Dual-regulated oncolytic adenovirus carrying *ERCC1*-siRNA gene possesses potent antitumor effect on ovarian cancer cells

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Abstract. Ovarian cancer is a multifactorial and deadly disease. Despite significant advancements in ovarian cancer therapy, its incidence is on the rise and the molecular mechanisms underlying ovarian cancer invasiveness, metastasis and drug resistance remain largely elusive, resulting in poor prognosis. Oncolytic viruses armed with therapeutic transgenes of interest offer an attractive alternative to chemical drugs, which often face innate and acquired drug resistance. The present study constructed a novel oncolytic adenovirus carrying *ERCC1* short interfering (si)RNA, regulated by *hTERT* and *HIF* promoters, termed Ad-siERCC1. The findings demonstrated that this oncolytic adenovirus effectively inhibits the proliferation, migration and invasion of ovarian cancer cells. Furthermore, the downregulation of *ERCC1* expression by siRNA ameliorates drug resistance to cisplatin (DDP) chemotherapy. It was found that Ad-siERCC1 blocks the cell cycle in the G_1 phase and enhances apoptosis through the PI3K/AKT-caspase-3 signaling pathways in SKOV3 cells. The results of the present study highlighted the critical effect of oncolytic virus Ad-siERCC1 in inhibiting the survival of ovarian cancer cells and increasing chemotherapy sensitivity to DDP. These findings underscore the potent antitumor effect of Ad-siERCC1 on ovarian cancers *in vivo*.

Introduction

Ovarian cancer is one of the most common malignant tumors affecting female reproductive organs worldwide and it has the highest mortality rate among gynecological tumors (1,2). Due to the lack of early diagnostic methods and inconspicuous clinical symptoms, most women are diagnosed at an advanced stage (corresponding to stages III and IV), resulting in 5-year survival rates ranging from 26-42% (3,4). Despite significant advancements in medical science and technology, the combination of surgery and chemotherapy has improved the therapeutic outcomes for ovarian cancer in recent years, but unfortunately, there has been no significant improvement in the 5-year survival rate (5). Therefore, it is imperative to explore new strategies to enhance the treatment of ovarian cancer.

The current front-line standard of care for ovarian cancer involves surgery followed by platinum-taxane maintenance chemotherapy (6,7). However, chemotherapy resistance remains a significant factor contributing to the high mortality rate in ovarian cancer (5). Various factors, such as decreased drug accumulation, elevated glutathione levels, increased metallothionein and enhanced DNA repair ability, have been implicated in the development of tumor cell resistance to paclitaxel or platinum. Among these factors, the most crucial one is the enhanced tolerance and repair of DNA damage through the nucleotide-excision-repair (NER) pathway (8,9). The NER pathway is highly conserved and represents one of the major DNA repair mechanisms in mammalian cells that counteract the formation of genetic damage (8). Understanding the involvement of the NER pathway in chemotherapy resistance could provide valuable insights for developing novel therapeutic strategies to combat ovarian cancer.

Nucleotide excision repair cross-complementary gene 1 (*ERCC1*) is a gene responsible for recognizing DNA damage and cleaving the DNA chain (7). This gene plays a crucial role in repairing the damaged platinum and DNA compounds of tumor cells, directly affecting the ability of tumor tissues to restore replication and proliferation (10). A number of studies have indicated that *ERCC1* serves as a marker for ovarian cancer resistance to platinum drugs (11-13). Research findings have suggested that individuals with low *ERCC1* expression tend to have an improved response to cisplatin chemotherapy compared with those with high expression levels (8,14,15). Moreover, using RNA interference (RNAi) to downregulate *ERCC1* mRNA expression in gastric cancer cells resistant to cisplatin (DDP) has been shown to restore susceptibility to treatment (16). Given these findings, inhibiting *ERCC1*

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expression emerges as a promising therapeutic strategy for improving the treatment of ovarian cancer. By targeting *ERCC1*, it may be possible to enhance the efficacy of platinum-based chemotherapy and overcome drug resistance in ovarian cancer patients.

Adenovirus vectors have emerged as one of the most widely studied and applied vectors for cancer gene therapy. They are designed to replicate preferentially in cancer cells and induce their destruction through the natural process of lytic virus replication (17). This approach allows for targeted delivery of the virus to tumor cells with specific genetic alterations or gene expression profiles, making them applicable only to tumors with the desired characteristics (18). However, despite the significant increase in the number of studies on oncolytic adenoviruses in recent years, research on ovarian cancer remains relatively limited. For example, according to a 2023 review by Lundstrom (19), only five out of ~150 studies involved ovarian cancer. This suggests that more exploration and research are needed in the field of ovarian cancer using oncolytic adenovirus therapy. In addition, the research mechanism of using oncolytic adenovirus vectors to carry therapeutic transgenes exhibits diversity. However, there has been no research report on exploring the use of oncolytic adenovirus vectors to target *ERCC1* gene. Given the important role of *ERCC1* gene in the resistance of ovarian cancer, exploring the use of oncolytic adenovirus vectors to carry interference RNA targeting *ERCC1* gene is a promising direction.

Furthermore, it is highly desirable to develop a repertoire of vectors that can exploit microenvironmental constraints specific to tumor growth, in addition to cell intrinsic gene expression profiles or genetic alterations. Hypoxia, characterized by a reduction in O_2 partial pressure, is a key element of the tumor microenvironment, commonly found in most solid tumors irrespective of their origin, location, or genetic makeup (20,21). Hypoxia plays a pivotal role in conferring resistance of cancer cells to radiotherapy and chemotherapy, promoting the selection of more aggressive tumor clones and facilitating metastasis predisposition (22,23). Thus, there is a critical need to devise innovative therapeutic strategies that can specifically target hypoxic regions within tumors. Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that governs cellular responses to hypoxia by binding to a hypoxia-response element (HRE) present within target genes (20,21). The HIF/HRE regulatory system becomes active under hypoxia or in response to genetic alterations during cell transformation. Therefore, harnessing the HIF/HRE system holds significant promise for selectively targeting therapeutic gene expression to tumor tissues.

The present study genetically engineered an oncolytic adenovirus armed with *ERCC1* short interfering (si)RNA, specifically regulated by hypoxia/HIF-dependent mechanisms. The experiments demonstrated that the recombinant adenovirus effectively induced sustained silencing of *ERCC1* and downregulation of its oncogenic signaling in *in vitro* models of ovarian cancer. Moreover, the adenovirus exhibits targeted killing of cancer cells resistant to DDP. These findings present a promising avenue for developing novel adjuvant chemotherapy approaches for ovarian cancer.

