



Thioredoxin 1 upregulates FOXO1 transcriptional activity in drug resistance in ovarian cancer cells



Jianlin Wang, Hao Yang, Wenjie Li, Huibi Xu, Xiangliang Yang, Lu Gan*

Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

ARTICLE INFO

Article history:

Received 10 September 2014
Received in revised form 24 November 2014
Accepted 1 December 2014
Available online 5 December 2014

Keywords:

Trx1
FOXO1
Drug resistance
ROS
Ovarian cancer cells

ABSTRACT

Drug resistance is the major cause of failure of cancer chemotherapy in ovarian cancer. However, the molecular mechanisms on the regulation of drug resistance are not fully understood. Here we showed that Trx1 and FOXO1 were involved in paclitaxel (PTX)-induced drug resistance in ovarian cancer A2780 cells. PTX induced reactive oxygen species (ROS) and resulted in Trx1 and FOXO1 nuclear translocation. We further found that Trx1 bound to FOXO1 and enhanced FOXO1 transcriptional activity; however Trx1 C69S mutant which is barely detected in the nucleus downregulated Trx1–FOXO1 interaction and Trx1-induced FOXO1 transcriptional activation. Silencing of FOXO1 abrogated Trx1-induced drug resistance. Trx1 increased FOXO1-induced drug resistance, while Trx1 C69S mutant completely abolished the regulation of FOXO1-mediated drug resistance by Trx1. These findings provided a novel mechanism on Trx1/FOXO1 signaling in drug resistance in ovarian cancer cells.

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1. Introduction

Ovarian carcinoma is the major cause of gynecological cancer-related mortality worldwide. The main limitation to a successful treatment for ovarian cancer is the development of drug resistance [1]. Several mechanisms on drug resistance in cancer have been elucidated, including increased DNA repair [2] and overexpression of drug efflux transporters, such as P-glycoprotein (P-gp) [3,4] and antiapoptotic proteins, such as Bcl-2 [5]. However the precise molecular mechanisms underlying drug resistance in ovarian cancer are far from fully elucidated.

Thioredoxin 1 (Trx1), a ubiquitously expressed small redox protein which has a conserved Cys-Gly-Pro-Cys redox catalytic site, plays critical roles in the regulation of cellular redox homeostasis [6]. Trx1 levels have been found to be overexpressed in a wide variety of human tumors

including ovarian cancer [7–9]. Elevated Trx1 is associated with increased tumor cell proliferation [10,11], inhibition of spontaneous and drug-induced apoptosis [12,13], aggressive tumor growth and decreased patient survival [14], which makes Trx1 an attractive target for cancer therapy [15]. It has been shown that Trx1 might regulate cell survival by activating a number of transcription factors, such as NF- κ B and AP-1 [16]. Trx1 also has been reported to bind to, and thus inhibit proapoptotic proteins including apoptosis signal regulating kinase-1 (Ask-1) [17] and the tumor suppressor PTEN, a protein which antagonizes the activity of the PI3K/Akt pathway [18].

Forkhead box Class O (FOXO) proteins, which include FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6 in humans, act downstream of the PI3K/Akt pathway and are involved in various cellular processes including cell cycle arrest, differentiation, apoptosis and resistance to oxidative stress and DNA damage [19]. FOXO function is modulated by phosphorylation, acetylation, ubiquitination and protein–protein interactions, which affects nuclear–cytoplasmic translocation, DNA binding and ultimately target gene expression [20]. Recent papers showed that FOXO proteins played an important role in protection of cancer cells against chemotherapy-induced apoptosis [21–24]. For example, Goto *T et al.* reported that FOXO1 contributed to paclitaxel (PTX)-induced drug resistance in ovarian cancer [24]. In the present study, we showed that the role of FOXO1 in PTX resistance was positively regulated by Trx1 through a protein–protein interaction and the effects might depend on Trx1 nuclear translocation which was mediated by PTX-induced reactive oxygen species (ROS) in ovarian cancer cells.

Abbreviations: FOXO, forkhead box class O; Trx1, thioredoxin 1; P-gp, P-glycoprotein; PTX, paclitaxel; ROS, reactive oxygen species; DTNB, 5,5'-dithio-bis(2-nitrobenzoic) acid; H₂DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; HPLC, high performance liquid chromatography; DHE, dihydroethidium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; BCA, bicinchoninic acid; GST, glutathione S-transferase; PI, propidium iodide; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; ChIP, chromatin immunoprecipitation; MnSOD, manganese superoxide dismutase; shRNA, short hairpin RNA; NAC, N-acetyl-cysteine; PARP, poly(ADP-ribose) polymerase

* Corresponding author at: College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China. Tel.: +86 27 87792147; fax: +86 27 87792234.

E-mail address: lugan@mail.hust.edu.cn (L. Gan).

