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PRECLINICAL STUDY

Downregulation of APE1 potentiates breast cancer cells to olaparib by inhibiting PARP-1 expression

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Abstract

Purpose Targeting DNA repair mechanisms to induce apoptosis may be a promising strategy for breast cancer treatment. Olaparib is proved to have anticancer effect by inhibiting DNA repairing protein poly (ADP-ribose) polymerase (PARP). However, the cytotoxicity of olaparib is very limited to homologous recombination-proficient cells. This study aims to examine the effect and mechanism of olaparib treatment in breast cancer cell lines.

Methods We investigated the cytotoxic effect of various doses of olaparib treatment to MCF-7 and ZR-75-1 cells in vitro. mRNA and protein levels of PARP and APE1 were examined by real-time PCR and western blot, respectively. APE1-deficient cell lines were created by RNA interference and used for in vitro cytotoxicity study as well as in vivo study.

Results 2 μM or higher concentrations of olaparib lead to significant cell death and ROS production. Moreover, olaparib treatment not only inhibits PARP1, but also reduces the expression of APE1 in both mRNA and protein levels. Deficiency of APE1 resulted in increased sensitivity of MCF-7 and ZR-75-1 cells to olaparib treatment. In vivo study showed that reduction of APE1 significantly reduced the volume and weight of MCF-7 xenografted tumors when treated with olaparib, which suggests the synergistic function of inhibition of APE1 in promoting antitumor effects of olaparib treatment.

Conclusion To acquire better benefits for HR-proficient breast cancer patients, developing chemotherapeutic drugs antagonize APE1 would be an effective strategy to improve the clinical outcome of PARP inhibitors.

Keywords Breast cancer · APE1 · PARP1 · Chemoresistance · ROS · Apoptosis

Introduction

Breast cancer is the most common cancer in women worldwide, with 249,260 new cases diagnosed in 2016 [1]. Chemotherapeutic improvements in breast cancer therapy have been achieved by targeting estrogen receptor signaling [2] and epidermal growth factor receptors [3]. However, the efficiency of chemotherapy is still challenged by secondary recurrence, metastasis, and drug resistance [4]. Among the factors that lead to poor survival rates of breast cancer patients, development of multidrug resistance (MDR) is a major hurdle. It is known that cancer is a disease with rapid DNA replication with aggressive DNA repair pathways which prevent genetic and epigenetic instability [5]. Failure of DNA repairing can lead to physical dissociation, fragmentation, and loss or rearrangement of chromosomes in cancer cells. Therefore, inhibition of DNA repair mechanisms may be a promising strategy to enhance anticancer therapy.

Poly(ADP-ribose) polymerase (PARP) is a family of highly abundant DNA binding protein that is involved in maintaining genomic integrity and modifying transcription when response to stresses that are toxic to the genome [6]. Inhibition of PARPs by genetic and pharmacologic methods disrupts base excision repair pathway and thus leads to cell death through synthetic lethality [2]. A number of PARP inhibitors with potential clinical application have since developed for targeted therapy; these inhibitors include

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rucaparib, veliparib, and olaparib [8]. Olaparib, also known as AZD2281 or KU-0059436, is a potent inhibitor of both PARP1 and PARP-2. The safety and efficacy of using olaparib as a monotherapy in breast cancer have been accessed by numerous studies [9–12]. After successful phase I studies where olaparib was tested as monotherapy for cancer patients, clinical trials have moved to the end of phase II [8]. However, an exception to this paradigm is found in homologous recombination (HR)-proficient cells, where olaparib showed close to no anticancer effect [7, 13]. About 30% of breast cancer patients are HR-proficient [14], for whom olaparib as a single agent offers a very limited therapeutic effect. Therefore, it is important to carry out further investigations on sensitizing breast cancer to olaparib, which are needed to establish a more rational design of the treatment course.

Apurinic endonuclease 1 (APE1) is a critical enzyme in the repair of oxidized bases, which cleaves the phosphodiester DNA backbone, generating a nick with 5′ sugar phosphate and 3′ hydroxyl group. This incision of DNA prevents replication fork to progress and thus induces DNA double strand breaks that are toxic to cells [15]. Previous studies suggest APE1 may have prognostic and/or predictive significance in cancer [16, 17]. Moreover, inhibition of APE1 has been demonstrated synthetic lethality in BRCA-deficient cancer [18]. Harris et al. reported a co-dependent expression between APE1 and PARP1, where downregulation of PARP1 resulted in deficiency of APE1, which indicates a synergistic interaction between APE1 and PARP1 [19].