Materials and methods

Cell lines and cell culture. The human ovarian cancer cell line SKOV3 was procured from the American Type Culture Collection. The 293 cells were acquired from Canada Microbix Biosystems Inc. All cell lines were cultured following the instructions provided by the suppliers in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Recombinant oncolytic adenoviruses construction. The dual-regulated adenovirus Ad-ERCC1-siRNA-hTERT-E1A-HRE-E1B (Ad-ERCC1) and its control adenovirus Ad-negative control (NC)-siRNA-hTERT-E1A-HRE-E1B (Ad-NC) were constructed by Shanghai GeneChem Co., Ltd. Briefly, fragments of the specific siRNA of ERCC1 (ERCC1-siRNA) gene were obtained which were designed based on Genbank's ERCC1 gene sequence by gene synthesis conducted by Shanghai GeneChem Co., Ltd. The sequence of ERCC1-siRNA was 5'-ccAAGCCCTTATTCCGATCTA-3' and the negative control sequence was 5'-TTCTCCGAACGTGTCACGT-3'. An overexpression vector was first constructed using PXC1 plasmid [PXC1 (hTERTp-E1A + HREp-E1B)], in which the human telomerase reverse transcriptase promoter (hTERT) was used to regulate the adenovirus *ELA* gene; the hypoxia regulatory element sequence (HRE) regulates the *E1B* gene. (Fig. 1A and B). ERCC1-siRNA and PXC1 were digested by *Bam*HI and *Kpn*I and then the products were combined by T4 DNA ligase to generate the PXC1-ERCC1-siRNA plasmid. The connected plasmids were transformed into competent cells, identified by PCR and sequenced and then extracted with a TIANprep Mini Plasmid Kit (Tiangen Biotech Co., Ltd.). The purified PXC1-ERCC1-siRNA plasmid was transfected into 293 cells with adenovirus packing plasmid (PBHGE3) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as follows: 5 μg of plasmid was added gently to the DMEM, the total volume adjusted to 50 μl and incubate at room temperature for 5 min. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.; 10 μl) was mixed with 50 μl of DMEM and incubated at room temperature for 5 min. the two solutions were gently mixed without shaking and incubated at room temperature for 20 min to form a DNA/Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) transfection complex. The transfection complex was slowly added to the 293 cell mixing well and incubate at 37˚C and in 5% CO_2. At 6 h after incubation, the medium containing the transfection mixture was discarded and the cells rinsed with sterile PBS and gently shaken to wash away the residual transfection mixture. Then 5 ml of DMEM containing 10% FBS was slowly added and incubation continued at 37˚C and in 5% CO_2. At 10-15 days after transfection, single virus plaques appeared in 293 cells, which were collected and confirmed by PCR analysis using the forward and reverse primers. The confirmed recombinant adenovirus was designated Ad-ERCC1. Ad-ERCC1 was amplified in 293 cells and purified by ultracentrifugation on cesium chloride (CsCl) gradients (40,000 x g; 2 h; 4˚C). Other viruses used were treated with the

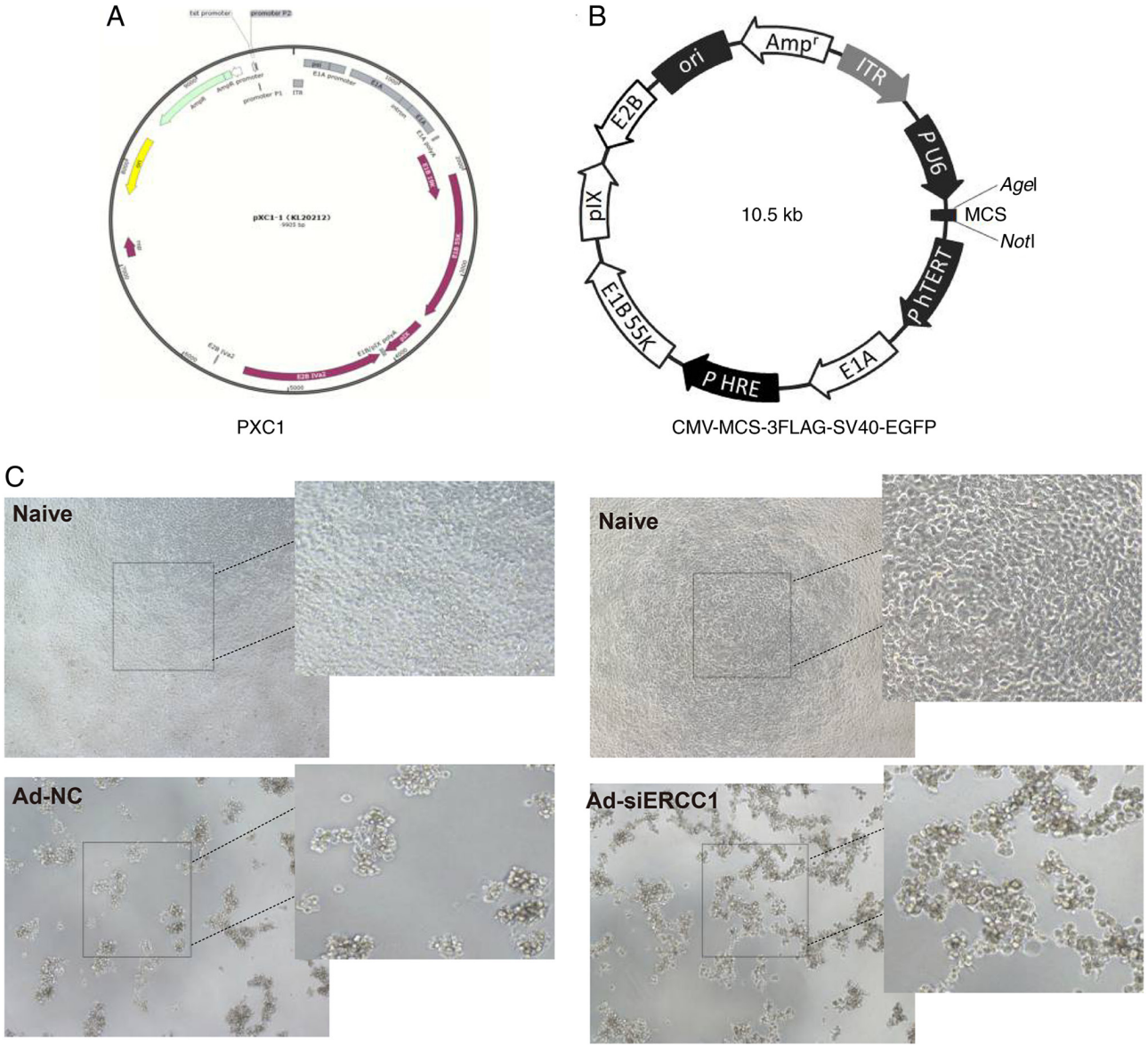

Figure 1. Schematic diagram of the recombinant adenovirus vectors and the inhibitory effect of the recombinant adenovirus on SKOV3 cells. (A-B) Vector plasmid carrying *ERCC1*-siRNA gene. (C) The recombinant adenovirus can inflect and kill tumor cells. Scale bars, left panels, 600 μm; right panels, 400 μm. si, short interfering; NC, negative control.

same method. EGFP gene was cloned into the viruses to determine the infectivity. Viral titer confirmation was performed by infecting 293 T cells using an End-point dilution method and was calculated by Spearman-Karber Method as follows: Viral titer=10 (x+0. 8) plaque-forming units (PFU)/ml) (x=the sum of positive rates of cytopathic effect in sequential dilution from 10^{-1} to 10^{-13}).

