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Cyclin D1 silencing impairs DNA double strand break repair, sensitizes BRCA1 wildtype ovarian cancer cells to olaparib

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HIGHLIGHTS

- Olaparib could reduce the expression level of cyclin D1 protein in ovarian cancer cells.
- Silencing of cyclin D1 could sensitize BRCA1 wildtype ovarian cancer cells to olaparib in vitro and in vivo.
- Silencing of cyclin D1 could mimic a BRCAness phenotype and induce G0/G1 cell cycle arrest in ovarian cancer cells.

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ABSTRACT

Objective. Poly(ADP-ribose) polymerase inhibitors (PARPi) are active in cancer cells that have impaired repair of DNA by the homologous recombination (HR) pathway. Strategies that disrupt HR may sensitize HR-proficient tumors to PARP inhibition. As a component of the core cell cycle machinery, cyclin D1 has unexpected function in DNA repair, suggesting that targeting cyclin D1 may represent a plausible strategy for expanding the utility of PARPi in ovarian cancer.

Methods. BRCA1 wildtype ovarian cancer cells (A2780 and SKOV3) were treated with a combination of CCND1 siRNA and olaparib in vitro. Cell viability was assessed by MTT. The effects of the combined treatment on DNA damage repair and cell cycle progression were examined to dissect molecular mechanisms. In vivo studies were performed in an orthotopic ovarian cancer mouse model. Animals were treated with a combination of lentivirus-mediated CCND1 shRNA and olaparib or olaparib plus scrambled shRNA. Molecular downstream effects were examined by immunohistochemistry.

Results. Silencing of cyclin D1 sensitized ovarian cancer cells to olaparib through interfering with RAD51 accumulation and inducing cell cycle G0/G1 arrest. Treatment of lentivirus-mediated CCND1-shRNA in nude mice statistically significantly augmented the olaparib response (mean tumor weight ± SD, CCND1-shRNA plus olaparib vs scrambled shRNA plus olaparib: 0.172 ± 0.070 g vs 0.324 ± 0.044 g, $P < 0.05$).

Conclusions. Silencing of cyclin D1 combined with olaparib may lead to substantial benefit for ovarian cancer management by mimicking a BRCAness phenotype, and induction of G0/G1 cell cycle arrest.

1. Introduction

Epithelial ovarian cancer has always been the most lethal malignancy of the gynecological cancers [1]. Except for improvements in surgical techniques and chemotherapeutic options, the long-term survival remains poor [2]. DNA repair-targeted therapy by poly(ADP-ribose) polymerase inhibitors (PARPi), is considered to be one of the most active and exciting new strategies for the treatment of ovarian cancer, and has demonstrated clinical activity against ovarian cancer, especially in deleterious germline BRCA mutation carriers and platinum-sensitive diseases [3]. Olaparib is the first US Food and Drug Administration-approved PARPi, licensed for use in previously treated germline BRCA mutation-associated ovarian cancer [4]. Despite the promising clinical results observed thus far for ovarian cancer, there have been barriers to clinical development of PARPi such as de novo and acquired homologous recombination (HR) proficiency [5–7]. A large portion of tumors

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are HR-proficient, which suggests that many ovarian cancer patients may not benefit from this drug [8–10]. In this case, drug combination approaches have been designed and evaluated in preclinical and early clinical trials.

PARP is a family of proteins involved in various cellular processes including chromatin organization, transcription, DNA repair, genomic integrity, differentiation, proliferation, cell death, and cancer progression. A recent report showed that inhibition of PARP-1(the founding member of the PARP enzyme family) could result in reduced level of cyclin D1 protein [11], and olaparib could decrease the expression of cyclin D1 in breast cancer cells [12]. No evidence has been collected yet on the relationship between cyclin D1 and PARPi's sensitivity in ovarian cancer.

Cyclin D1 is a product of the CCND1 gene that is often amplified in several human cancers, including ovarian cancer [13]. Overexpression of cyclin D1 was previously observed in 14–89% of ovarian cancer cases [14–16], and was an independent prognostic indicator of overall survival in patients with ovarian cancer [17,18]. Cyclin D1 is known to play a pivotal role in the regulation of progression from the G1 to S phase of the cell cycle. It is generally considered that accurate repair by HR is restricted to the S and G2 phases of the cell cycle [19,20]. A proteomic screen for cyclin D1 protein partners, performed in different types of human tumors, has demonstrated that cyclin D1 plays a kinase-independent function in DNA repair and interacts with proteins implicated in DNA repair machinery such as RAD51 [21].

We hypothesized that PARPi-induced cytotoxicity may be augmented by inhibition of cyclin D1. This study evaluated the preclinical efficacy of silencing CCND1 in combination with olaparib in BRCA1 wildtype ovarian cancer cells. Our results indicate that cyclin D1 silencing impairs double-strand DNA damage repair and robustly sensitized ovarian cancer cells to PARP inhibition in vitro and in vivo.

2. Materials and methods

2.1. Cell culture and reagent

BRCA1 wildtype A2780 and SKOV3 cell lines [22] were kind gifts from Prof. Yuquan Wei of Sichuan University. Cells were cultured as previously described [1,23]. The cells we used were 3 passages since origin. Olaparib was from Sigma-Aldrich (MCE, USA).

2.2. MTT assay and cell cycle analysis

For olaparib treatment, the cells were treated with a titration of olaparib for 5 days, with the medium and drug were replenished at day 3. Cells transfected with indicated plasmids or siRNAs were incubated overnight at 37 °C, then treated with a titration of olaparib for 5 days, with the medium and olaparib were replenished at day 3. Cell viability was estimated using the MTT reagent (Sigma Chemical, St. Louis, MO). For IC50 assay, the concentration of olaparib was 2, 4, 8, 16, 32, 64, 128, 256, 512 (μM). Cell survival was calculated by normalizing the absorbance to that of untreated controls. Cell cycle analyses were carried out by flow cytometric analysis using PI staining method, as previously described [1].

2.3. Plasmids and transfection

Sequence encoding human CCND1 was amplified from cDNA production of SKOV3 cells, then inserted into *Nhe*I/*Xba*I-digested pcDNA3.1 plasmid. Small interfering RNA (siRNA) directed against homo CCND1 was designed and synthesized by GenePhama (Shanghai, China). The scrambled and CCND1-shRNA were synthesized according to siRNA sequence and connected to pLKO.1 plasmid. CCND1-shRNA pLKO.1 plasmid was constructed as previously described [24]. For cell transfection, pcDNA3.1 or pcDNA3.1-CCND1 plasmid, scrambled or CCND1-shRNA pLKO.1 plasmid was transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols. Negative control and CCND1 siRNAs were transfected using Liopfectamine RNAiMAX regent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 20 nM in 6-well plates according to the manufacturers' protocols. Total RNA and protein were extracted 48 h after transfection.

