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Inhibition of PARP1 by small interfering RNA enhances docetaxel activity against human prostate cancer PC3 cells

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ABSTRACT

Though poly(ADP-ribose) polymerase 1 (PARP1) inhibitors have benefits in combination with radiotherapy in prostate cancers, few is known about the exactly role and underlying mechanism of PARP1 in combination with chemotherapy agents. Here our data revealed that inhibition of PARP1 by small interfering RNA (siRNA) could enhance docetaxel's activity against PC3 cells, which is associated with an accelerate repression of EGF/Akt/FOXO1 signaling pathway. Our results provide a novel role of PARP1 in transcription regulation of EGFR/Akt/FOXO1 signaling pathway and indicate that PARP1 siRNA combined with docetaxel can be an innovative treatment strategy to potentially improve outcomes in CRPC patients.

1. Introduction

Prostate cancer is the most frequently diagnosed cancer and becomes second-leading cause of cancer-related deaths in men worldwide [1,2]. However, the castration-resistant prostate cancer (CRPC) is still hard to treat successfully. Chemotherapy is one of the main means for the treatment of CRPC, but the toxicity of chemotherapy drugs and the emergence of drug resistance limit the tolerated dose and reduce the therapeutic efficacy, so developing a means to sensitize prostate cancer cells to cytotoxic chemotherapy are urgently needed. One possible approach is to knockdown proteins involved in cancer cell survival or proliferation using specific RNA interference.

As the most abundant member of the PARP family, PARP1 is activated by binding to DNA breaks and involved in base excision repair. Previous studies have shown that PARP1 is involved in various cellular processes, including DNA repair and transcriptional regulation as well as being a master component of several transcription factors involved in tumor genesis and development, which are not universally dependent upon its enzymatic activity [3,4]. Recently, it was reported that PARP inhibitors could enhance the cytotoxic effects of ionizing radiation and DNA-damaging chemotherapy agents, especially for the cell lines which are defective in homologous recombination [5,6]. Although the relevance of PARP1 in cancer treatment has increased in recent years, the mechanisms and impact underlying PARP1 mediated transcriptional regulation in human malignancy including prostate cancer remain unexplored.

In the present study, we show that siRNA-mediated inhibition of PARP1 could inhibit the proliferation and viability of CRPC PC3 cell lines, and sensitize PC3 cells to docetaxel treatment through accelerated inhibition of EGFR/Akt/FOXO1 signal transduction pathway. Furthermore, our data represent a novel role of PARP1, which is different from PARP1 inhibitor, in the regulation of Akt/FOXO1 pathway.

2. Materials and methods

2.1. Cell culture and drugs

The CRPC cell line PC3 was purchased from ATCC (USA) and cultured in DMEM/F12 medium supplemented with 10% (V/V) fetal bovine serum (GIBCO), 8×10^5 U/L penicillin and 0.1 g/L streptomycin in a humidified incubator containing 5% CO_2 at 37 °C. EGF, docetaxel and ABT888 were purchased from R&D Systems, Sigma–Aldrich and Enzo Life Science, respectively.

2.2. RNA interference and transfection

Small interfering RNA (siRNA) was synthesized by RiBoBio Co. Ltd (China). The sequences of human PARP1 siRNA were: 5′-GAGGAAGGUAUCAACAAAUTT-3′ (siRNA-1706), 5′-GAGCACUUCAUGAAAUUAUTT-3′ (siRNA-2003) and 5′-GAGACCC AAUAGGCUUAAUTT-3′ (siRNA-2907). The sequence of unrelated siRNA was 5′-UUCUC

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CGAACGUGUCACGUTT-3′ (siRNA-NC). The cultured PC3 cells were transfected with 50 pM siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