Reverse transcription-quantitative (RT-q) PCR for mRNA analysis. For quantitative RT-PCR, total RNA was extracted from cells of different groups (6-well plate with 80% cell density) using SuperfecTRI, Total RNA Isolation Reagent (cat. no. 3101-400; Shanghai Pufei Biotechnology Co., Ltd.). cDNA was synthesized from 400 ng of RNA with the Promega M-MLV kit according to the manufacturer's protocol (cat. no. M1705; Promega Corporation). RT-qPCR) were performed in triplicate according to the kit protocol of Bulge-Loop miRNA qRT-PCR Starter Kit (cat. no. C10211-1; Guangzhou RiboBio Co., Ltd.) using a LightCycler 480 II (Roche Diagnostics) with SYBR green PCR reagents. Experiments were replicated four times and the data are shown as fold changes. The primer sequences were: *ERCC1:* 5'-CTA CGCCGAATATGCCATCTC-3', 3'-GTACGGGATTGCCCC TCTG-5'; *GAPDH*: 5'-TGACTTCAACAGCGACACCCA-3', 3'-CACCCTGTTGCTGTAGCCAAA-5'. The PCR program included 95˚C for 30 s, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The relative amount of mRNA for target gene was determined by the $2^{-\Delta\Delta Cq}$ method (24) and presented as changes in fold in the target gene expression normalized to two endogenous reference genes (*GAPDH*).

Detection of the inhibition rate in ovarian cancer cells by MTT assay. The cancer cell inhibition rate was evaluated by a standard MTT assay. Briefly, SKOV3 cells were plated in

96-well plates at a density of $2x10^4$ cells/well. After incubation for 24 h, the cells were infected with of different multiplicity of infection (MOI)s of Ad-siERCC1 (0, 0.001, 0.01, 0.1, 1, 5, 10, 50, 100 and 500 for 48 or 72 h) or different concentrations of DDP (0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 μmol for 24 h) or both (10 MOI Ad-siERCC1 first for 24 h, 10 μmol DDP for anther 24 h). After treatment, 20 μl (5 mg/ml) MTT solution (cat. no. JT343; Genview Corp.) was added to each well. After 4 h incubation at 37˚C, the supernatant was discarded and 100 μl DMSO was loaded in every well for 2-5 min. Blank control wells were also set. The optical density (OD) of each well was measured with a Microplate Reader (cat. no. M2009PR; Tecan infinite; Tecan Group, Ltd.) at 490 nm. The cell growth inhibitory rate (I%) was calculated according to the following equation: inhibitory rate%=$(OD_{control}-OD_{sample})/(OD_{control}-OD_{blank})$ x 100%, where $OD_{control}$ is the absorbance of the untreated cells, OD_{sample} is the absorbance of the cells exposed to Ad-siERCC1, DDP or both and A_{blank} is the absorbance of the media. A total of three reduplicate wells were measured at each group and every experiment was performed at least 3 times.

Wound healing migration assay. Cells were counted in a Neubauer chamber slide using the trypan blue exclusion method. Viable cells were plated at $3x10^5$ cells/well in 6-well culture plates using growth media containing 10% FBS for 24 h. The cells were washed with DPBS and pretreated with either DDP or recombinant adenovirus in serum-free media. To control wells, only serum-free media was added. The cells were scratched perpendicular to the horizontal line with a 200 μl sterile pipette tip to create a cell-free wound area. After washing away suspended cells, fresh serum-free media was added and images were captured immediately (time 0 h) using an Olympus CX41 inverted microscope (Olympus Corporation). The cells were cultured with 37˚C and 5% CO_2 and images were captured again after 24 h at the same position. The measurement of cell scratch was done by ImageJ software (version 1.47; National Institutes of Health). Within each assay the experiments were performed in triplicates. Data shown are representative of minimum three independent experiments.

Matrigel invasion assay. A total of $3x10^5$ cells in 100 μl serum-free medium were seeded in a Transwell chamber (MilliporeSigma) with a coating of Matrigel (MilliporeSigma). The Matrigel was placed on ice and thawed overnight at 4˚C in the freezer. It was applied diluted with DMEM at 1:5 to the insert cell growth surface at 150-200 μl/cm^2 at 37˚C for 30 min. Briefly, SKOV3 cells were seeded into the upper chamber in a serum-free medium and treated with either DDP or recombinant adenovirus. A volume of 0.5 ml of medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, the upper surface of the membrane was wiped off with a cotton swab. Cells that invaded the lower surface of the porous membrane were fixed with 4% formaldehyde in PBS containing 4% sucrose for 10 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature. Stained cells were counted in 20 random fields per filter, in a total of three filters (n=3). Invasion was presented as percentage of invasion=(number treated cells/number of control cells) x100.

Cell cycle analysis. SKOV3 cells were transfected with Ad at an MOI of 10 for 6 h. Following DDP treatments for 24 h, cells were detached with trypsinization, washed with Dulbecco's phosphate buffered saline (DPBS) and the cells collected. For assessment of DNA contents, cells were stained with propidium iodide (PI; 50 μg/ml; cat. no. P4170; MilliporeSigma) with 0.5 mg/ml RNase (cat. no. EN0531; Thermo Fisher Scientific, Inc.) in DPBS + 0.1% Tween (pH 7.4) in the dark for 30 min and then monitored by a fluorescence-activated cell sorter (FACS) using a BD C6 PLUS (BD Biosciences). The FACS data were analyzed using NovoExpress (ACEA Biosciences, Inc.) to calculate the fraction of cells in G_1, S and G_2 phases.

Cell apoptosis assay. Annexin V-APC staining and flow cytometry analysis were used to detect cell apoptosis. Annexin V Apoptosis Detection Kit APC (cat. no. 88-8007; eBioscience; Thermo Fisher Scientific, Inc.) was used to measure cell apoptosis according to the manufacturer's instructions. Briefly, after washing with PBS, cells were resuspended in Annexin-V binding buffer, stained with the Annexin V-APC for 15 min at room temperature in the dark and analyzed using FACSCalibur (C6 PLUS; BD Biosciences) and the apoptotic rate was calculated as the numbers of early + late apoptotic cells/total numbers of cell.

Protein extraction and immunoblotting. Cells were harvested and re-suspended in a RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitor cocktail (cat. no. GK10014; GlpBio). After determining the protein concentration using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology), the obtained lysates (20 μg/well) were subjected to 4-10% SDS-PAGE to separate the proteins, which were subsequently transferred to PVDF membranes (cat. no. A29562259; Cytiva). The membranes were incubated with the following primary antibodies: anti-PI3K (1:1,000; cat. no. 4257; Cell Signaling Technology, Inc.), phosphorylated (p)-Akt (Ser473; 1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), anti-AKT (1:1,000; cat. no. ab179463; Abcam), anti-caspase-3 (1:1,000; cat. no. sc-7272; Santa Cruz Biotechnology, Inc.), cleaved caspase-3 (1:1,000; cat. no. 9661S; Cell Signaling Technology, Inc.), GAPDH (1:2,000; cat. no. sc-32233; Santa Cruz Biotechnology), β-Actin (1:2,000; cat. no. sc-8432; Santa Cruz Biotechnology). After blocking for 1 h at room temperature in non-fat milk in TBST (Tris-buffered saline with 0.1% Tween-20), membranes were incubated overnight at 4˚C with primary antibodies in blocking buffer. After rinses with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, cat. no. 7074; goat anti-mouse, cat. no. 7076; Cell Signaling Technology, Inc.) for 1.5 h at room temperature and detected using 20X LumiGLO reagent and 20X peroxide (cat. no. 7003; Cell Signaling Technology, Inc.) and film exposure. Optical densities of the bands from the original image were measured with NIH ImageJ software (version 1.47; National Institutes of Health).