2.4. Quantitative real-time polymerase chain reaction analysis (qRT-PCR)

Total RNA was isolated with a miRNeasy mini kit (Qiagen). The concentration and purity of RNA were determined by measuring the absorbance at 260 and 280 nm in NanoDrop. Reverse transcription was performed using RevertAid First strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol. Reverse transcription of mRNA was carried out for 10 min at 70 °C, 5 min at 4 °C, 60 min at 42 °C, and 5 min at 85 °C. PCR was followed by 40 cycles (10 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C for cyclinD1 and GAPDH). Real-time polymerase chain reaction (RT-PCR) assays were performed using LightCycler 480 SYBR Green I Master (Roche). GAPDH was used for normalization controls. Data were analyzed by using the $2^{-\Delta\Delta ct}$ method.

2.5. Western blot analysis

Cells or tissues used for western blot were treated with RIPA Lysis Buffer, and protein concentrations were quantitated by BCA assay kit (Beyotime, Shanghai, China). Primary GAPDH antibody (mouse) (ata10168, AtaGenix) and Rabbit polyclonal CCND1 antibody (ATApla7592, AtaGenix) were purchased from Atagenix Laboratory. In brief, 20 μg of whole-cell lysate from each sample was loaded onto a 12% polyacrylamide gel for electrophoresis and transferred to NC membrane. The membrane was blocked by 5% nonfat milk in 1 × Tris-buffered saline solution (pH 7.6) containing 0.05% Tween-20 and probed with primary antibodies at a concentration of 1:3000 (for GAPDH) or 1:1000 (for CCND1). The secondary antibodies were used at a concentration of 1:5000. The proteins were visualized using a Western Bright ECL detection kit (K-12043-D10, Junengbio) and analyzed by the chemiluminescence imaging analysis system (Bio-Rad, ChemiDocXRS+, USA). The density of specific bands was quantified using Image J software.

2.6. Immunofluorescence assay

Sections were fixed with 4% paraformaldehyde for 20 min, blocked with 3% bovine serum albumin containing 0.1% Triton X-100 for 30 min, and incubated with antibodies against RAD51 (ab133534; Abcam; dilution 1:300) or γ-H2AX (ab26350; Abcam; dilution 1:300) for 2 h at room temperature. The sections were then incubated with species-specific Alexa 488 (Proteintech; dilution 1:100) conjugated secondary antibody for one hour at room temperature. Nuclei were counterstained with 4′ 6-diamidino-2-phenylindole (DAPI). Phase images were captured by a Nikon C2-ER confocal microscope system at a magnification of 1380×. The fluorescence images were captured using a Nikon C2-ER GaAsP imaging system.

2.7. Recombinant lentivirus particles production

Recombinant lentivirus particles were produced as previously described [25]. Briefly, 40%–60% percent confluent HEK 293T cells in 10 cm cell culture dishes were co-transfected with 15 μg pLKO.1 and 12 μg helper plasmid deta8.9 and 8 μg pVSVG, 48 and 72 h later, the supernatant medium was harvested separately and centrifuged at 3000 rpm for 30 min and filtered through a 0.45 μm filter to get the products. The lentivirus was concentrated using a Lentivirus Concentration Kit (BioGeek, Beijing China) according to the manufacturer's protocols.

2.8. In vivo animal treatment

Our research procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University and were in compliance

with all regulatory guidelines. Female athymic nude mice (BALB/c, 6–8 weeks old, nonfertile, 18–20 g each) were used to establish the intraperitoneal carcinomatosis model. SKOV3 ovarian cancer cells were administered intraperitoneally as previously described [23]. One week after tumor cell injection, mice were randomly separated into four treatment groups ($n = 5$ mice per group): (a) Control, 100 μl of 5% glucose solution treated; (b) lentivirus-mediated scrambled shRNA (100 μl/20 g) treated; (c) olaparib (50 mg/kg) + lentivirus-mediated scrambled shRNA (100 μl/20 g) treated; and (d) olaparib + lentivirus-mediated CCND1 shRNA (100 μl/20 g) treated. All groups received intraperitoneal administration of drugs. Lentivirus-mediated shRNA was given every other day and olaparib was solubilized as previously described [26] and given every day for four weeks. When treatment was over, the mice were killed and tumors were harvested. Tumor weights, numbers, and locations were recorded.

2.9. Immunohistochemistry assay

This was down as previously described [1]. Reagents were as follows: rabbit anti-human monoclonal antibody against CCND1 (ab134175, Abcam; dilution 1:100), rabbit anti-human monoclonal antibody against RAD51 (ab133534, Abcam; dilution 1:300), mouse anti-human monoclonal antibody against γ-H2AX (ab26350; Abcam; dilution 1:300) and enVision + System HRP rabbit (KIHC-5, Proteintech). A minimum of 3 slides per group and 3 high power fields and 3 low power fields per slide were evaluated. Scale bar = 20 μM, *** $P < 0.001$. Data represented the mean ± SD from three independent experiments.

2.10. Statistical analysis

Data were means ± standard deviation of at least three independent experiments. The two-sided Student's *t*-test was used for comparisons of two independent groups. *P* value of <0.05 was defined as statistically significant. All statistical analyses were done using SPSS 17.0 (SPSS Inc., Chicago, IL) and R 2.10.0.

3. Results

3.1. Olaparib treatment decreased CCND1 expression and increased the proportion of ovarian cancer cells in G0/G1 phase

A2780 and SKOV3 ovarian cancer cells were treated with olaparib for 5 days, MTT assay was conducted. Results showed that cell survival rate decreased in a dose-dependent manner (Fig. 1A). We chose the