2.3. RNA amplification and quantitative real-time PCR analysis

Total RNA was isolated from untreated and siRNA-transfected cells by the Trizol (Takara) extraction method. An equal amount of RNA was reverse-transcribed to cDNA and amplified by PCR with the RNA PCR Kit (Takara) according to the manufacturer's protocol. Real-time PCR was performed with SYBR Green PCR Master mix and following the manufacturer's instruction. The primers were as follows: PARP1: 5′-AAGGCGAATGCCAGCGTTAC-3′ (Forward), 5′-GGCACTCTTGGAGACCATGTCA-3′ (Reverse); GAPDH: 5′-GCACCG TCA AGGCTGAGAAC-3′ (Forward), 5′-AUAAUUUCAUGAAGUGC-UCTT-3′ (Reverse). The real-time PCR reaction was maintained at 95 °C for 5 min, followed by 40 cycles of: 95 °C for 15 s, 57 °C for 30 s and 72 °C for 20 s. All samples were processed in triplicate and all values were normalized for the GAPDH expression levels. The relative quantification was determined by the comparative Ct method.

2.4. Western blot analysis

PC3 cells (5×10^5) were seeded onto 6-well plates. Forty-eight hours after transfection, cells were collected and washed twice by cold PBS, and each well was treated with 60 μL lysis buffer (2 mmol Tris–HCl, pH 7.4, 50 mmol NaCl, 25 mmol EDTA, 50 mmol NaF, 1.5 mmol Na_3VO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/L pepstatin, 10 mg/L aprotinin and 5 mg/L leupeptin) (Sigma). Protein concentrations were determined using the DC™ protein assay regents (Bio-Rad, USA). Equal amounts of protein (50 μg) were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were blocked in 5% non-fat dry milk in TBS-T (0.1% Tween-20, 150 mM NaCl, 25 mM Tris–HCl, pH 7.6) for 2 h at room temperature and incubated with primary antibodies appropriately diluted in TBS-T overnight at 4 °C. After three washes for 10 min with TBS-T, membranes were incubated for 1 h with secondary antibody appropriately diluted in TBS-T. Membranes were developed for chemiluminescence detection by using ECL detection kit (Pierce, USA) and Universal Hood II image system (Bio-Rad, USA) as recommended by the manufacturer. Quantity one software was used to quantify the intensities of bands. The primary antibodies used included those against PARP1, GAPDH (Santa Cruz), EGFR, phospho-EGFR (Tyr1068), phospho-Akt (Ser473), Akt, FOXO1 (Cell Signaling Technology), phospho-IGF-1R (Tyr1165/Tyr1166, SAB), and phospho-FOXO1 (Ser256, SAB). The secondary antibodies were anti-mouse and anti-rabbit IgG conjugated with HRP (Santa Cruz).

2.5. Cell proliferation and viability assay

Cell proliferation and viability was assessed by the Cell Titer 96® AQueous One Solution Cell Proliferation Assay kit (MTS, Promega). For the MTS assay, at the end of the experiment (96 h), 10 μL of MTS (5 mg/mL in PBS) were added and the cells were incubated for 2 h in the humidified incubator containing 5% CO_2 at 37 °C. Relative cell viability was obtained by scanning with an ELISA reader with a 490 nm filter.

2.6. Statistical analysis

Data are reported as means ± SD of at least three independent experiments. Mean differences were compared by ANOVA and the Student *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. siRNA mediated PARP1 inhibition inhibits the proliferation and viability of PC3 cells

To knock down the expression of PARP1 in PC3 cells, three small interfering RNAs and an unrelated RNA were synthesized and transfected into PC3 cells in accordance with the manufacturer's recommendations. The knockdown level of PARP1 was verified by real-time PCR and western blot. As shown in Fig. 1A (Top), comparing to untreated control PC3 cells, PARP1 siRNA 1706, 2003 and 2907 significantly decreased PARP1 mRNA levels to 16.61 ± 7.58%, 26.98 ± 8.95% and 41.75 ± 6.78% at 96 h post-transfection, respectively, but no significant difference was detected between control and unrelated siRNA transfected cells. Meanwhile, PARP1 protein levels were also obviously decreased by PARP1 siRNAs, (Fig. 1A, bottom). Then siRNA-1706 and siRNA-2003 were chosen to assess the impact of PARP1 expression on the proliferation and viability of PC3 cells through MTS assay. As shown in Fig. 1B and C, comparing to the control group, both siRNA-1706 and siRNA-2003, but not unrelated siRNA, significantly inhibited the proliferation and decreased the cell viability of PC3 cells.