Statistical analysis. All data are expressed as mean ± SEM. The statistical comparisons for two groups were made with unpaired Independent-Samples t test. Multiple comparisons

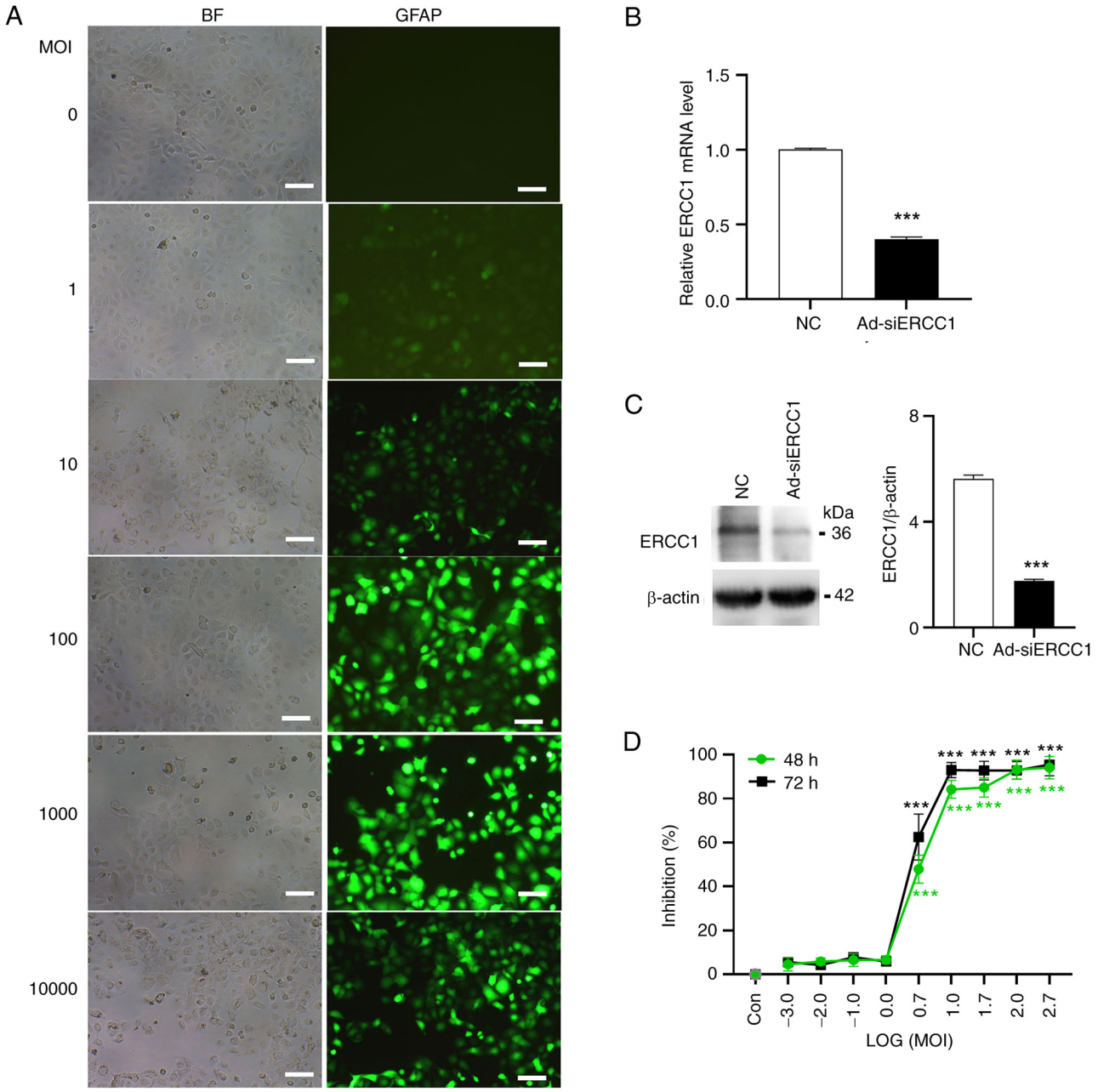

Figure 2. Infection and inhibitory effect of the recombinant adenovirus on SKOV3 cells. (A) The recombinant adenovirus can infect cells effectively. The most efficient MOI=0. Scale bars, 600 μm. The recombinant adenovirus reduced the (B) mRNA and (C) protein levels of ERCC1 on SKOV3 cells (n=4 in each group). (D) The recombinant adenovirus decreased the cell viability (n=3 in each group). Data are shown as the mean ± SEM. ***P<0.001 vs. corresponding controls. MOI, multiplicity of infection; NC, negative control.

were made with one-way ANOVA followed by between-group comparisons using the Bonferroni comparisons or Dunnett's T3 comparisons post hoc tests, according to whether the data were normally distributed or not, using SPSS 13.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Recombinant adenovirus can effectively inhibit the proliferation of ovarian cancer cells. In the present study, the recombinant adenovirus effectively infected and killed tumor cells (Fig. 1C). Subsequently, the transfection efficiency of the recombinant adenovirus on ovarian cancer cells was evaluated. Cultured SKOV3 cells were infected with the recombinant adenovirus expressing green fluorescent protein (GFP) with ERCC1-siRNA (Ad-ERCC1-siRNA, Ad-siERCC1) or the empty vector (Ad-NC-siRNA, Ad-NC) at varying MOI levels ranging from 1-10,000. It was determined that the most efficient MOI value was 10 (Fig. 2A). At MOI=10, the mRNA and protein levels of ERCC1 were validated using RT-qPCR and immunoblotting techniques, revealing a decrease in ERCC1 mRNA (~60% less than control; Independent-Samples t test; $t_{(6)}$=36.842; P<0.001; Fig. 2B) and protein (>3-fold less than control) (Independent-Samples t test; $t_{(6)}$=24.036; P<0.001; Fig. 2C) upon Ad-siERCC1 treatment.