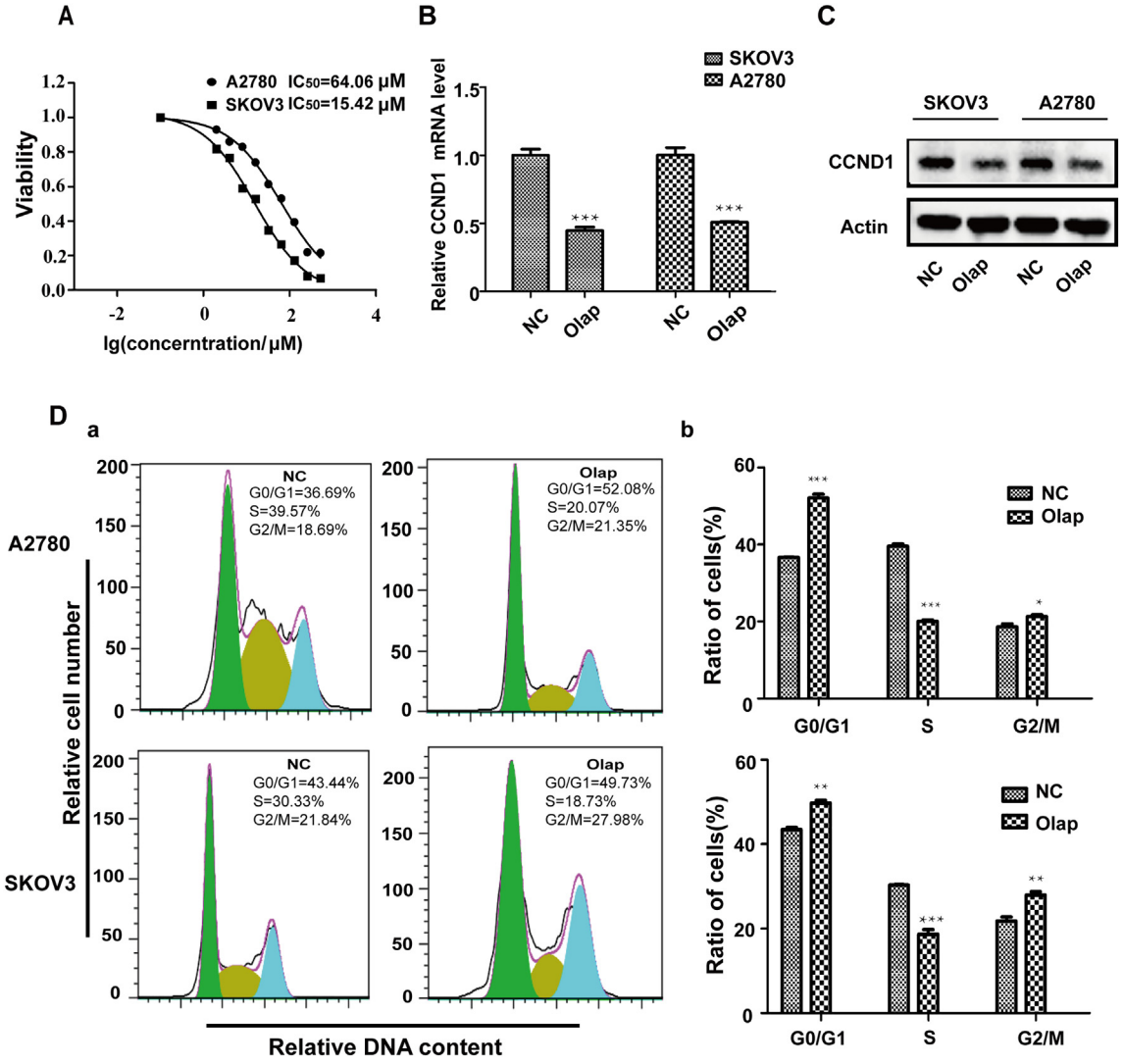

Fig. 1. Olaparib treatment decreased CCND1 expression and increased the proportion of ovarian cancer cells in G0/G1 phase. A) Cell viability was analyzed by MTT assay. B and C) A2780 and SKOV3 cells were treated with IC50 doses of olaparib for 5 days. The expression of CCND1 mRNA and cyclin D1 protein level in A2780 and SKOV3 cells was analyzed by RT-qPCR and western blot after olaparib treatment at IC50 concentrations. D) A2780 and SKOV3 cells were treated with olaparib at IC50 concentrations for 5 days, cell cycle analyses were conducted by flow cytometry. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-sided Student's *t*-test.

IC50s of both cell lines (A2780 was 64.06 μM and SKOV3 was 15.42 μM) for further study. A2780 and SKOV3 cells were treated with olaparib (64 μM or 15 μM) or saline for five days, respectively. RT-qPCR found that olaparib treatment decreased CCND1 expression in both cell lines (Fig. 1B). Western blot (WB) analysis of collected cell extracts also showed that olaparib was efficient in reducing expression of CCND1 in both cell lines (Fig. 1C). Cell cycle analyses showed that olaparib treated cells were blocked at G1/G0 phase, and concomitantly the percentage of S-phase cells was reduced (Fig. 1D). For the ratio of G1/G0 phase cells, NC group vs olaparib group was 36.69% ± 0.09% vs 52.08% ± 0.98 ($P < 0.001$) in A2780 cells and 43.44% ± 0.49% vs 49.74% ± 0.65 ($P < 0.01$) in SKOV3 cells. Furthermore, both SKOV3 and A2780 cells treated with olaparib showed, to a variable extent, an increase in G2-phase when compared with untreated cells. This increase in G2-phase may suggest that these cells manifest a G2 delay-like state reflecting replicative stress caused by olaparib. The same as in the previous report [27], cell-cycle analysis revealed that olaparib treatment could not statistically significantly increase sub-G1 cell subpopulation, suggesting that cells were not going through apoptosis (data not shown).

3.2. Knockdown or overexpression of CCND1 changed olaparib sensitivity in vitro

pcDNA3.1-CCND1 plasmid and siRNA targeting CCND1 were constructed to over express or knock down CCND1 gene in vitro. RT-qPCR and western blot assays indicated that SKOV3 cells transfected with pcDNA3.1-CCND1 or CCND1-siRNAs resulted in enhanced or decreased expression of CCND1 compared with the controls (Fig. 2A–B). siRNA3 was the most effective among the three siRNAs and was chosen for further study. To determine the effect of CCND1 on ovarian cancer cell proliferation, A2780 and SKOV3 cells were transfected with pcDNA3.1, pcDNA3.1-CCND1, NC siRNA, or CCND1 siRNA3, and 24 h later were incubated with olaparib for 5 days at the indicated concentrations (A2780 was 0, 16, 32, 64,128 μM, and SKOV3 was 0, 4, 8, 16, 32 μM). MTT assay