3.2. siRNA mediated PARP1 inhibition enhances docetaxel's activity against PC3 cells

Docetaxel is widely used for the therapy of patients with advanced PCa [7,8]. Since docetaxel alone has been reported to cause severe mitotic arrest and cellular apoptosis [9,10], we designed a sequential treatment regime that involved initial pretreatment with PARP1-siRNA for 72 h followed by the addition of increasing concentrations of docetaxel for 24 h. As shown in Fig. 2A and B, docetaxel alone treatment inhibited the cell proliferation and viability of PC3 cells in a dose-dependent manner. Comparing to the cells treated with docetaxel alone, siRNA-mediated inhibition of PARP1 significantly increased docetaxel-induced inhibition of cell proliferation and viability. The effects of PARP1 siRNAs on docetaxel-treated cells were sequence-specific because no effect was observed for the unrelated siRNA transfected cells.

3.3. siRNA mediated PARP1 inhibition enhances docetaxel's activity against EGFR/Akt/FOXO1 signaling pathway

The EGFR/Akt/FOXO1 pathway is implicated in a number of cellular processes, including cell proliferation, survival, angiogenesis, DNA damage response and repair. We postulated that PARP1-siRNA inhibits the cell proliferation and viability through inhibiting EGF associated signals. As shown in Fig. 3A, EGF (100 ng/mL) could obviously induce the phosphorylation of EGFR, Akt and FOXO1, whereas has no effect on the expression of PARP1. In addition, both PARP1 siRNA-1706 and siRNA-2003 inhibited EGF-induced phosphorylation of EGFR as well as that of Akt and FOXO1. Furthermore, similar to their inhibitory effects on the EGFR/Akt/FOXO1 signal pathway, PARP1-siRNAs could inhibit IGF-1-induced phosphorylation of IGF-IR, Akt and FOXO1 (Fig. 3B). These results suggest that PARP1-siRNA-induced inhibition of proliferation and viability could be mediated through different signal transduction pathways.

We then examined the combination effect of PARP1 siRNA and docetaxel on the transduction of EGFR/Akt/FOXO1 signaling pathway. As shown in Fig. 3C, comparing to the treatment with PARP1-siRNAs or docetaxel alone, pretreatment with PARP1-siRNAs to knockdown PARP1 expression followed by the addition of docetaxel, could significantly further enhance the inhibition of EGF-induced phosphorylation of EGFR and its downstream targets Akt and FOXO1, which is dependent on the intensity of the PARP1 blocking by PARP1-siRNAs. In addition, co-treatment of docetaxel