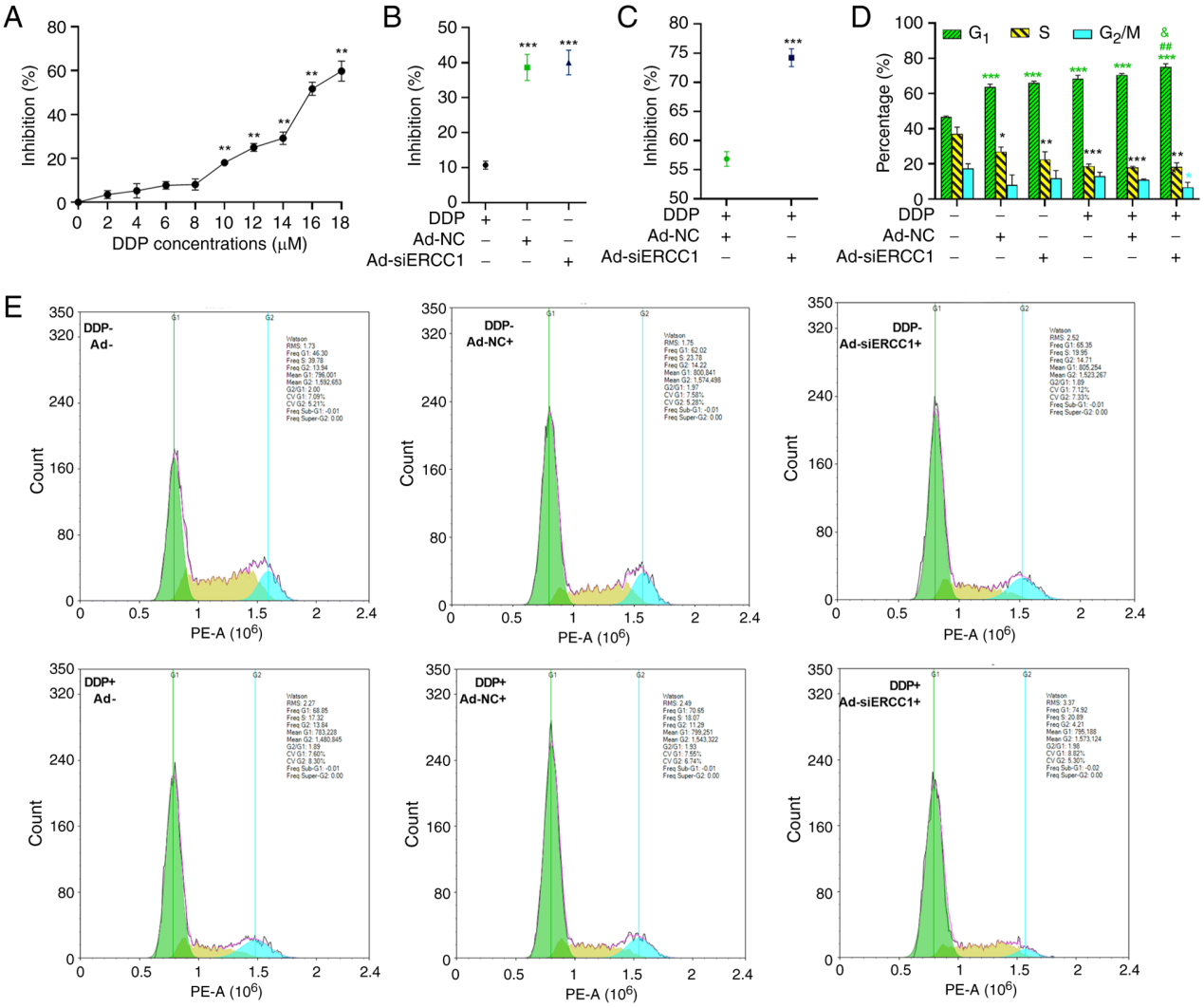

Figure 3. The recombinant adenovirus expressing *ERCC1* siRNA significantly enhance chemosensitivity to DDP on SKOV3 cells. (A) DDP decreased the cell viability (n=4 in each group). (B) Recombinant adenovirus was more effective in cell mortality than DDP treatments (n=4 in each group). (C) Combined with DDP, the cell viability was lower in cancer cells transduced with Ad-siERCC1 compared with those transduced with Ad-NC (n=4 in each group). (D-E) Cell block in G_1 phase was more obvious in Ad-siERCC1 combined with DDP (n=3 in each group). Data are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. corresponding controls; $^{\#\#}$P<0.01 vs. DDP groups; $^{\&}$P<0.05 vs. DDP combined with Ad-NC groups. si, short interfering; DDP, cisplatin; NC, negative control.

Oncolytic viruses specifically target and replicate within tumor cells, ultimately lysing them and releasing viral progeny that can propagate among tumors, eventually leading to the destruction of all tumors and thereby inhibiting tumor cell proliferation and survival (16). Considering this, it was further assessed using MTT assay whether the recombinant adenovirus would induce inhibition of cancer cell survival following transduction. As demonstrated in Fig. 2D, the recombinant adenovirus caused a significant increase in cell mortality from MOI 5 (~47.87% for 48 h and 62.49% for 72 h) to MOI 500 [~94.03% for 48 h and 95.43% for 72 h; one-way ANOVA; 48 h: *F (9,20)*=362.834; P<0.001; vs. the control group, LOG (5): P<0.001; LOG (10): P<0.001; LOG (50): P<0.001; LOG (100): P<0.001; LOG (500): P<0.001; 72 h: *F (9,20)*=313.047; P<0.001; vs. the control group, LOG (5): P<0.001; LOG (10): P<0.001; LOG (50): P<0.001; LOG (100): P<0.001; LOG (500): P<0.001].

ERCC1 silencing can significantly enhance chemosensitivity to DDP of ovarian cancer cells. DDP is a widely used anticancer drug in combination regimens and it exerts its activity by inducing the formation of various types of DNA adducts, leading to the inhibition of DNA synthesis, function and transcription (25). According to our previous study (26), a systematic inhibition of SKOV3 cell survival was observed with increasing concentrations of DDP from 0-18 μM after 24 h of treatment, as determined by MTT assay (one-way ANOVA; F (9,30)=258.978; P<0.001; Fig. 3A). Markedly, the inhibitory effect had a significantly higher expression of 18.05, 24.98, 29.18, 51.73 and 59.75% for 10,12,14,16,18 μM DDP (vs. the 0 μM DDP group, 10 μM DDP: P=0.001; 12 μM DDP: P=0.001; 14 μM DDP: P=0.002; 16 μM DDP: P=0.001; 18 μM DDP: P=0.001). Therefore, this concentration was chosen for subsequent experiments. Subsequently, the inhibitory rates were compared among DDP, Ad-NC and Ad-siERCC1. The results demonstrated that the recombinant adenovirus [both Ad-NC (38.63%) and Ad-siERCC1 (40%)] exhibited greater effectiveness in inducing cell mortality vs. DDP alone (10.7%; one-way ANOVA; F (2,9)=258.978,