We hypothesized that the effect of PARP inhibition on breast cancer cells would be enhanced by co-targeting APE1 expression. We investigate the cytotoxic effect of olaparib treatment to MCF-7 and ZR-75-1 cells in vitro and found a lethal dose of 2 μM olaparib or higher leads to significant cell death and ROS production. Moreover, we demonstrated that olaparib treatment not only inhibits PARP1, but also reduce the expression of APE1 in both mRNA and protein levels. Deficiency of APE1 resulted in increased sensitivity of MCF-7 and ZR-75-1 cells to olaparib treatment. This observation was also supported by in vivo model that APE1 downregulation significantly reduced the volume and weight of xenografted tumors when treated with olaparib, which suggests the synergistic function of inhibition of APE1 in promoting antitumor effects of olaparib treatment.

Materials and methods

Cell culture

Human breast cancer cell line MCF-7 was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Hyclone). ZR-75-1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin (all from Hyclone). Cells were passaged once every 4 days and cultured at 37 °C in a humidified 5% CO_2 atmosphere. For olaparib treatment, MCF-7 cells with 70% confluency were exposed to various concentrations of olaparib (Selleck Chemicals) dissolved in DMSO (Sigma-Aldrich) for 3 days before subsequent tests were carried out.

MTT assay

Cell viability was determined by a Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) as previously reported [20]. Briefly, after olaparib treatment, 20 μL of MTT (5 mg/mL) solution was incubated with cells in each well of a 96-well plate for 4 h at 37 °C. Then the medium was removed and 100 μL of the SDS–HCl solution was added to the well. Incubate the plate again at 37 °C for 4 h and read absorbance at 570 nm by a Synergy 2 multi-mode plate reader (Bio-Tek).

TUNEL

A DeadEnd Fluorometric TUNEL Detection System (Promega) was used to determine apoptosis according to manufacturer's protocol. Briefly, cells were fixed in PBS containing 4% formaldehyde for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS twice, cells were incubated with a fluorometric terminal deoxytransferase (TdT) mixture at 37 °C for 1 h, followed by twice PBS wash. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured using a Nikon Eclipse Ti microscope.

Detection of ROS accumulation

Intracellular ROS production was measured by staining with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Thermo Fisher Scientific; 8 mg/mL in DMSO), a specific probe to ROS. MCF-7 cells treated with olaparib were incubated with H_2DCFDA for 30 min in dark at 37 °C. Then cells were rinsed twice with PBS and trypsinized, and fluorescence intensity was measured by flow cytometry in a FACSAria™ III cytometer (BD Biosciences). Data analysis was performed with BD FACSDiva software with a minimum of 10,000 cells acquired for each sample.

Western blot

Total protein extracts were collected in RIPA buffer (Thermo Fisher Scientific) and quantified by a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Equivalent amounts of protein (100 µg) from each sample were loaded to SDS–polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane (Millipore). Blocking was performed by incubating the membrane with 5% skimmed milk in TBS-Tween (TBST) for 1 h at room temperature followed by overnight incubation at 4 °C with the target primary antibodies. In this study, the following primary antibodies were used: rabbit anti-APE1 (1:500, ab175315, Abcam), rabbit anti-cleaved PARP (1:1000, ab32064, Abcam), and rabbit anti-GAPDH (1:10,000, ab181602, Abcam). After rinsing the membrane with TBST for 3 times, membrane was further incubated with Alexa Fluor® 680-conjugated goat anti-rabbit IgG antibody (1:10,000, ab175773, Abcam) for 1 h at room temperature. After 3 washes with TBST, the protein bands were scanned using a Amersham Typhoon Biomolecular Imager (GE Healthcare). Quantification of protein levels was performed using Image J software (Version 1.30, National Institutes of Health).

RNA interference

Cells were transfected with APE1 siRNA (80 nM; sc-29,470; Santa Cruz Biotechnology) using Lipofectamine™ RNAiMAX™ Transfection Reagent (Thermo Fisher Scientific) in a 10:3 ratio of RNA: Lipofectamine RNAiMAX according to the manufacturer's manual. Scrambled nonspecific siRNA (80 nM; sc-37,007; Santa Cruz Biotechnology) was used as control. Forty-eight hours after transfection, cells were treated as described.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's protocol. For each sample, 500 ng total RNA was reverse-transcribed using an Advantage® RT-for-PCR Kit (Clonetech). Real-time PCR reactions were performed by a SYBR Fast qPCR Mix (Takara) as recommended by manufacturer's guide. To detect the corresponding gene expression, the following primers were used: human GAPDH, 5′-GAAGATGGTGATGGGATTTC-3′ and 5′-GAAGGTGAAGGTCGGAGTC-3′; human APE1, 5′-GATCTCGCGAGTAGGGCA-3′ and 5′-GCGGCCGTCTTACTCTTCTT-3′; human PARP1, 5′-TTCAACAAGCAGCAAGTGCC-3′ and 5′-CCTTTGGGGTTACCCACTCC-3′. Reactions were quantified by a CFX Connect™ Real-Time PCR Detection System (Bio-rad) for 5 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 15 s at 60 °C. GAPDH was used as loading control. Relative expression was calculated using the comparative Ct method.