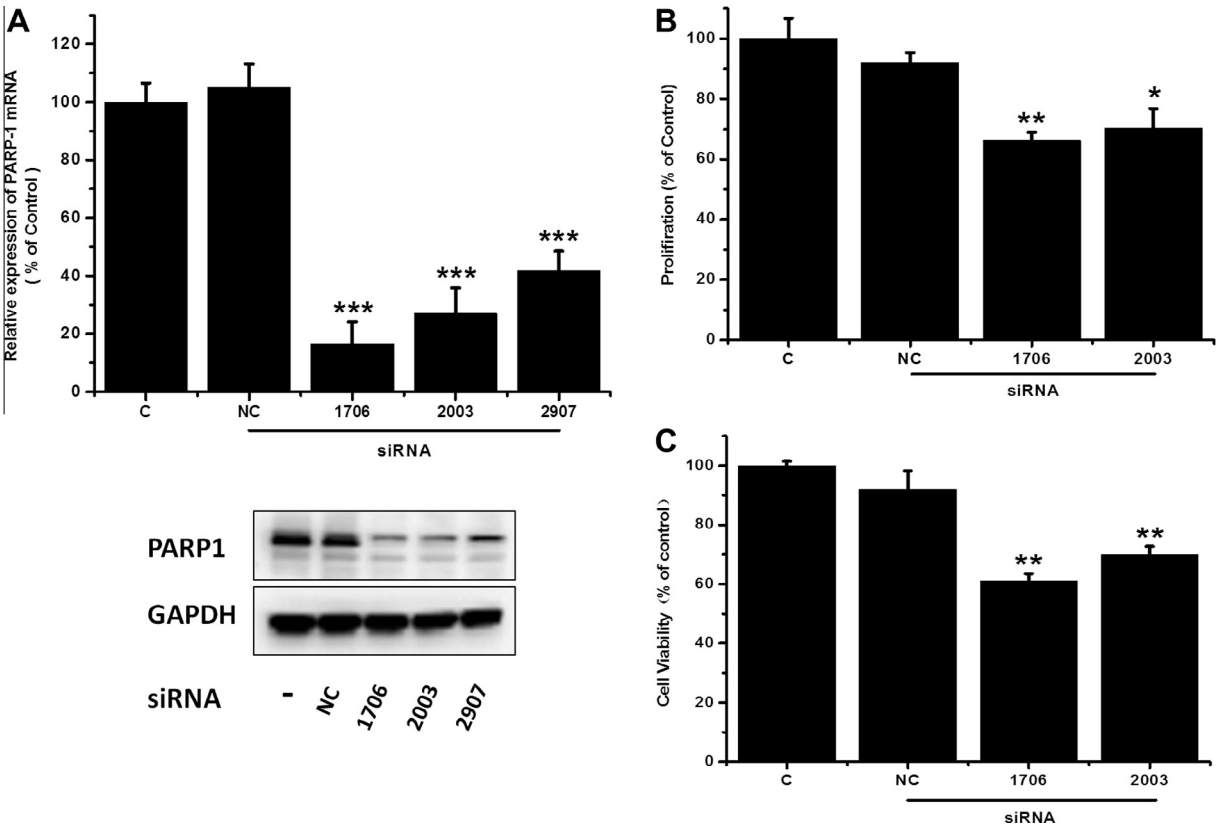

Fig. 1. siRNA-mediated PARP1 inhibition inhibits cell proliferation and viability in PC3 cells. (A) Ninety-six hours after transfection with the indicated siRNA, the expression of PARP1 and GAPDH were detected by real-time PCR and western blot, respectively. (B) and (C) Seventy-two hours after transfection with the indicated siRNA, equal amount cells were seeded in the 96-wells plate and cultured in the medium supplemented with 10% FBS (or 1% FBS) for 24 h, then cell proliferations (or viabilities) were detected by MTS array and were expressed as a percent of the untransfected cells. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the control group.