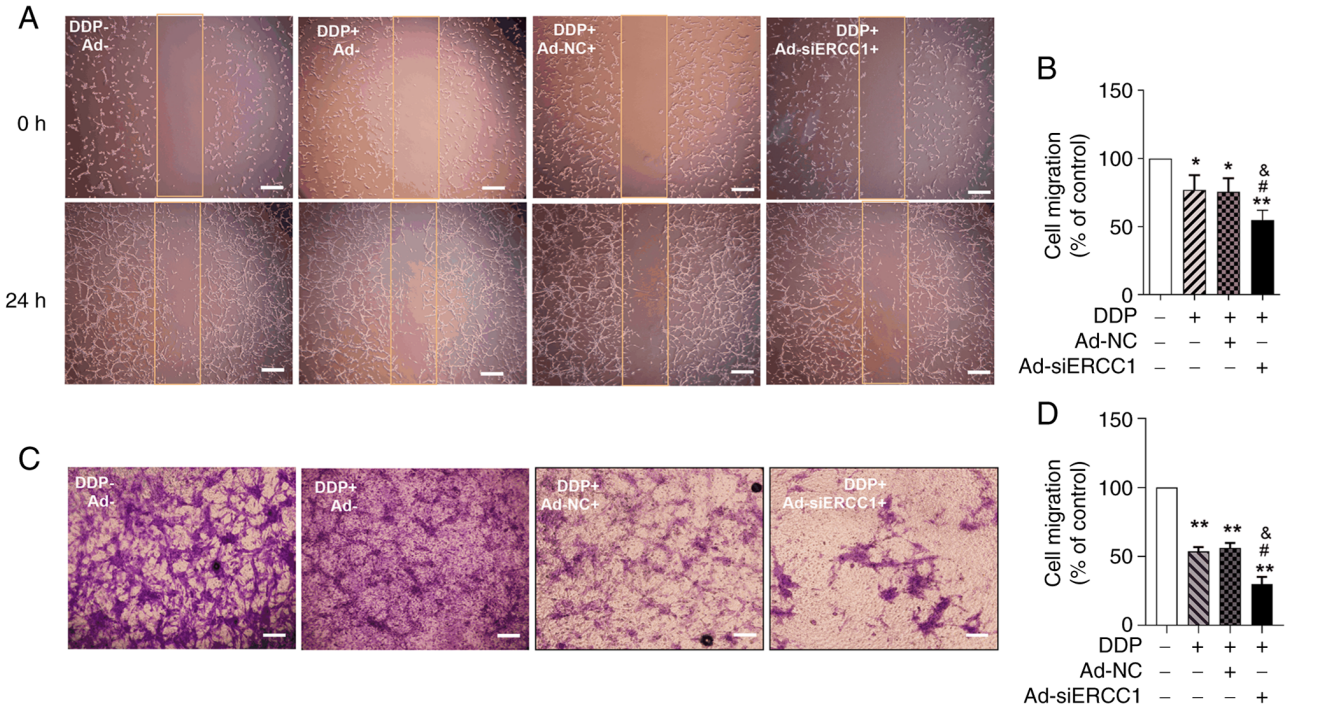

Figure 4. Effects of the recombinant adenovirus on SKOV3 cells migration and invasion. (A) Representative images of cells scratch assay. Scale bars, 400 μm. (B) Cells treated with Ad-siERCC1 combined with DDP showed the lowest migration (n=5 in each group). (C) Representative images of cells, Transwell assay. Scale bars, 400 μm. (D) Suppression of SKOV3 cells invasion after treatment with DDP and Ad-siERCC1 (n=5 in each group). All measurements were performed in triplicate. Data are shown as the mean ± SEM. *P<0.05, **P<0.01 vs. corresponding controls; $^{\#}$P<0.05 vs. DDP groups, $^{\&}$P<0.05 vs. DDP combined with Ad-NC groups. si, short interfering; DDP, cisplatin; NC, negative control.

P<0.001; vs. DDP group, Ad-NC: P<0.001; Ad-siERCC1: P<0.001, Fig. 3B).

Resistance, however, has limited the efficacy of these drugs in most ovarian cancer patients. Among the various mechanisms contributing to cisplatin (DDP) resistance, enhanced tolerance and repair of DNA damage through the NER pathway have been recognized as the most crucial resistance mechanism to platinum drugs (7,8). To further investigate whether *ERCC1* silencing could enhance the cancer cell-killing effect following chemotherapy, MTT assays were performed on SKOV3 cells treated with recombinant adenovirus and DDP. As depicted in Fig. 3C, in the presence of DDP, cancer cells transduced with Ad-siERCC1 (74.19%) exhibited significantly lower cell viability compared with those transduced with Ad-NC (56.85%) six days post-transduction (Independent-Samples t test; $t_{(6)}$=-7.420; P<0.001). Additionally, cell cycle analysis revealed that SKOV3 cells in all groups were arrested in the G_1 phase when compared with the control group (control group: 46.60%; Ad-NC: 63.79%; Ad-siERCC1: 66.05%; DDP: 68.42%; DDP + Ad-NC: 70.56%; DDP + Ad-siERCC1: 75.12%; one-way ANOVA, G_1: *F (5,12)*=148.758, P<0.001; vs. the control group, Ad-NC: P<0.001; Ad-siERCC1: P<0.001; DDP: P<0.001; DDP + Ad-NC: P<0.001; DDP + Ad-siERCC1: P<0.001). However, the G_1 phase cell block was more pronounced in the Ad-siERCC1 combined with cisplatin group (vs. DDP group, DDP + Ad-siERCC1: P=0.001; vs. DDP + Ad-NC group, DDP + Ad-siERCC1: P=0.027) and the proportion of cells in the G_2/M phase was the lowest (Fig. 3D and E; one-way ANOVA, S: *F (5,12)*=16.442, P<0.001; vs. the control group, Ad-NC: P=0.036; Ad-siERCC1: P=0.001; DDP: P<0.001; DDP + Ad-NC: P<0.001; DDP + Ad-siERCC1: P<0.001; G_2/M: *F (5,12)*=3.697, P=0.029; vs. the control group, DDP + Ad-siERCC1: P<0.036).

To assess the effect of the recombinant adenoviruses on SKOV3 cell migration, a scratch wound-healing migration assay was performed. The change in scratch width reflected cell mobility. Ad-siERCC1 combined with DDP resulted in the most potent inhibition of cell migration to 54.69%. (one-way ANOVA, *F (3,16)*=24.970, P<0.001; vs. the control group, DDP: P=0.038; DDP + Ad-NC: P=0.024; DDP + Ad-siERCC1: P=0.001; vs. DDP group, DDP + Ad-siERCC1: P=0.034; Vs. DDP + Ad-NC group, DDP + Ad-siERCC1: P=0.035; Fig. 4A and B). In the Transwell invasion assay, it was observed that the recombinant adenovirus and DDP inhibited the migration ability of SKOV3 cells (one-way ANOVA, *F (3,16)*=69.035, P<0.001; vs. the control group, DDP: P=0.001; DDP + Ad-NC: P=0.001; DDP + Ad-siERCC1: P=0.001; Fig. 4C and D). Moreover, in the presence of DDP, the effect of Ad-siERCC1 was significantly superior to the DDP group (control group: 100.00%; DDP: 53.62%; DDP + Ad-NC: 56.18%; DDP + Ad-siERCC1: 29.93%; vs. DDP group, DDP + Ad-siERCC1: P=0.034; vs. DDP + Ad-NC group, DDP + Ad-siERCC1: P=0.021). These findings indicated that the recombinant adenovirus significantly enhanced the inhibitory effect on migration and invasion of ovarian cancer cells and Ad-siERCC1 can improve the chemotherapy sensitivity of DDP.