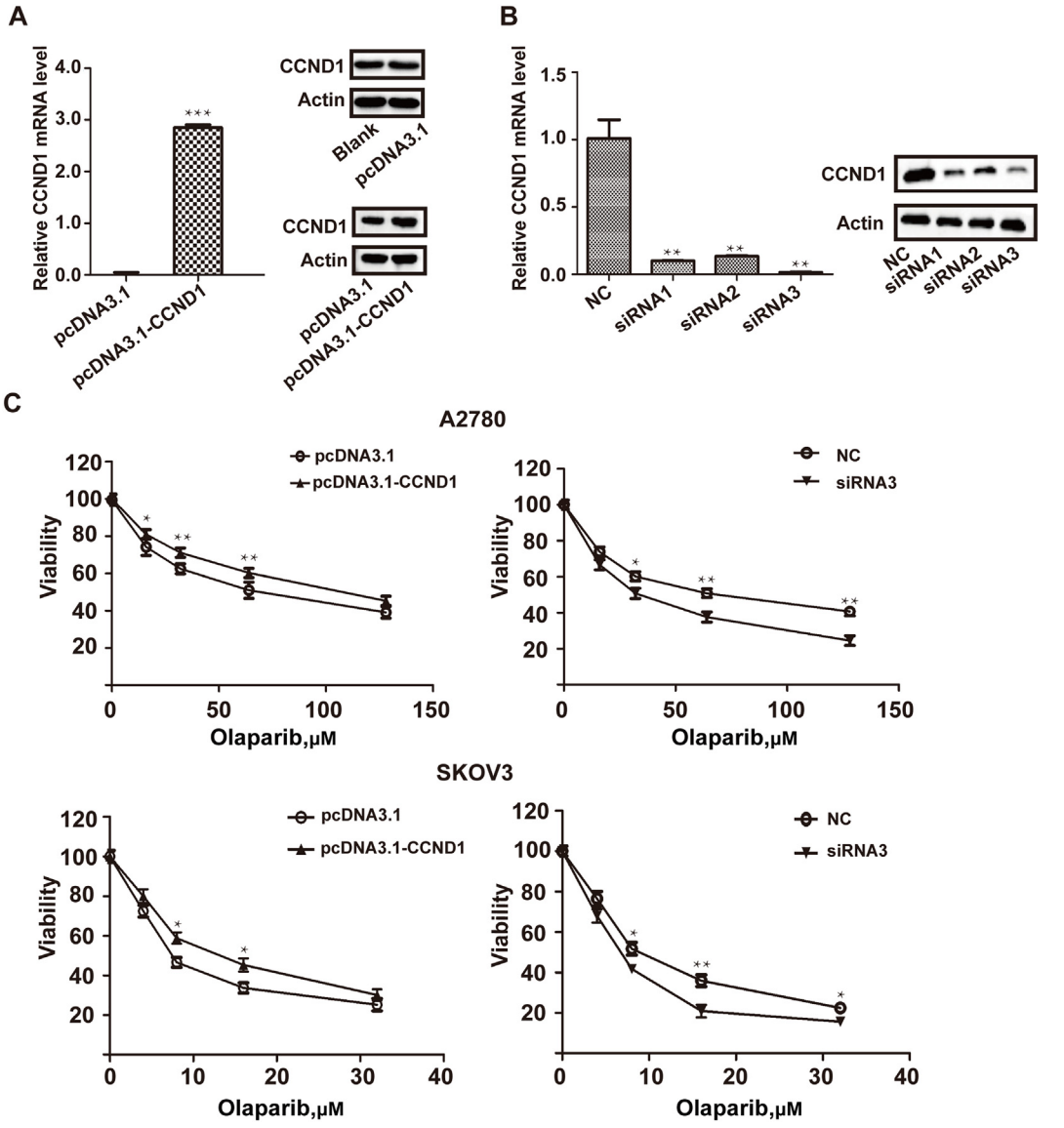

Fig. 2. Overexpression or knockdown of CCND1 changed olaparib's sensitivity in A2780 and SKOV3 cells. A) SKOV3 cells were transfected with pcDNA3.1 or pcDNA3.1-CCND1 for 48 h respectively. The expression of CCND1 mRNA and cyclin D1 protein level were analyzed by RT-qPCR and western blot. B) SKOV3 cells were transfected with NC siRNA or three siRNA targeting CCND1 for 48 h respectively. The expression of CCND1 mRNA and cyclin D1 protein level were analyzed by RT-qPCR and western blot. C) A2780 and SKOV3 cells were transfected with pcDNA3.1, pcDNA3.1-CCND1, NC siRNA, or CCND1 siRNA3 and 24 h later treated with olaparib for 5 days at indicated concentrations. The effects of CCND1 on olaparib treated A2780 and SKOV3 cell proliferation were analyzed by MTT assay. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-sided Student's *t*-test.

was used to validate changes of olaparib sensitivity. As expected, CCND1 siRNA treated A2780 and SKOV3 ovarian cancer cells were more sensitive to olaparib than the control siRNA. Conversely, A2780 and SKOV3 cells transfected with pcDNA3.1-CCDN1 plasmid gained resistance to olaparib compared with pcDNA3.1 empty plasmid transfected cells (Fig. 2C).

3.3. Knockdown or overexpression of CCND1 affected cell cycle in olaparib treated cells

To clarify the mechanisms underlying the effect of CCND1 on olaparib sensitivity, A2780 and SKOV3 ovarian cancer cells were transfected with pcDNA3.1 plasmid, pcDNA3.1-CCND1 plasmid, NC siRNA, or CCND1 siRNA3, and 24 h later were incubated with olaparib for 5 days at IC50 concentrations. Cell cycle analyses showed that silencing of cyclin D1 significantly increased the proportion of olaparib treated cancer cells in G0/G1 phase (NC vs siRNA was 54.10 ± 0.58% vs 64.05% ± 0.19% in A2780 cells, $P < 0.001$; and 53.97% ± 0.99% vs 64.57% ± 0.54% in SKOV3 cells, $P < 0.001$) and decreased cancer cells in S phase (NC vs siRNA was 22.96 ± 0.58% vs 17.89% ± 0.40% in A2780 cells, $P < 0.01$; and 21.70% ± 0.39% vs 14.55% ± 0.46% in SKOV3 cells, $P < 0.001$). Conversely, the G0/G1 phase of olaparib treated cells significantly decreased when CCND1 gene was overexpressed (pcDNA3.1 vs pcDNA3.1-CCND1 was 54.12% ± 0.45% vs 46.92% ± 0.40% in A2780 cells, $P < 0.001$; and 54.40% ± 0.50% vs 48.15% ± 0.60% in SKOV3 cells, $P < 0.01$). However, only olaparib treated SKOV3 cells showed significant increase in S phase cells (pcDNA3.1 vs pcDNA3.1-CCND1 was 21.87% ± 0.97% vs 26.05% ± 0.80%, $P < 0.05$) when CCND1 gene was overexpressed (Fig. 3A–B).