In vivo study

Five-week-old female SCID mice were obtained from experimental animal center of Xi'an Jiaotong University and randomly assigned to each group ($n = 10$). All animal procedures were approved by the Xi'an Jiaotong University IACUC committee. To establish xenograft models, 1×10^6 MCF-7 cells transfected with siAPE1 or scramble siRNA were suspended in 100 µL PBS and subcutaneously injected into the left flank of SCID mice. Four days after transplantation, 50 mg/kg/day olaparib or vehicle was administered intravenously via lateral tail vein, daily for 16 days. All mice were monitored daily for activity, body weight, and tumor volume. Mice were euthanized by cervical dislocation on day 20, and the tumors were excised and weighed.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed by GraphPad Prism 5.0 software. Statistical analysis was performed using independent two-sample Student's t test or one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistical significant.

Results

Cytotoxic effect of olaparib treatment in breast cancer cell lines

To assess the dose-dependent susceptibility of breast cancer to olaparib, we first examined the viability of MCF-7 and ZR-75-1 cells under the treatment of olaparib with various doses of olaparib (0.1 to 10 µM) for 3 days. Our MTT result showed that the MCF-7 cell viability decreased in a olaparib dose-dependent manner (Fig. 1a), whereas olaparib induced a significant cytotoxic effect to MCF-7 cells at 2 µM ($66.5 \pm 8.2\%$), 5 µM ($53.7 \pm 9.7\%$), and 10 µM ($38.9 \pm 7.9\%$). The olaparib has similar effect on the ZR-75-1 (Fig. 1b).

High *concentration* of olaparib treatment leads to ROS production in MCF-7 and ZR-75-1 cells

It has been reported that PARP1 inhibition leads to enhanced reactive oxygen species (ROS) formation, which damages DNA cancer cell lines [21]. In order to elucidate the role of ROS in the cytotoxic effect of olaparib, the production of ROS was evaluated in MCF-7 and ZR-75-1 cells pretreated with various doses of olaparib (0.1 to 10 µM) for 3 days. Our

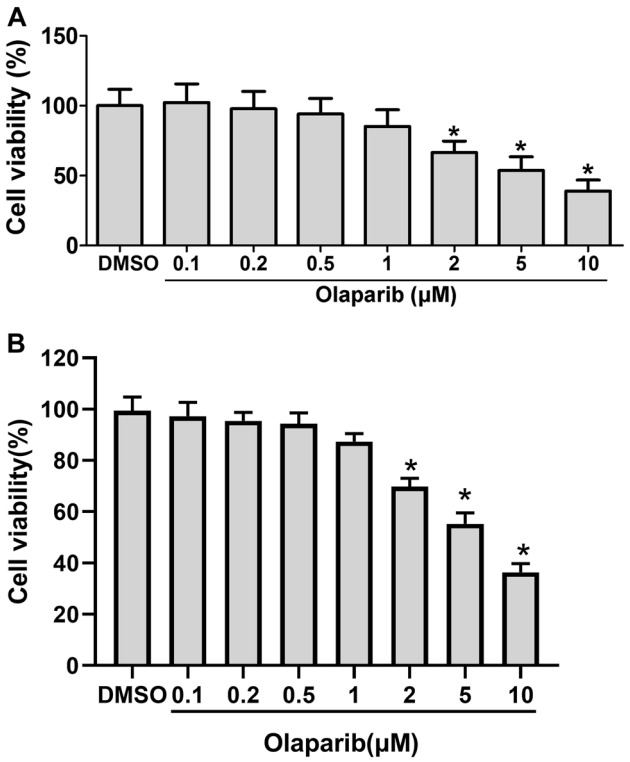

Fig. 1 Sensitivity of breast cancer cell lines to olaparib. MCF-7 (**a**) and ZR-75-1 (**b**) cells were treated with various doses of olaparib (0.1 to 10 μM) for 3 days. Cell viability was determined using MTT assay. Equal volume of DMSO was used as control. Cell viabilities were normalized to control and shown as mean ± SD of three independent repeats. $^*P < 0.05$

result showed that MCF-7 and ZR-75-1 treated with 2 μM or higher concentration of olaparib exhibited remarkable ROS accumulation as shown by H_2DCFDA staining (Fig. 2a, b). Analysis of cellular H_2DCFDA fluorescence intensities by flow cytometry revealed a consistent result that significant increase of ROS production was found only in cells treated with 2 μM or higher concentration of olaparib (Fig. 2c, d).