Recombinant adenovirus improves drug resistance to DDP by enhancing apoptosis through the PI3K/AKT-caspase-3 signaling pathways in ovarian cancer cells. Flow cytometry with Annexin V-APC single-dye method was used to detect cells arrested in early apoptosis. As shown in Fig. 5A and B

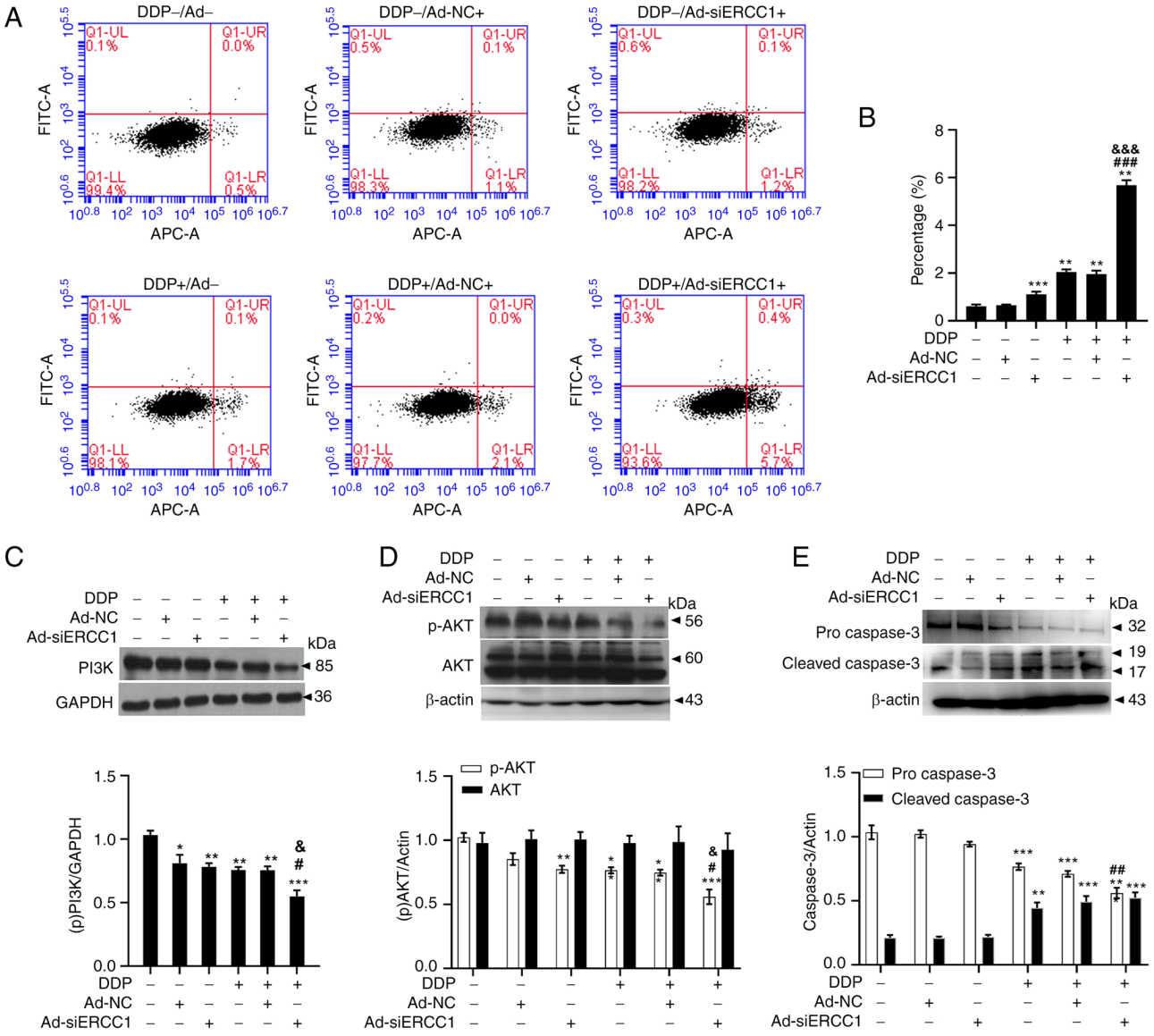

Figure 5. Recombinant adenovirus enhancing apoptosis through PI3K/AKT-caspase-3 ameliorates resistance to DDP on SKOV3 cells. (A) The representative images of the Annexin V-APC staining assay. (B) Recombinant adenovirus and/or DDP promoted the apoptosis of ovarian cancer cells and the apoptosis rate in combination group was significantly higher than that in DDP group (n=4 in each group). (C) PI3K was significantly suppressed by recombinant adenovirus and/or DDP and Ad-siERCC1 could greatly enhanced the effect of DDP (n=4 in each group). (D) AKT was significantly suppressed by recombinant adenovirus and (or) DDP and Ad-siERCC1 could greatly enhanced the effect of DDP (n=4 in each group). (E) Procaspase-3 was significantly reduced after recombinant adenovirus and/or DDP incubation, whereas cleaved caspase-3 was considerably increased (n=4 in each group). Data are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. corresponding controls; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. DDP groups, $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 vs. DDP combined with Ad-NC groups. DDP, cisplatin; si, short interfering; NC, negative control; p-. phosphorylated.

(one-way ANOVA, F (5,18)=892.196, P<0.001), recombinant adenovirus and/or DDP induced apoptosis in SKOV3 cells. The mean fluorescence intensity was 0.61±0.07 on control cells, 0.65±0.03 on Ad-NC cells, 1.11±0.11 on Ad-siERCC1 cells, 2.05±0.11 on DDP cells, 1.96±0.14 on DDP + Ad-NC cells and 5.68±0.21 on DDP + Ad-siERCC1 cells. (Vs. the control group, Ad-NC: P<0.001; Ad-siERCC1: P<0.001; DDP: P<0.001; DDP + Ad-NC: P<0.001; DDP + Ad-siERCC1: P<0.001) and Ad-siERCC1 significantly enhanced the apoptotic effect of DDP (>2.7-fold more than control; vs. DDP group, DDP + Ad-siERCC1: P<0.001; vs. DDP + Ad-NC group, DDP + Ad-siERCC1: P<0.001). To gain further insights into this phenomenon, immunoblotting was performed to examine the proteins involved in apoptosis. As depicted in Fig. 5C (one-way ANOVA, F (5,18)=14.040, P<0.001; vs. the control group, Ad-NC: P=0.019; Ad-siERCC1: P=0.006; DDP: P=0.002; DDP + Ad-NC: P=0.002; DDP + Ad-siERCC1: P<0.001; vs. DDP group, P=0.034; vs. DDP + Ad-NC group, P=0.037). PI3K were significantly suppressed by recombinant adenovirus and/or DDP and Ad-siERCC1 greatly enhanced the effect of DDP (DDP + Ad-siERCC1: 0.55±0.10 vs. DDP: 0.76±0.05). Recombinant adenovirus and/or DDP reduced the phosphorylation level of p-AKT. Ad-siERCC1 enhanced the effect of DDP on p-AKT [DDP + Ad-siERCC1: 0.56±0.11 vs. DDP: 0.77±0.05; one-way ANOVA, p-AKT: F (5,18)=16.062, P<0.001; vs. the control group, Ad-siERCC1: P=0.003; DDP: P=0.003; DDP + Ad-NC: P=0.001; DDP + Ad-siERCC1: P<0.001; vs. DDP group, P=0.012; vs. DDP + Ad-NC group,

DDP + Ad-siERCC1: P=0.035. AKT: F (5,18)=0.107, DDP + Ad-siERCC1: P=0.989]. Concurrently, procaspase-3 levels were notably reduced after incubation with recombinant adenovirus and/or DDP (control: 1.03±0.11 vs. DDP: 0.77±0.05 vs. DDP + Ad-NC: 0.71±0.04 vs. DDP + Ad-siERCC1: 0.56±0.08), whereas the cleaved caspase-3, the activated form of procaspase-3, exhibited a substantial increase (control: 0.21±0.04 VS DDP: 0.44±0.08 vs. DDP + Ad-NC: 0.49±0.04 vs. DDP + Ad-siERCC1: 0.52±0.08; one-way ANOVA, procaspase-3: F (5,18)=36.689, P<0.001; vs. the control group, DDP: P<0.001; DDP + Ad-NC: P<0.001; DDP + Ad-siERCC1: P<0.001; vs. DDP group, P=0.003. cleaved caspase-3: F (5,18)=22.502, P<0.001; vs. the control group, DDP: P=0.001; DDP + Ad-NC: P<0.001; DDP + Ad-siERCC1: P<0.001, Fig. 5E). These results indicated that recombinant adenovirus ameliorates drug resistance to DDP by promoting apoptosis through the PI3K/AKT-caspase-3 signaling pathways in ovarian cancer cells.