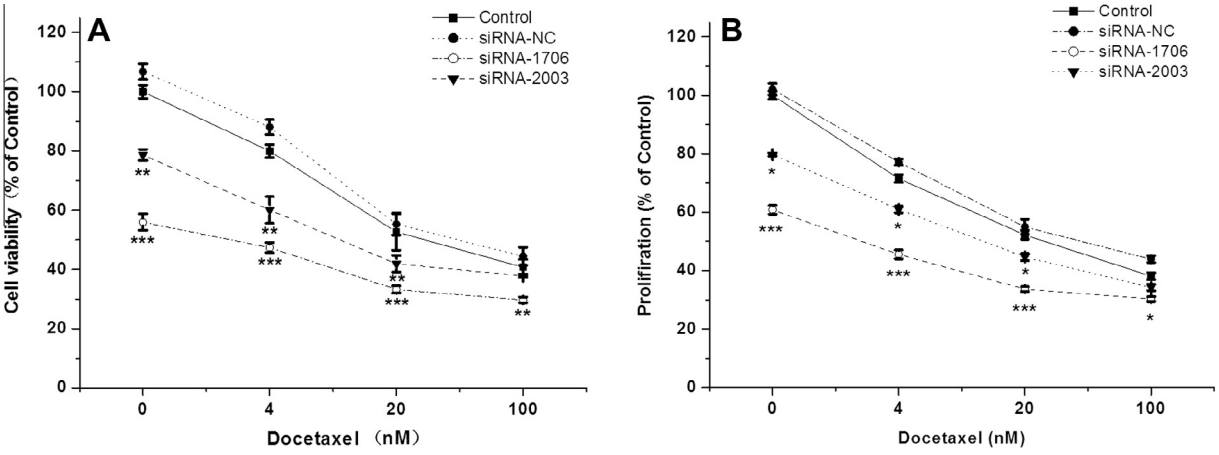

Fig. 2. Effects of siRNA-mediated PARP1 inhibition on docetaxel's activity against PC3 cells. (A) and (B) Seventy-two hours after transfection with the indicated siRNA, PC3 cells were incubated in the medium supplemented with 1% FBS (or 10% FBS) and increasing concentrations of docetaxel for 24 h, then the cell viabilities (or proliferations) were detected by MTS array and were expressed as a percent of the untransfected cells. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$with respect to the Control group.

with the PARP1 siRNA could further increase FOXO1 expression but decrease EGFR expression, whereas had no significant effect on the expression of Akt.

3.4. PARP1-siRNA, but not ABT888, inhibits the phosphorylation of Akt and FOXO1

Previous studies have shown that PARP inhibitor, such as DPQ, PJ-34, olaparib and ANI (4-amino-1,8-naphthalimide), could induce the phosphorylation of Akt, which contributed to the cytostatic resistance in different carcinoma cell lines [11–14]. As shown in the Fig. 4B, similar to the previous reports, ABT888, another well characterized PARP inhibitor, decreased the expression of PAR and EGFR, but had no effect on the expression of PARP1. In addition, ABT888 decreased the phosphorylation of EGFR but increased the phosphorylation of Akt and FOXO1 in a dose dependent manner, while having no significant effects on the expression of Akt and FOXO1. In contrast, siRNA-1706 inhibited the expression of both PAR and PARP1. Meanwhile, siRNA-1706 not only decreased the phosphorylation and expression of EGFR, but also inhibited the phosphorylation of Akt and FOXO1 in a dose dependent manner. Furthermore, siRNA-1706 increased the expression of FOXO1