Inhibition of PARP1 by olaparib downregulates APE1 expression in MCF-7 and ZR-75-1 cells

To evaluate the influence of PARP1 inhibition on the expression of another critical base excision repair enzyme APE1, we treated MCF-7 and ZR-75-1 cells with 5 μM olaparib for 3 days, and the protein level of APE1 was examined by western blot (Fig. 3a, b). Our results demonstrated a significant reduction of APE1 in response to PARP1 deficiency (Fig. 3c, d), suggesting co-dependence between the stability of PARP1 and APE1 proteins. In order to determine whether the decreased levels of PARP1 and APE1 in olaparib-treated MCF-7 and ZR-75-1 cells were resulted from posttranscriptional or transcriptional regulation, we examined the mRNA levels of PARP1 and APE1 by real-time PCR. As shown in Fig. 3e, f, compared with control, the expression levels of both PARP1 and APE1 mRNAs were significantly downregulated in MCF-7 and ZR-75-1 treated with 5 μM olaparib. It indicated that inhibition of PARP1 leads to the reduction of APE1 in both transcript and protein levels.

Downregulation of APE1 synergizes the cytotoxic effect of olaparib in MCF-7 and ZR-75-1

After confirming the co-dependence between PARP1 and APE1 proteins, we next examined the role of APE1 expression in olaparib-induced cytotoxicity. We first confirmed the efficiency of APE1 RNA interference in MCF-7 and ZR-75-1 cells. Our real-time PCR result showed that both siRNAs targeting APE1 reduced its mRNA level over 50%, whereas scrambled siRNA showed no change in APE1 expression after transfection (Fig. 4a, b). Chemoresistance was further determined in MCF-7 and ZR-75-1 cells transfected with APE1-siRNAs, followed by treatment of olaparib at a non-lethal dose of 1 μM for 3 days, and cell viability was tested by MTT assay. Downregulation of APE1 dramatically increased the sensitivities of MCF-7 and ZR-75-1 cells to low concentration of olaparib treatment (Fig. 4c, d), indicating downregulation of APE1 is capable to potentiate breast cancer cells to olaparib-induced cytotoxicity.

We further investigated the expression of PARP1 in MCF-7 and ZR-75-1 cells transfected with two APE1-siRNAs, and our results showed that APE1 downregulation was capable of reducing the both mRNA and protein levels of PARP1 without olaparib treatment (Fig. 5). Interestingly, with the treatment of olaparib at a non-lethal dose (1 μM), the expression of PARP1 further decreased in both APE1-silenced cells, indicating downregulation of APE1 and olaparib treatment have a synergistic effect to reduce PARP1 expression.

Downregulation of APE1 enhances the effect of olaparib in xenograft model

According to our results, olaparib induced a significant reduction in MCF-7 cell viability in vitro when APE1 was knocked down. To further investigate the synergistic effect between APE1 downregulation and olaparib treatment in vivo, we established xenografted tumors by injecting MCF-7 cells transfected with siAPE1 or scramble siRNA into athymic nude mice ($n = 10$). Olaparib treatment was started four days after transplantation for 16 days. In accordance to our in vitro observations, when treated with olaparib, APE1-downregulated xenografts exhibited a significant decreased volume compared to tumors xenografted from MCF-7 cells transfected with scrambled

