary, CDCA8 over-expression resulted in increased expression of cyclin A in EC cells, while the expressions of cyclin B1, cyclin D1, CDC2 (CDK2) and CDK6 were not significantly changed. The expression of cyclin A increased after CDCA8 overexpression, but no changes in S phase were found after CDCA8 overexpression. The reason may be that although cyclin A functions in phase S, phase S alteration requires not only cyclin A, but also co-action with CDK and CDKI. These results imply that CDCA8 knockdown can lead to cell cycle arrest and inhibit DNA synthesis, but CDCA8 overexpression can promote DNA synthesis and cell cycle progression.

In summary, CDCA8 is over-expressed in EC and related to clinical characteristics. CDCA8 plays a role in the cell growth of EC through regulating cell cycle and P53/Rb signaling pathway (**Figure 11**), indicating that it might be a target for the treatment of EC.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.

[2] Moore K and Brewer MA. Endometrial cancer: is this a new disease? Am Soc Clin Oncol Educ Book 2017; 37: 435-442.

[3] Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM and Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 2014; 74: 2913-2921.

[4] Siegel RL, Miller KD, Fuchs HE and Jemal A. Cancer statistics, 2022. CA Cancer J Clin 2022; 72: 7-33.

[5] Makker V, Green AK, Wenham RM, Mutch D, Davidson B and Miller DS. New therapies for advanced, recurrent, and metastatic endometrial cancers. Gynecol Oncol Res Pract 2017; 4: 19.

[6] Clarke MA, Long BJ, Del Mar Morillo A, Arbyn M, Bakkum-Gamez JN and Wentzensen N. Association of endometrial cancer risk with postmenopausal bleeding in women: a systematic review and meta-analysis. JAMA Intern Med 2018; 178: 1210-1222.

[7] Arend RC, Jones BA, Martinez A and Goodfellow P. Endometrial cancer: molecular markers and management of advanced stage disease. Gynecol Oncol 2018; 150: 569-580.

[8] Aristizabal P, Graesslin O, Barranger E, Clavel-Chapelon F, Haddad B, Luton D, Darai E, Rouzier R and Koskas M. A suggested modification to FIGO stage I endometrial cancer. Gynecol Oncol 2014; 133: 192-196.

[9] Cancer Genome Atlas Research Network, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, Shen H, Robertson AG, Pashtan I, Shen R, Benz CC, Yau C, Laird PW, Ding L, Zhang W, Mills GB, Kucherlapati R, Mardis ER and Levine DA. Integrated genomic characterization of endometrial carcinoma. Nature 2013; 497: 67-73.

[10] Wenzel ES and Singh ATK. Cell-cycle checkpoints and aneuploidy on the path to cancer. In Vivo 2018; 32: 1-5.

[11] Hindriksen S, Meppelink A and Lens SM. Functionality of the chromosomal passenger complex in cancer. Biochem Soc Trans 2015; 43: 23-32.

[12] Zhang C, Zhao L, Leng L, Zhou Q, Zhang S, Gong F, Xie P and Lin G. CDCA8 regulates meiotic spindle assembly and chromosome segregation during human oocyte meiosis. Gene 2020; 741: 144495.

[13] Haase J, Bonner MK, Halas H and Kelly AE. Distinct roles of the chromosomal passenger complex in the detection of and response to errors in kinetochore-microtubule attachment. Dev Cell 2017; 42: 640-654, e5.

[14] Dai C, Miao CX, Xu XM, Liu LJ, Gu YF, Zhou D, Chen LS, Lin G and Lu GX. Transcriptional activation of human CDCA8 gene regulated by transcription factor NF-Y in embryonic stem cells and cancer cells. J Biol Chem 2015; 290: 22423-22434.

[15] Bu Y, Shi L, Yu D, Liang Z and Li W. CDCA8 is a key mediator of estrogen-stimulated cell proliferation in breast cancer cells. Gene 2019; 703: 1-6.

[16] Bi Y, Chen S, Jiang J, Yao J, Wang G, Zhou Q and Li S. CDCA8 expression and its clinical relevance in patients with bladder cancer. Medicine (Baltimore) 2018; 97: e11899.

[17] Phan NN, Wang CY, Li KL, Chen CF, Chiao CC, Yu HG, Huang PL and Lin YC. Distinct expression of CDCA3, CDCA5, and CDCA8 leads to shorter relapse free survival in breast cancer patient. Oncotarget 2018; 9: 6977-6992.

[18] Wang Y, Zhao Z, Bao X, Fang Y, Ni P, Chen Q, Zhang W and Deng A. Borealin/Dasra B is overexpressed in colorectal cancers and contributes to proliferation of cancer cells. Med Oncol 2014; 31: 248.