3.4. Knockdown or overexpression of CCND1 affected homologous recombination-mediated repair in olaparib treated cells

To assess whether silencing of cyclin D1 sensitized ovarian cancer cells to olaparib through promoting DNA damage and impairing HR-mediated DNA repair, pcDNA3.1, pcDNA3.1-CCND1, NC siRNA, or CCND1 siRNA3 transfected A2780 and SKOV3 cells were treated with olaparib for 5 days at IC50 concentrations, and the number of γH2AX and RAD51 foci inside the cells were examined. γ-H2AX is a biomarker for DNA double-strand breaks, while RAD51 plays a vital role in HR during double strand break repair [28–30]. Knockdown of CCND1 plus olaparib treatment led to more γ-H2AX formation (NC vs siRNA was 28.13% ± 0.92% vs 40.37% ± 1.21% in A2780 cells, $P < 0.01$; and 27.44% ± 1.16% vs 44.15% ± 2.04% in SKOV3 cells, $P < 0.01$) and less RAD51 foci accumulation (NC vs siRNA was 26.21% ± 1.18% vs 15.97% ± 0.90% in A2780 cells, $P < 0.01$; and 23.63% ± 1.04% vs 13.36% ± 0.51% in SKOV3 cells, $P < 0.01$) than NC siRNA plus olaparib treatment, indicating the defects in DNA repair function. On the other hand, overexpression of CCND1 by transfecting pcDNA3.1-CCDN1 plasmid together with olaparib treatment led to less γ-H2AX formation (pcDNA3.1 vs pcDNA3.1-CCND1 was 27.64% ± 0.89% vs 21.60% ± 1.89% in A2780 cells, $P < 0.05$; and 30.32% ± 1.08% vs 17.24% ± 1.66%

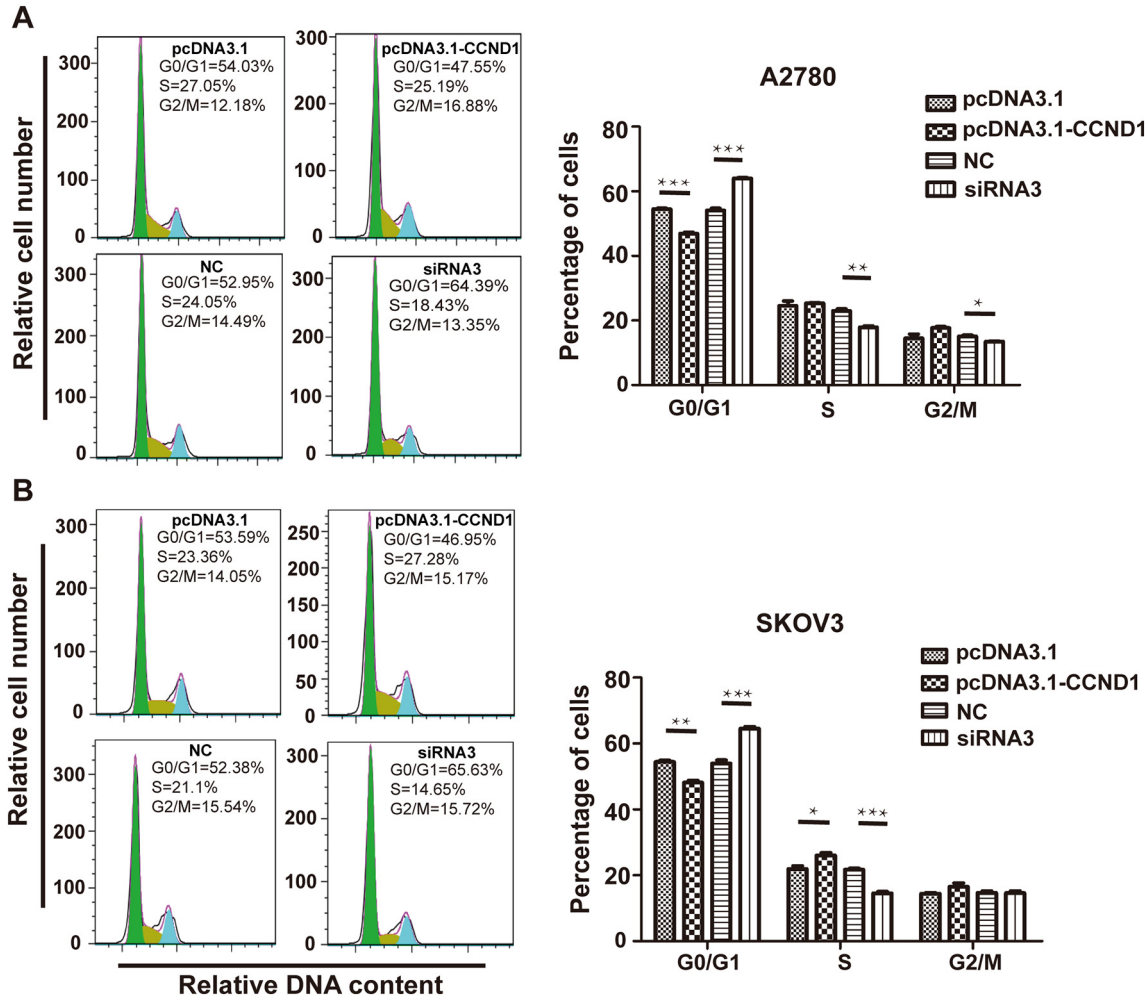

Fig. 3. Overexpression or knockdown of CCND1 affected cell cycle in olaparib treated cells. A2780 and SKOV3 cells were transfected with pcDNA3.1, pcDNA3.1-CCND1, NC siRNA, or CCND1 siRNA3 and 24 h later treated with olaparib for 5 days at IC50 concentrations. Cell cycle analyses were conducted by flow cytometry. A) Results for A2780 cells, B) Results for SKOV3 cells. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-sided Student's *t*-test.

in SKOV3 cells, $P < 0.01$) and more RAD51 foci accumulation (pcDNA3.1 vs pcDNA3.1-CCND1 was 26.02% ± 1.33% vs 37.9% ± 1.69% in A2780 cells, $P < 0.01$; and 25.63% ± 1.13% vs 38.79% ± 2.18% in SKOV3 cells, $P < 0.01$) than control plasmid plus olaparib treatment, indicating the rescue of DNA repair ability in ovarian cancer cells (Fig. 4).