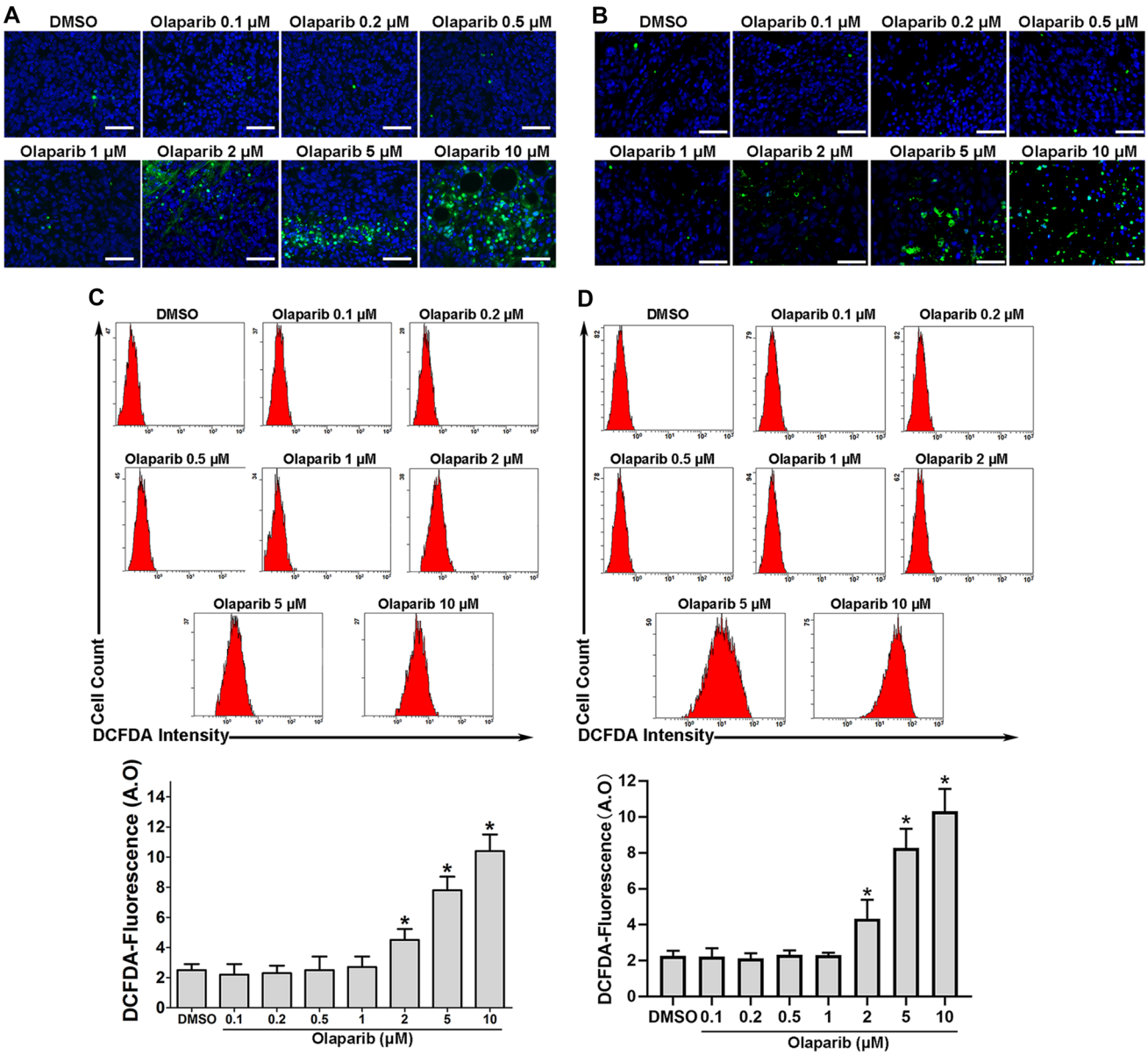

Fig. 2 Olaparib induces ROS production in HR-proficient breast cancer cells. Cells were treated with various doses of olaparib (0.1 to 10 μM) for 3 days and intracellular ROS levels were evaluated by H2DCFDA staining. Equal volume of DMSO was used as control. Representative fluorescent images of MCF-7 (**a**) and ZR-75-1 (**b**) cells. Nuclei were counterstained with DAPI. Scale bars = 50 μm. The fluorescent intensities of DCFDA in MCF-7 (**c**) and ZR-75-1 (**d**) were measured by flow cytometry. Values were shown as mean arbitrary units ± SD of three independent repeats. *$P < 0.05$

siRNAs (Fig. 6a, b). These data were further corroborated by the end-point analysis of explanted tumor weight (Fig. 6c). Taken together, these data suggest knockdown of APE1 significantly improved the sensitivity of MCF-7 cells to olaparib treatment in vivo, thus confirming that targeting APE1 in breast cancer cells is able to overcome chemoresistance to olaparib by triggering apoptosis and thereby leading to tumor shrinkage.

Discussion

Selective targeting PARP activity in cancer deficient in DNA repair pathways has emerged, especially in breast and ovarian cancers, the two malignancies most frequently associated with BRCA mutations [22, 23]. However, its application is greatly limited due to the induction of chemoresistance in HR-proficient cells [24, 25]. Clinical trials