Discussion

The present study developed a recombinant oncolytic adenovirus regulated by the hTERT/HIF dual system, which effectively inhibited the proliferation of ovarian cancer cells. Furthermore, using siRNA technology to downregulate *ERCC1* gene expression improved drug resistance to DDP by enhancing apoptosis through the PI3K/AKT-caspase-3 signaling pathways in ovarian cancer cells.

Ovarian cancer predominantly implants and metastasizes in the pelvic cavity. However, effective drug concentration delivery intravenously to the pelvic cavity is challenging, resulting in poor therapeutic outcomes. Additionally, the resistance of tumor cells to platinum drugs hinders the anti-tumor effect, leading to a low 5-year postoperative survival rate (3,4). Thus, the development of a new treatment strategy that targets tumors effectively and reverses drug resistance is a crucial research objective in gynecologic oncology. Tumor-specific proliferative oncolytic adenovirus carriers have garnered attention due to their high tumor targeting and lethality. These carriers infect tumor cells, replicate, multiply, lyse and kill tumor cells, releasing more virus that can infect other tumor cells, resulting in a chain killing reaction. Replication and proliferation are limited to tumor cells, with minimal damage to normal host tissues (16). The present study constructed a recombinant oncolytic adenovirus regulated by the hTERT/HIF dual system, expressing GFP with *ERCC1*-siRNA (Ad-siERCC1). It was demonstrated that Ad-siERCC1 effectively inhibited the proliferation, migration, invasion and survival of ovarian cancer cells. Furthermore, compared with DDP alone, Ad-siERCC1 significantly increased the inhibitory effect on SKOV3 cells, possibly due to its amelioration of drug resistance to DDP.

Ovarian cancer is a heterogeneous disease categorized into several major morphological subtypes: Epithelial carcinoma, germ cell tumor, sex cord-stromal tumor and Krukenberg's tumor (2,25,27). The most common subtype is serous ovarian carcinoma (28). The present study focused on the inhibition ratio of the recombinant oncolytic adenovirus (Ad-siERCC1) on SKOV3 cells, derived from serous cystadenoma carcinoma. The results revealed that Ad-siERCC1 had a marked ability to kill tumor cells. However, the effect of Ad-siERCC1 on other subtypes of ovarian tumors was not assessed. Considering that the subtype of ovarian cancer varies with the age of onset and epidemiological differences exist between races and countries due to various factors, including genetics and economic factors (2,25,27), it is essential to investigate the effect of Ad-siERCC1 on different subtypes of ovarian cancer in future research.

Viral vectors in gene therapy in the treatment of ovarian cancer, according to a review by Lundstrom in 2023 (19); the five projects listed involve four vector systems, including p-dpp-VSVMP [a nanoparticle that carries the gene for the matrix protein of the vesicular stomatitis virus (VSVMP) and encapsulates paclitaxel] (29), VSVMP (the gene for VSVMP enclosed in a lipid complex) (30), SIN AR339 (an RNA virus belonging to the Togavirus genus) (31) and MV-CEA (a measles virus Edmonston vaccine strain expressing the CEA peptide) (32). The p-dpp-VSVMP was found to cause tumor regression in both subcutaneous and orthotopic ovarian cancer mouse models. When combined with the JAK1/2 inhibitor ruxolitinib, the treatment outcome was further improved. The VSVMP system demonstrated a high tumor suppression rate of 87-98% in mouse tumor xenografts models, leading to an increase in survival. These non-replicating vector particles induce apoptosis in cancer cells by disrupting cell cytoskeletal elements and suppressing host cell gene expression. Another study (31) found that intraperitoneal injection of the oncolytic adenovirus SIN AR339 led to tumor cell killing and regression in ovarian cancer. In clinical applications, the MV-CEA particle has been evaluated in a phase I clinical trial for recurrent ovarian cancer patients (32). This treatment showed no dose-limiting toxicities and all nine patients who received the treatment achieved disease stabilization. The median overall survival rate for these patients was 12.15 months, twice the expected survival time. However, this treatment is only suitable for patients with normal CEA levels. Compared to these studies, our research still has a long way to go, is focusing on exploring the application of ERCC1 gene in oncolytic adenovirus therapy. It is well known that ERCC1 is the most critical factor contributing to platinum resistance in ovarian cancer and is one of the most studied DNA repair genes (33). *ERCC1*, located on human chromosome 19, is involved in the cleavage and recognition of DNA chains damaged by platinum-based drugs. The level of *ERCC1* mRNA reflects the ability of tumor tissue to repair DNA helix twist induced by platinum-based drugs and serves as an important indicator of tumor patients prognosis and the effectiveness of platinum-based chemotherapy (12). In the present study, interference with *ERCC1* gene expression significantly improved the efficiency of DDP chemotherapy, consistent with numerous studies showing that higher ERCC1 expression in patients receiving platinum-based chemotherapy is associated with worse chemotherapy outcomes and patient prognosis (8,14,15). However, Bösmüller *et al* (34) reported that ERCC1 expression is not related to platinum chemosensitivity in ovarian cancer patients. The discrepancies could be attributed to limited small-scale and small-sample research on chemotherapy and ERCC1 gene or protein expression in ovarian cancer, lacking large-sample multicenter clinical studies.

Although oncolytic viruses are considered a promising new class of anticancer drugs, they present unique challenges.

Oncolytic viruses are live viruses that proliferate upon administration, resulting in variable effective doses (35). Currently, limited data are available on correlating viral dose with *in vivo* replicative potential and therapeutic response. Further investigations relating viral replication to clinical response are crucial. Moreover, while a number of clinical trials have shown that replication-defective and replication-competent adenovirus vectors are safe and have therapeutic activity (36-39), the replicative potential of oncolytic viruses and immunoreaction require further study. Additionally, the safety and immunological response to oncolytic viruses still need to be fully evaluated. The present study constructed a novel Dual-regulated oncolytic adenovirus carrying ERCC1-siRNA gene that can infect and replicate in ovarian cancer cells. This recombinant oncolytic adenoviruses effectively suppressed cancer cell proliferation, especially when combined with DDP. The findings of the present study suggested that the recombinant oncolytic adenovirus exerts its anti-cancer effect by manipulating the PI3K/Akt signaling pathway, a well-known survival and growth pathway in cancer cells (40,41). However, further investigation is required to confirm these results and to address any potential safety concerns or off-target effects. Overall, oncolytic viruses show great potential in the treatment of ovarian cancer, but more research is needed to optimize their use in clinical settings.

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Availability of data and materials

The data generated in the present study are included in the figures of this article.

Authors' contributions

WY, YM, LX, TZ initiated and directed the study; TZ, RZ, XZ, QS performed the research and wrote the first draft of the manuscript; TZ, XX, WC analyzed the data. WY and YM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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