3.5. Effect of cyclin D1 on sensitivity to olaparib in ovarian cancer in vivo

To further assess the effect of CCND1 on olaparib sensitivity, we tested the therapeutic efficacy of lentivirus-mediated CCND1 shRNA and olaparib combined treatment in an established ovarian cancer model. SKOV3 cells infected with scrambled lentivirus or CCND1 shRNA lentivirus were analyzed by RT-qPCR and WB to confirm the shRNA efficacy before in vivo test. Relative CCND1 mRNA level in treatment groups reduced to 36%, and the corresponding protein level reduced to 48% of the control level, respectively (Fig. 5A). We then administered SKOV3 cells intraperitoneally to set up an intraperitoneal ovarian carcinomatosis model in nude mice. Intraperitoneal treatment groups were as follows: (a) Control; (b) scrambled shRNA lentivirus treated; (c) olaparib + scrambled shRNA lentivirus treated; and (d) olaparib + CCND1-shRNA lentivirus treated. Compared to the scrambled shRNA treated group (mean tumor weight = 0.964 ± 0.239 g), we observed statistically significant reduction in tumor burden in the group treated with olaparib + scrambled shRNA (mean tumor weight = 0.324 ± 0.044 g, $P < 0.01$). However, a marked reduction of tumor weight was observed in the group treated with combination of CCND1-shRNA and olaparib (mean tumor weight = 0.172 ± 0.070 g, $P < 0.05$) compared to the olaparib + scrambled shRNA treatment. There is no statistically significant difference in tumor burden between scrambled shRNA treated mice (mean tumor weight = 0.964 ± 0.239 g) and control mice (mean tumor weight = 0.712 ± 0.093 g, $P > 0.05$) (Fig. 5B–C). These results showed that knockdown of CCND1 may serve as a potential olaparib sensitizer for ovarian cancer. Cyclin D1 expression was tested again in tumor samples by RT-qPCR, WB and immunohistochemistry assay. Results showed that CCND1 expression was substantially decreased in CCND1-shRNA and olaparib co-treated tumors, compared to tumors in olaparib + scrambled shRNA treated group (Fig. 5D–F). Furthermore, RAD51 and γ-H2AX expression was examined by immunohistochemistry assay in samples of SKOV3 tumors. Consistent with in vitro results, CCND1 shRNA and olaparib co-treated tumors showed lower RAD51 expression (Average IHC score = 1.67) and higher expression of γ-H2AX (Average IHC score = 5.33) compared with olaparib + scrambled shRNA treatment, (Average IHC score = 7.67 for RAD51, $P < 0.05$; Average IHC score = 1.33 for γ-H2AX, $P < 0.05$, respectively) (Fig. 6A–B). Together, these data suggest a greater sensitivity to olaparib upon RAD51 downregulation mediated by knockdown of CCND1 in vivo.

4. Discussion

The emergence of the DNA repair pathway as a rational target in various cancers led to the development of the PARPi [31]. Of the many targeted therapies currently under evaluation, PARPi is one of the most promising strategies developed thus far for ovarian cancer. Future development of PARPi will need further studies to better understand which combination of treatment strategies potentiates PARP inhibitor's antitumor activity [32]. In this regard, several approaches have been evaluated in preclinical and early clinical trials, such as combinations of PARPi with epigenetic drugs [33] or PI3K (phosphatidylinositol 3-kinase)/AKT inhibitors [34] or suppressing of RAD51 foci formation [35], etc.

Dysregulation of normal cell cycle control has been implicated in the pathogenesis of most human cancers. Cyclin D1 is one of the most frequently altered cell cycle regulating proteins in cancers and, therefore, is a potential therapeutic target [36]. It has been reported that olaparib reduces S-phase cells of human osteosarcoma, resulting in a dramatic decrease in homologous DNA damage repair (HDR) activity [27], and olaparib could also decrease the expression of cyclin D1 in breast cancer

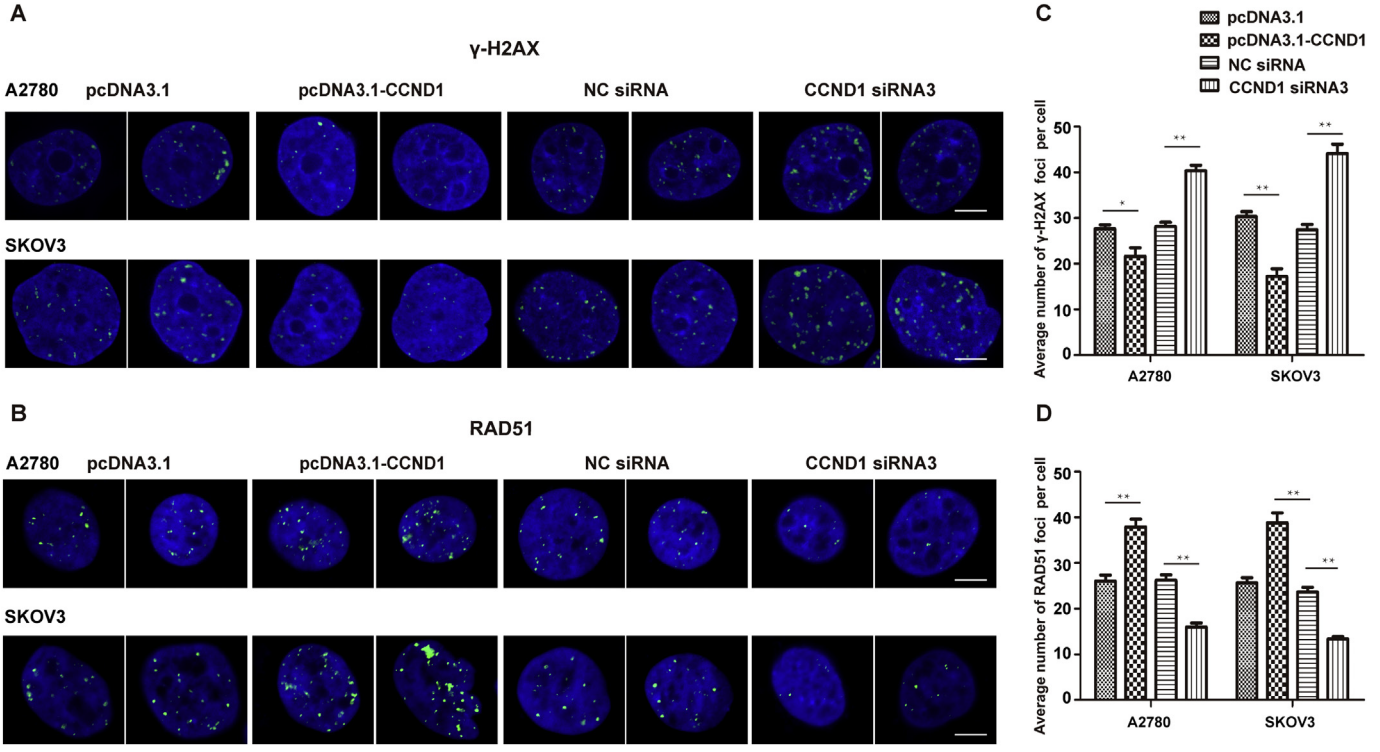

Fig. 4. Effects of overexpression or knockdown of CCND1 on DNA double-strand break repair after olaparib treatment. A2780 and SKOV3 cells were transfected with pcDNA3.1, pcDNA3.1-CCND1, NC siRNA, or CCND1 siRNA3 and 24 h later treated with olaparib for 5 days at IC50 concentrations. A and B) The number of γ-H2AX and RAD51 foci were examined by laser confocal fluorescence microscope. C and D) Quantification of the number of γ-H2AX and RAD51 foci in A2780 and SKOV3 cells in treatment groups, scale bar = 13 μm. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-sided Student's *t*-test.