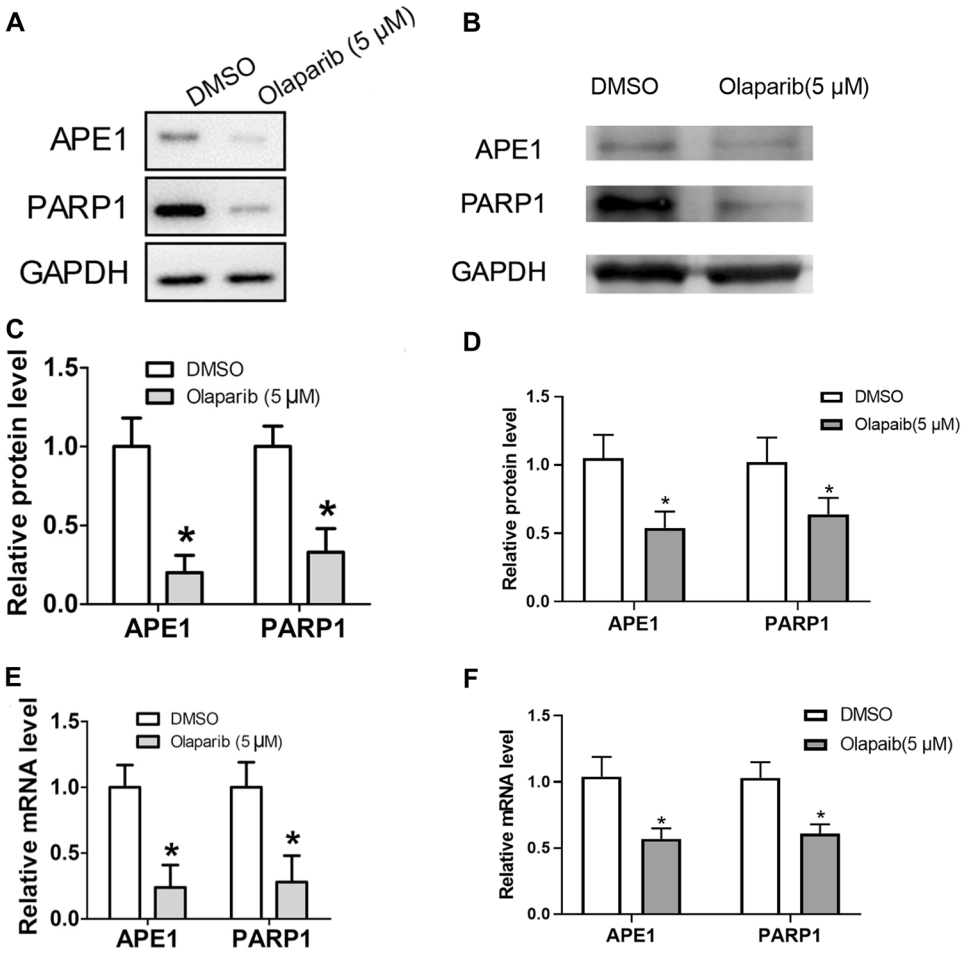

Fig. 3 Olaparib treatment decreases intracellular APE1 and PARP1 level. MCF-7 (**a**) and ZR-75-1 (**b**) cells were treated with 5 μM olaparib for 3 days and intracellular APE1 and PARP1 expression was assessed by western blotting. Equal volume of DMSO was added as vehicle control. GAPDH was used as loading control. **c, d** Protein levels were quantified using Image J software (Version 1.30, National Institutes of Health) and normalized to those in vehicle controls. Data are presented as mean ± SD ($n = 4$). *$P < 0.05$. Transcript levels of PARP-1 and APE1 in MCF-7 (**e**) and ZR-75-1 (**f**) cells were analyzed by real-time PCR. Relative mRNA level was normalized to MCF-7 treated with DMSO only. Data are shown as mean value ± SD of three independent repeats

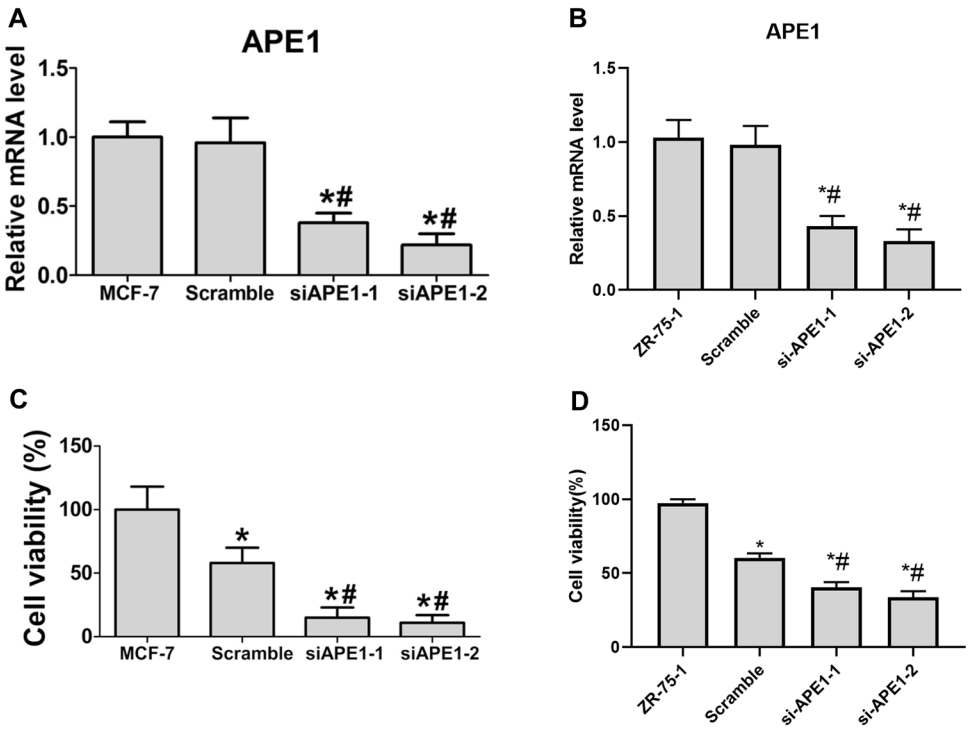

Fig. 4 Downregulation of APE1 reduces chemoresistance of HR-proficient breast cancer cells to olaparib treatment. MCF-7 (**a**) and ZR-75-1 (**b**) cells were transfected with two APE1-siRNAs or scramble siRNA, and APE1 mRNA level was determined at 48 h posttransfection by real-time PCR. Relative mRNA level was normalized to MCF-7 without transfection. Data are shown as mean value ± SD of three independent repeats. MCF-7 (**c**) and ZR-75-1 (**d**) cells transfected with two APE1-siRNAs or scramble siRNA were treated with 5 μM olaparib for 3 days and cell viability was determined using MTT assay. Data are shown as mean ± SD of three independent repeats. *$P < 0.05$ compared to MCF-7; $^{\#}P < 0.05$ compared to scramble control