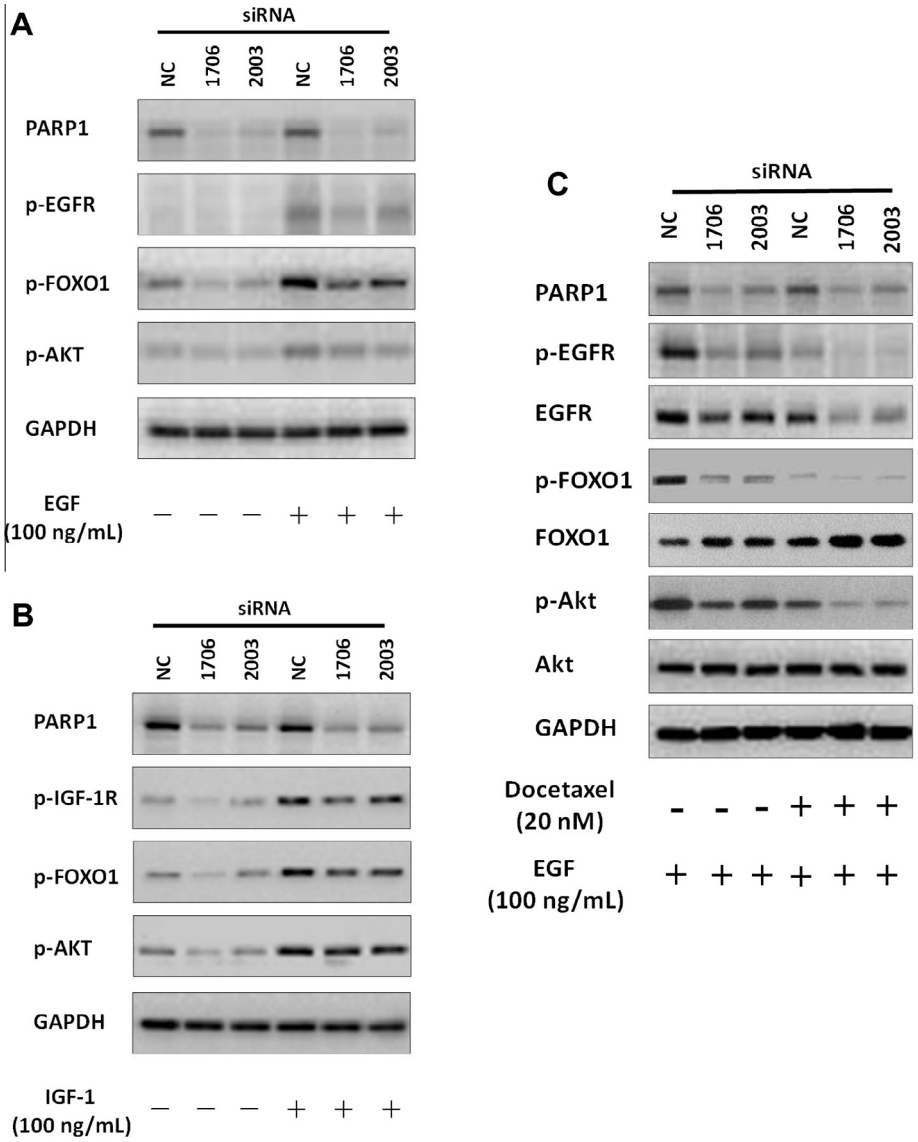

Fig. 3. Effects of siRNA-mediated PARP1 inhibition on docetaxel's activity against EGFR/Akt/FOXO1 signal transduction pathway. (A) and (B) Ninety-six hours after transfected with the indicated siRNA, PC3 cells were incubated in serum-free medium for 1 h, then EGF (100 ng/mL) or IGF-1 (100 ng/mL) was added and incubated for 15 min, then the expression of indicated proteins were detected by means of western blot. (C) Seventy-two hours after transfection with the indicated siRNA, PC3 cells were incubated with docetaxel (20 nM) for 24 h followed by the addition of EGF (100 ng/mL) for 15 min, then the expression of indicated proteins were detected by means of western blot.

(Fig. 4A). These results indicate that the transduction mechanisms of Akt and FOXO1 mediated by PARP inhibitor and PARP1-siRNA are different.

4. Discussion

As its prognosis is extremely poor and no efficient cure is available for the "castrate-resistant" stage of the disease, prostate cancer has become a major health problem in men worldwide and alternatives are urgently required to sensitize prostate cancer cells to cytotoxic chemotherapy or radiotherapy. Recent studies have shown that PARP1 inhibitors can enhance the sensitivity of both radiotherapy and various chemotherapeutic agents in multiple carcinoma models, such as the cell lines of breast cancer, lioblastoma, lung cancer, melanoma, colorectal and cervical carcinoma, but the sensitizing effects and underlying mechanism of PARP inhibition in combining with chemotherapy regents in prostate cancer is still unclear [3,5,15]. In the present study, our results first indicated that PARP1-siRNA could sensitize human prostate cancer PC3 cells to docetaxel treatment, which is associated with an accelerated inhibition of EGFR/Akt/FOXO1 signal transduction pathway, and combining PARP1-siRNA with docetaxel may provide a potential effective therapy for human CRPC.

Docetaxel is an antimitotic cytotoxic drug that widely used in the treatment of advanced PCa patients. Its anticancer effects are associated with its ability against cell proliferation and apoptosis [7,8,16]. However, docetaxel-based chemotherapy has unexpected side effects that limit the tolerated dose which may reduce its therapeutic efficacy [17], suggesting that combination therapy result in a reduction of the required dose of docetaxel would be beneficial to PCa therapy. One possible approach is to knockdown the proteins involved in cancer cell proliferation or survival using small interfering RNA (siRNA), or inhibit its transduction activity using specific inhibitor. Sakamaki et al. [18] showed that PARP1 inhibition mediated by siRNA led to a significant decrease in cell proliferation of HEK293 cells. In addition, both the addition of PARP1 inhibitor (ANI) and depletion by siRNA significantly enhanced the growth