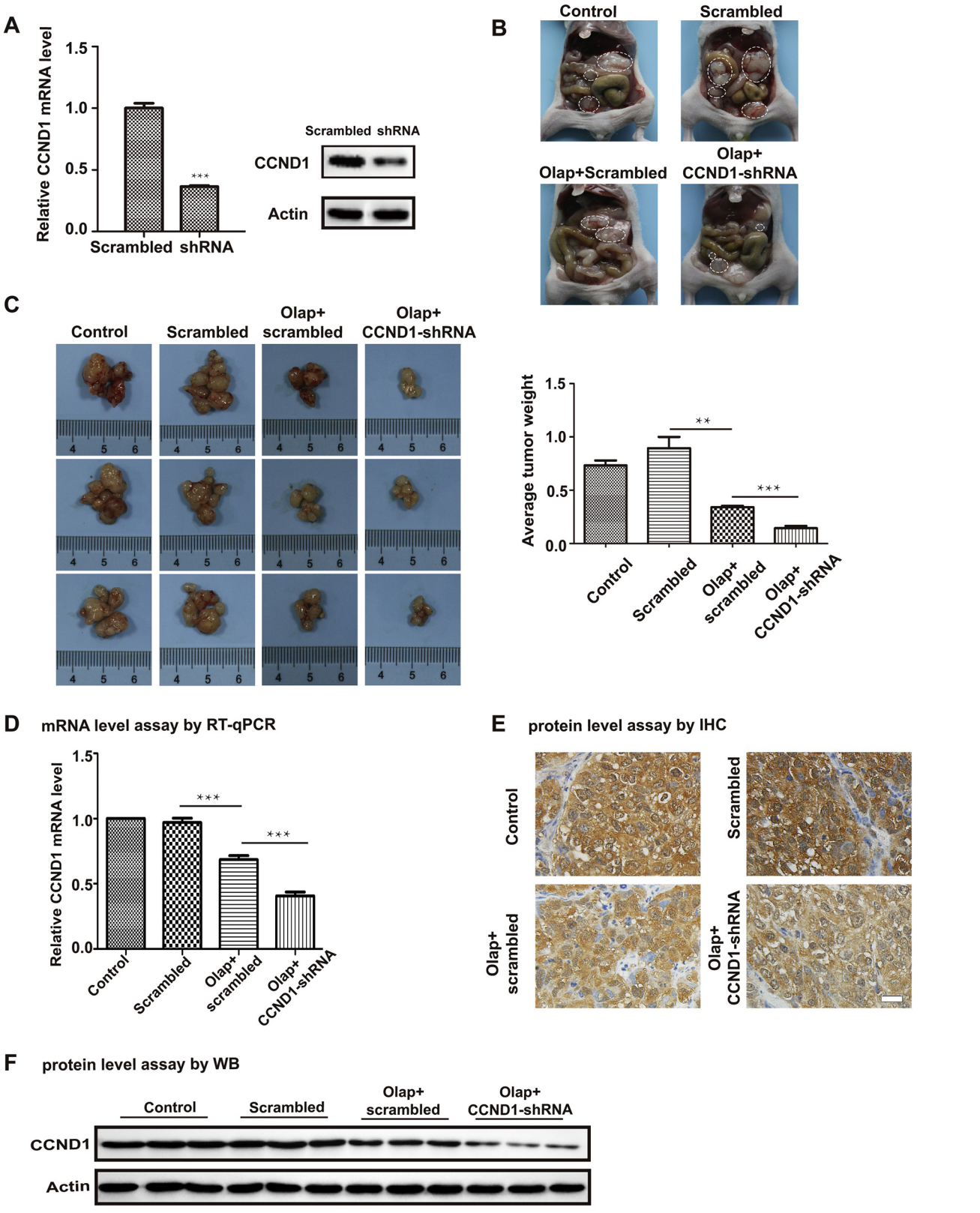

Fig. 5. Effect of cyclin D1 on sensitivity to olaparib in ovarian cancer in vivo. A) SKOV3 cells were infected with scrambled shRNA or CCND1-shRNA lentivirus. Relative CCND1 mRNA and cyclin D1 protein level were analyzed by RT-qPCR and western blot. B and C) Nude mice were treated with 5% glucose solution (control), scrambled shRNA lentivirus, olaparib + scrambled shRNA lentivirus, or olaparib + CCND1-shRNA lentivirus, respectively. Images of the representative gross tumors and tumor weight from different treatment groups are shown. D) Relative CCND1 mRNA level in tumor samples of different treatment groups were analyzed by RT-qPCR. E) Immunohistochemistry for CCND1 was performed in tumor samples, scale bar = 20 μm. F) CCDN1 protein level in tumor samples of different treatment groups were analyzed by western blot. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$ ** $P < 0.01$, * $P < 0.05$, one way ANOVA.