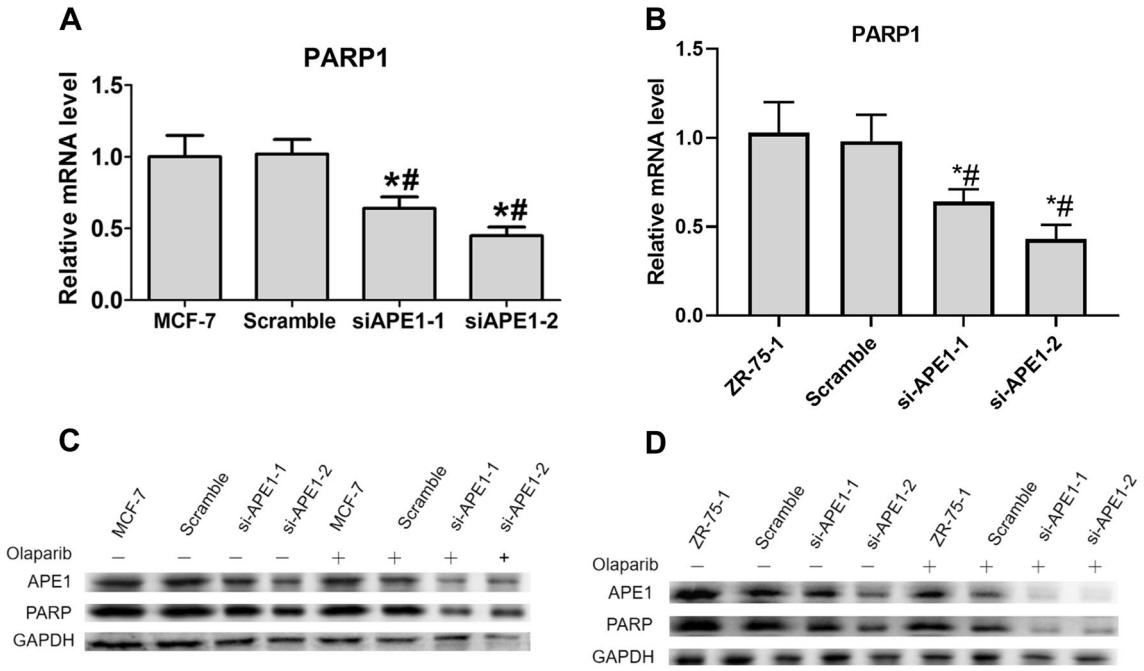

Fig. 5 PARP1 is downregulated by loss-of-function of APE1 in olaparib-treated MCF-7 and ZR-75-1 cells. MCF-7 (**a**) and ZR-75-1 (**b**) cells were transfected with two APE1-siRNAs or scramble siRNA, and PARP1 mRNA level was determined at 48 h posttransfection by real-time PCR. Relative mRNA level was normalized to MCF-7 without transfection. Data are shown as mean value ± SD of three independent repeats. MCF-7 (**c**) and ZR-75-1 (**d**) cells transfected with two APE1-siRNAs or scramble siRNA were treated with 5 μM olaparib for 3 days and APE1 and PARP1 protein level was determined by western blot. GAPDH was used as loading control

also demonstrate significant variations in the response to PARP inhibitors even in BRCA1/2-mutated patient cohorts [7]. These discrepancies highlight further investigations on sensitizing breast cancer to PARP inhibitors to establish a more rational strategy of the treatment course. In this study, we examined the possibility of potentiating the response of MCF-7 and ZR-75-1, two HR-proficient breast cancer cell lines, to PARP1 inhibition by downregulating APE1 expression. To our knowledge, the present study is the first to show synergistic effects between reduced APE1 expression and PARP1 inhibition. Our data suggest that downregulating APE1 by RNA interference enhances the cytotoxicity of non-lethal dose of olaparib, which indicates a more effective therapeutic strategy based on co-targeting the expression of PARP1 and APE1.