[19] Ci C, Tang B, Lyu D, Liu W, Qiang D, Ji X, Qiu X, Chen L and Ding W. Overexpression of CDCA8 promotes the malignant progression of cutaneous melanoma and leads to poor prognosis. Int J Mol Med 2019; 43: 404-412.

[20] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.

[21] Li WX, Zhou RQ, Liu Y and Zhang GY. Expression and clinical relevance of cell division cycle associated 8 (CDCA8) gene in endometrial cancer patients. Eur J Gynaecol Oncol 2021; 42: 278-285.

[22] Jeon TW, Ko M, Seo YR, Baik I, Hwang I, Ryoo HM, Kim JY, Park K and Lee YH. Abstract 3113: knockdown of cell division cycle-associated 8 (CDCA8) suppresses hepatocellular carcinoma growth via the upregulation of tumor suppressor ATF3. Cancer Res 2017; 77: 3113.

[23] Hu C, Wu J, Wang L, Liu X, Da B, Liu Y, Huang L, Chen Q, Tong Y and Jiang Z. miR-133b inhibits cell proliferation, migration, and invasion of lung adenocarcinoma by targeting CDCA8. Pathol Res Pract 2021; 223: 153459.

[24] Gao CT, Ren J, Yu J, Li SN, Guo XF and Zhou YZ. KIF23 enhances cell proliferation in pancreatic ductal adenocarcinoma and is a potent therapeutic target. Ann Transl Med 2020; 8: 1394.

[25] Qi G, Zhang C, Ma H, Li Y, Peng J, Chen J and Kong B. CDCA8, targeted by MYBL2, promotes malignant progression and olaparib insensitivity in ovarian cancer. Am J Cancer Res 2021; 11: 389-415.
[26] Wang X, Wang H, Xu J, Hou X, Zhan H and Zhen Y. Double-targeting CDCA8 and E2F1 inhibits the growth and migration of malignant glioma. Cell Death Dis 2021; 12: 146.
[27] Li S, Liu X, Liu T, Meng X, Yin X, Fang C, Huang D, Cao Y, Weng H, Zeng X and Wang X. Identification of biomarkers correlated with the TNM staging and overall survival of patients with bladder cancer. Front Physiol 2017; 8: 947.
[28] Liu J, Zhou S, Li S, Jiang Y, Wan Y, Ma X and Cheng W. Eleven genes associated with progression and prognosis of endometrial cancer (EC) identified by comprehensive bioinformatics analysis. Cancer Cell Int 2019; 19: 136.
[29] Gao X, Wen X, He H, Zheng L, Yang Y, Yang J, Liu H, Zhou X, Yang C, Chen Y, Chen M and Zhang S. Knockdown of CDCA8 inhibits the proliferation and enhances the apoptosis of bladder cancer cells. PeerJ 2020; 8: e9078.
[30] Li Q, Liang J and Chen B. Identification of CDCA8, DSN1 and BIRC5 in regulating cell cycle and apoptosis in osteosarcoma using bioinformatics and cell biology. Technol Cancer Res Treat 2020; 19: 1533033820965605.
[31] Shuai Y, Fan E, Zhong Q, Chen Q, Feng G, Gou X and Zhang G. CDCA8 as an independent predictor for a poor prognosis in liver cancer. Cancer Cell Int 2021; 21: 159.
[32] Jeon T, Ko MJ, Seo YR, Jung SJ, Seo D, Park SY, Park KU, Kim KS, Kim M, Seo JH, Park IC, Kim MJ, Bae JH, Song DK, Cho CH, Lee JH and Lee YH. Silencing CDCA8 suppresses hepatocellular carcinoma growth and stemness via restoration of ATF3 tumor suppressor and inactivation of AKT/β-catenin signaling. Cancers (Basel) 2021; 13: 1055.
[33] Wang X, Zhu L, Lin X, Huang Y and Lin Z. MiR-133a-3p inhibits the malignant progression of oesophageal cancer by targeting CDCA8. J Biochem 2022; 170: 689-698.
[34] Barnum KJ and O'Connell MJ. Cell cycle regulation by checkpoints. Methods Mol Biol 2014; 1170: 29-40.
[35] Gao SW and Liu F. Novel insights into cell cycle regulation of cell fate determination. J Zhejiang Univ Sci B 2019; 20: 467-475.
[36] Chong QY, Kok ZH, Bui NL, Xiang X, Wong AL, Yong WP, Sethi G, Lobie PE, Wang L and Goh BC. A unique CDK4/6 inhibitor: current and future therapeutic strategies of abemaciclib. Pharmacol Res 2020; 156: 104686.