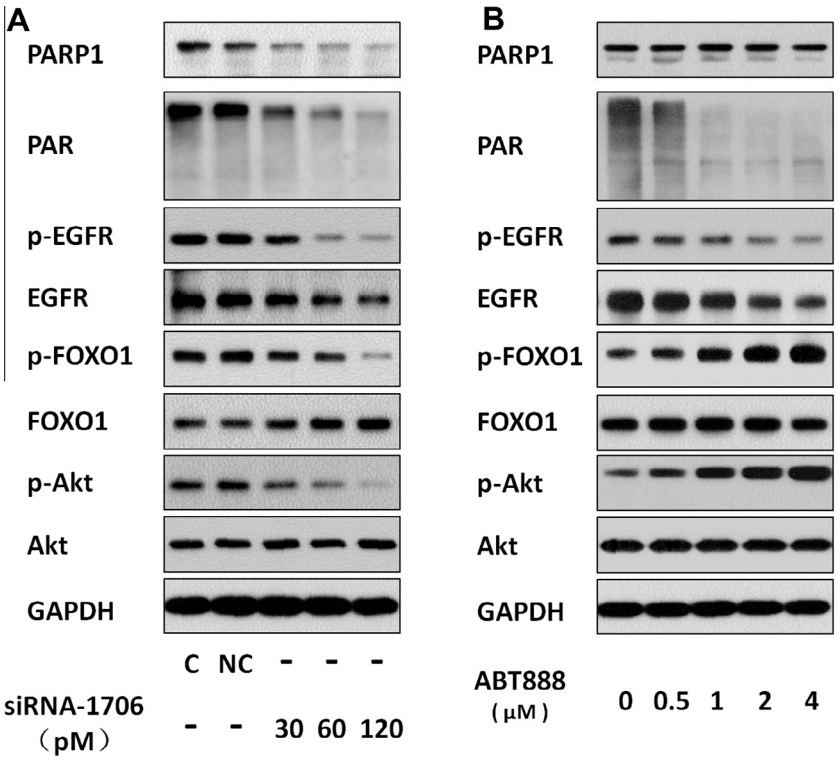

Fig. 4. Different effects of PARP1 siRNA and ABT888 on the EGFR/Akt/FOXO1 signaling pathway. (A) Ninety-six hours after transfection with the indicated siRNA, the expression of indicated proteins were detected by means of western blot. (B) PC3 cells were incubated in the medium supplemented with 1% FBS and indicated concentrations of ABT888 for 48 h, then the expression of indicated proteins were detected by means of western blot.

inhibition induced by doxorubicin and sensitized human liver cancer cell lines to doxorubicin treatment [12]. Though PARP inhibitor ABT-888 (Veliparib) can enhance the response of prostate PC3 cells and tumors to ionizing radiation (IR) which may partially depend on a competent senescence response to accumulated DNA damage [19], few is known about the effects of PARP1 inhibitor or PARP1-siRNA combining with cytotoxic chemotherapy regents in prostate cancer.

Previous clonogenic assays have indicated that ABT-888 alone induced a significant inhibition in colony formation and increased the G2-M fraction in PC3 cells [19]. In contrast, Matthew et al. [20] demonstrated that inhibition of PARP1 activity by ABT888 only resulted in reduced growth of AR-positive prostate cancer cells and no effect was seen in AR-negative prostate cancer cells, such as PC3 cells and DU145 cells. The different concentrations of ABT888, 10 and 2.5 μM, used in the two studies may account for the contradictory results. To confirm the role of PARP1 in the cell proliferation and viability of PC3 cells, the effects of PARP1 inhibition mediated by siRNAs were detected in this study. Similar to the former report, our data indicated that PARP1-siRNA mediated PARP1 inhibition induced a significant decrease in cell proliferation and viability of PC3 cells, suggesting that PARP1 is indeed involved in the regulation of cell proliferation and viability in PC3 cells. Furthermore, siRNA-mediated inhibition of PARP1 significantly increased docetaxel's activity against cell proliferation and viability in PC3 cells.