It has been shown that HR-proficient breast cancer cells are insensitive to the inhibition of PARP, thus limiting the synthetic lethality caused by chromosome instability cell cycle arrest after olaparib treatment [13]. To study the cytotoxic effect of olaparib in HR-proficient MCF-7 and ZR-75-1 cells, a dose–response trend was obtained to determine the lowest effective concentration (Fig. 1): 5 μM and 1 μM olaparib were chosen as lethal and non-lethal concentrations for subsequent assays performed in APE1-downregulated cells. In accordance to the cytotoxicity result, olaparib treatment with concentrations at 2 μM or higher induced significant ROS production and accumulation in MCF-7 and ZR-75-1 cells (Fig. 2a, b). It was reported that cancer cells have a higher baseline level of ROS compared to healthy cells [26], which makes them more dependent on ROS protective and neutralizing signaling to maintain redox balance [27, 28]. Recently, APE1 has become a promising target for enhancing cancer therapy due to its involvement in two major biological functions: redox regulation of gene expression and DNA repair. APE1 redox is associated with the activity of a number of transcription factors, such as P53, NF-κB, cAMP response element binding protein, hypoxia-induced factor-1α, and STAT3, many of which are central regulative factors in the process of proliferation and apoptosis [29]. We found that olaparib treatment increased intracellular ROS level in a dose-dependent manner (Fig. 2a, b). The accumulated ROS may contribute cell death in the absent of APE1 redox, as evidenced by the increased sensitivity to olaparib treatment in APE1-silenced cells (Fig. 4c, d).

Since APE1 serves as a critical enzyme that is responsible for repairing DNA damage induced by radiation, oxidative stress, and chemotherapeutic drugs, inhibition of APE1 has shown antitumor effects in various cancers. Our data indicated that besides the inhibition of PARP1, olaparib remarkably decreases both mRNA and protein levels of APE1 (Fig. 3). Therefore, we further investigated whether downregulation of APE1 combined with olaparib could enhance

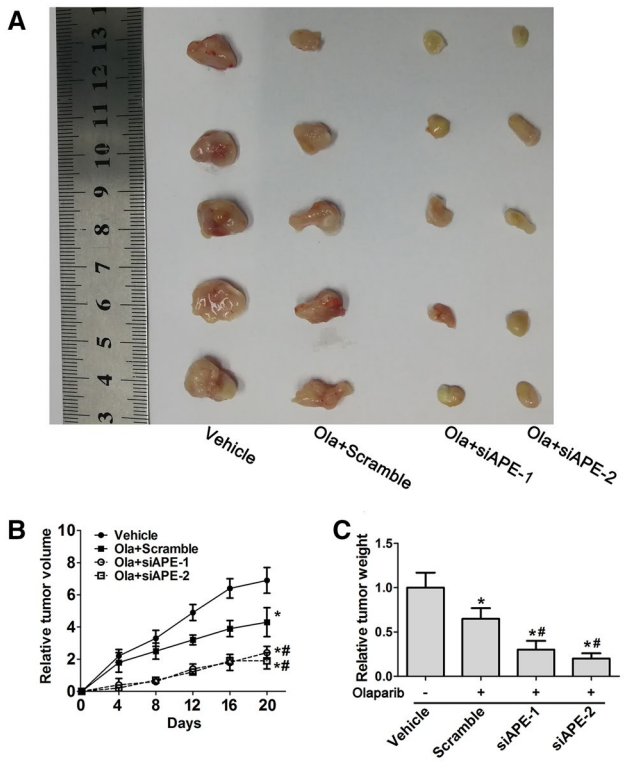

Fig. 6 Downregulation of APE1 sensitizes MCF-7 cells to olaparib in vivo. Forty-eight hours after transfected with two APE1-siRNAs or scramble siRNA, 1 × 106 MCF-7 cells were subcutaneously injected into left flank of SCID mice. Normal MCF-7 cells were used as control (Vehicle). Chemotherapeutic groups were intraperitoneal injected 50 mg/kg/day olaparib or vehicle via lateral tail vein, daily for 16 days. **a** Representative macroscopic overview of xenografted tumors. Tumor volume (**b**) and weight (**c**) were decreased in APE1-downregulated mice. $^{*}P < 0.05$ compared to vehicle control; $^{\#}P < 0.05$ compared to scramble siRNA

antitumor effect in the two HR-proficient breast cancer cells. Our results showed that deficient of APE1 significantly improved the inhibition of PARP1 induced by olaparib at non-lethal concentration, thus contributing to increased cancer cell death (Figs. 4, 5). Our in vivo test confirmed the synergistic effect between APE1 downregulation and olaparib treatment in the MCF-7 xenograft model, where APE1-downregulated xenografts exhibited significantly decreased volume and weight compared to control tumors (Fig. 6a, b).

Conclusion

The reduction of APE1 plus olaparib treatment enhances apoptosis in MCF-7 and ZR-75-1 cells in vitro, and suppresses MCF-7 xenografted tumor development in vivo. Thus, to acquire better benefits for HR-proficient breast cancer patients, developing chemotherapeutic drugs antagonize APE1 could be an effective strategy to improve the clinical outcome of PARP inhibitors. More works are needed to unveil the mechanism of the synergistic interaction between APE1 downregulation and PARP inhibitors.

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Data availability The datasets used during the present study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethics approval and Informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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