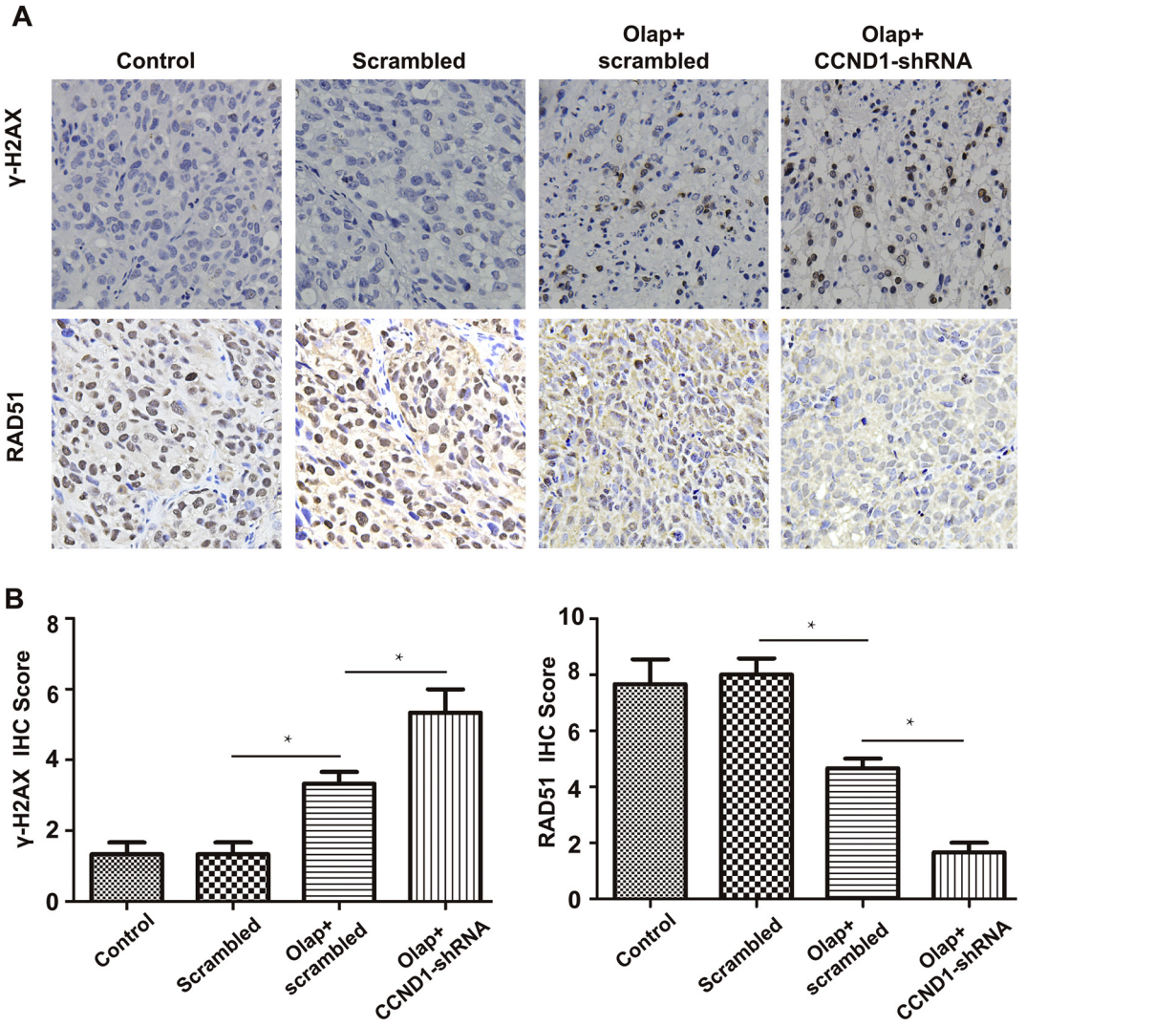

Fig. 6. Immunohistochemistry of RAD51 and γ-H2AX in samples of SKOV3 tumors. A) Representative sections were taken from tumor tissue of 5% glucose solution treated (control), scrambled shRNA treated, olaparib + scrambled shRNA treated, or olaparib + CCND1-shRNA treated mice. B) Expression of RAD51 and γ-H2AX proteins were calculated as immunohistochemical staining scores (0–9), i.e. scores equaled to the intensity of the signal (0, negative; 1, light yellow; 2, light brown; 3, dark brown) multiply the percentage of positive cells (0, no signal, 0%; 1, weak signal, <25%; 2, intermediate signal, 25–50%; and 3, strong signal, >50%). Scale bar = 20 μm. Data represented the mean ± SD of three independent experiments. *** $P < 0.001$ ** $P < 0.01$, * $P < 0.05$, one way ANOVA.

cells [12]. In this study, we demonstrated that olaparib decreased cyclin D1 expression, increased the proportion of ovarian cancer cells in G0/G1 phase and decreased cancer cells in S phase, which is consistent with previously published data. Our in vitro study showed that knockdown of CCND1 could sensitize BRCA1 wildtype SKOV3 and A2780 ovarian cancer cells to olaparib, while overexpression of CCND1 reduced olaparib's sensitivity. Moreover, silencing of cyclin D1 could significantly increase the proportion of olaparib treated SKOV3 and A2780 ovarian cancer cells in G0/G1 phase, and reduce S phase cells. Conversely, the proportion of olaparib treated cells in G0/G1 phase was significantly decreased by overexpression of cyclin D1. Consistent with the results of in vitro experiments, knockdown of CCND1 by lentivirus-mediated CCND1-shRNA effectively enhanced the effect of olaparib in an orthotopic ovarian cancer model and resulted in statistically significantly lower tumor weight than olaparib treatment alone.

There is a widespread agreement that PARPi can affect BRCA-related cancers by using the inherent HR defectiveness of the tumors in a synthetically lethal manner [37,38]. Cancer cells deficient in HR also showed sensitivity to PARP inhibitors [35]. Strategies that disrupt HR in cancer cells may sensitize HR-proficient tumors to PARP inhibition. Moreover, acquired HR proficiency is one of the most common mechanisms for PARPi resistance [39]. HR is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA, known as double-strand breaks (DSB). RAD51 plays a critical role in this pathway, and the accumulation of activated H2AX protein was an indication of DNA-DSBs damage [30]. Several studies have reported correlation between cyclin D1 overexpression and perturbation of the DNA repair machinery, such as cyclin D1 plays a kinase-independent function in DNA repair and increases sensitivity of pRB-negative cancer cells to radiation [21], silencing of CCND1 sensitized androgen independent prostate cancer cells to radiation through impairing DNA repair [40]. Consistent with previous studies, our in vitro and in vivo study showed that sensitization of olaparib induced by silencing of cyclin D1 depended on increased accumulation of DNA-DSBs damage, as suggested by the accumulation of activated H2AX protein and decreased RAD51 protein level.

Our data support the idea that silencing of cyclin D1 combined with PARPi may lead to substantial benefit for ovarian cancer management by mimicking a BRCAness phenotype and induction of G0/G1 cell cycle arrest. But there are still many issues that need to be further investigated, such as the mechanisms by which cyclin D1 influences the RAD51 protein level and the significance of the physical interaction

between cyclin D1 and other members of DNA-DSBs repair machinery. Despite these limitations, this is the first report demonstrating the therapeutic potential of cyclin D1 targeted therapy in combination with olaparib in ovarian cancers that carry the wildtype BRCA1 gene. The combination treatment showed decreased tumor burden and cell viability, arrested cell cycle and impaired DNA repair ability.

This study provides new expectation for the development of targeted personalized cancer therapy and expands the number of patients that could benefit from PARP inhibitors. The next step would be to establish methods that can target cyclin D1 directly and effectively.

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Contribution of the authors

Dr. Yang participated in the study design, supervised students in the lab, and edited the manuscript. Dr. Hu participated in the study design and interpretation of results. Dr. Li Qiao conducted literature search. Dr. Li Jinke did statistical analysis. Dr. Yi edited the manuscript. I conceptualized the study, conducted the experiments, interpreted results and drafted the manuscript. All authors read and approved the final manuscript and have contributed to it in a meaningful way.

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