Abnormally elevated expression and activity of EGFR are associated with most common cancers, including prostate cancer. EGFR-driven intracellular signaling controls not only cell proliferation but also the processes that are important for tumor progression, including apoptosis, invasion, angiogenesis, and metastasis [21,22]. Elevated EGFR expression and activity have been associated with the progressive and metastatic growth of prostate cancer [22–26], thus, inhibition of EGFR expression or activity is promising for successful treatment of prostate cancer. Previous studies have demonstrated that both PARP1 inhibition mediated by PARP inhibitors or specific siRNA could led to a decrease expression and activity of EGFR in live or breast cancer [12,13]. In addition, PARP inhibitor ABT888 could reduce radiation-induced nuclear EGFR and augment head and neck tumor response to radiotherapy [27]. Noticeably, as PARP inhibitors are designed to compete with NAD^+ at the enzyme active site, they universally have the potential to inhibit other enzymes that use NAD^+, including other members of the PARP family, though the extent to which they do so is still largely unknown. Since PARP inhibitors inhibit the enzymatic activity but not the expression of PARP1, the exactly role of PARP1 in the transcriptional regulation which is not universally dependent upon its enzymatic activity is still unclear [28,29]. Good in consistent with the previous studies, our results also showed that PARP1 siRNA, including both siRNA-1706 and siRNA-2003, decreased EGFR expression and inhibited EGF-induced phosphorylation of EGFR.

Akt, a key downstream target of EGFR, is an important regulator of cell proliferation and survival which contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis [30]. Previous studies [11,13] have demonstrated that treatment of PARP inhibitor, such as PJ34 and olaparib, resulted in increased phosphorylation of Akt that may contribute resistance to chemotherapy regents in T24 human bladder carcinoma cells and BRCA1-related breast cancer, respectively. Interestingly, our results showed that treatment of PARP1 siRNA inhibited but not accelerated the phosphorylation of Akt, whereas ABT888 accelerated the phosphorylation of Akt in PC3 cells. Moreover, our data revealed that the levels of PARP1 were associated with the phosphorylation levels of Akt in PC3 cells. As PARP inhibitors are designed to compete with NAD^+ at the enzyme active site and have no effect on the expression of PARPs, together with their potential effect on other members of the PARP family is largely unknown, we hypotheses that the potency and specificity of PARP inhibitors to

PARP1 and their invalid effect on the PARP1 expression are, at least in part, account for the different effects of PARP inhibitor and PARP1-siRNA on the phosphorylation of Akt.

Docetaxel's anticancer effect is associated with its ability to induce the expression and nuclear accumulation of FOXO1, a key downstream of Akt that becomes inactive when phosphorylated by Akt, in breast cancer, ovarian cancer and 22Rv1 CRPC cells [31]. In addition, PARP1 could directly bind to FOXO1 and repress its transactivation function independently of PARP enzymatic activity [18]. Furthermore, knockdown of PARP1 resulted in inhibition of cell proliferation in a FOXO1-dependent manner in HEK293 cells, suggesting PARP1 functions as a negative regulator of FOXO1 transcription factor. In the present study, co-treatment of docetaxel with the PARP1 depletion by siRNA significantly reduced FOXO1 expression, as well as enhanced inhibition of EGF-induced phosphorylation of EGFR, Akt and FOXO1 in PC3 cells. Moreover, the levels of PARP1 in tumoural cells were also associated with cell proliferation and viability, together with the FOXO1 expression and phosphorylation of EGFR, Akt and FOXO1. These data indicate siRNA-mediated PARP1 depletion augment docetaxel's activity to prostate cancer PC3 cells through, at least in part, EGFR/Akt/FOXO1 transduction signaling pathway.

Induction of accelerated senescence is a novel therapeutic approach in clinical trials. Our data demonstrate for the first time that siRNA-mediated PARP1 depletion augments the antitumoral effects of docetaxel through accelerated inhibition of EGFR/Akt/FOXO1 signal pathway, which is partly different from PARP inhibitors, suggesting that combination of PARP1-siRNA and docetaxel can be an innovative treatment strategy to potentially improve outcomes in CRPC patients. Though previous studies have shown that PARP inhibitors are strong candidates for becoming reliable senescence-inducing agents, their potency and specificity still need further improvement.

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