2. Materials and methods

2.1. Cell culture

The human ovarian cancer cell line A2780 was kindly provided by Dr. Ding Ma (Huazhong University of Science and Technology, Wuhan, China). The PTX-resistant A2780 cells (A2780/PTX) were purchased from KeyGen Biotech Co. Ltd (Nanjing, China). A2780 cells were cultured in RPMI 1640 medium and A2780/PTX cells were cultured in RPMI 1640 medium containing 900 nM PTX at 37 °C in 5% CO₂ atmosphere. All media contained 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2. Plasmids, short hairpin RNAs (shRNAs), and chemicals

Plasmids for wild type FLAG-FOXO1, V5-FOXO1, the *Renilla* luciferase reporter vector and the luciferase reporter construct, 3 × IRS, which contains three copies of the FOXO response element in the promoter of the IGFBP1 gene were kindly provided by Dr. Haojie Huang (Mayo Clinic, Rochester, MO, USA). Constructs for GST-FOXO1 recombinant proteins were described earlier [25]. The full-length cDNA of Trx1 was amplified from A2780 cells and then cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and p3 × FLAG-CMV-7.1 (Sigma-Aldrich, St Louis, MO, USA). Plasmids expressing Trx1 C69S and Trx1 C32/35S were generated by PCR-based mutagenesis according to the manufacturer's instruction (Stratagene, La Jolla, CA, USA) and verified by sequencing. For construction of Trx1 shRNA and FOXO1 shRNA plasmids, sequences used to knock down endogenous Trx1 (5'-ATGACTGTTCAGGATGTTGC-3') and FOXO1 (5'-CCAGATGCCTATACAAACA-3') were inserted into pCMS4-H1p-EGFP which contains an H1 promoter for shRNA expression, a CMV promoter for expression of shRNA-resistant cDNAs, and an SV40 promoter controlling EGFP expression and then identified by sequencing [26]. PTX was purchased from Taihua Natural Plant Pharmaceutical Co. Ltd (Xi'an, China).

2.3. Gene transfection and luciferase reporter assay

Transient transfection of the cells was done by electroporation as described [25]. Transfection efficiencies (~75–90%) were routinely achieved. For luciferase reporter assays for FOXO1 transcriptional activity [25], cells were transfected with the indicated plasmids in combination with plasmids for 3 × IRS-Luc firefly and *Renilla* luciferase reporter genes. After 36 h transfection, cells were harvested and the activities of firefly and *Renilla* luciferase in cell lysates were measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). *Renilla* luciferase activities of cells were used as internal control.

2.4. Trx1 activity assay

The activity of Trx1 in cell lysates was determined using insulin disulfides as substrate [27]. Briefly, 30 µg of sample proteins was pre-incubated with 1 µl of 1 mM DTT at 37 °C for 20 min. The samples were then incubated with buffer containing 85 mM HEPES (pH 7.5), 660 µM NADPH, 3 mM EDTA, 0.3 mM insulin and 0.08 unit (as defined by the manufacturer) of rat recombinant Trx reductase (TrxR, Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 2 h in a total volume of 50 µl. For each sample, a blank containing all reagents except TrxR was incubated and treated in the same manner. The reaction was terminated by addition of 0.5 ml of 8 M guanidine-HCl and 0.4 mg/ml of 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) in 0.2 M Tris-Cl (pH 8.0). Absorbance at 412 nm was measured with a UV-VIS spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) and the absorbance of the blank was subtracted from that of the sample.

2.5. Measurement of ROS generation

Intracellular ROS levels were measured using the oxidant-sensitive fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Molecular Probes, Eugene, OR, USA) [28]. Briefly, after treatment with different concentrations of PTX for 24 h, the cells were stained with 10 µM H₂DCF-DA in phosphate buffered saline (PBS) at 37 °C for 30 min, then washed with PBS twice to remove the extracellular H₂DCF-DA. The intracellular ROS were analyzed with F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan).

2.6. Hydrogen peroxide (H₂O₂) measurement

Extracellular H₂O₂ was detected using Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen, Carlsbad, CA, USA) [29]. Briefly, after treatment with different concentrations of PTX for 24 h, cells were suspended in Krebs-Ringer phosphate glucose (KRPG; 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl₂, 1.22 mM MgSO₄ and 5.5 mM D-glucose, pH 7.35) containing Amplex red reagent. Fluorescence was measured with excitation at 530 nm and emission at 590 nm using a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 37 °C.

2.7. Detection of intracellular superoxide formation

Superoxide levels were quantified using high performance liquid chromatography (HPLC) analysis of 2-hydroxyethidium formation as described [30]. Briefly, after treatment with different concentrations of PTX for 24 h, the cells were washed with PBS and then treated with 10 µM dihydroethidium (DHE, Sigma-Aldrich, St Louis, MO, USA) in the dark for 30 min. The cells were harvested and cell pellets were lysed in 250 µl lysis buffer (0.1% Triton X-100 in PBS, pH7.4). After n-butanol extraction and drying in 100% N₂, sample residues were reconstituted in 100 µl H₂O, centrifuged and the supernatants were transferred to amber-colored HPLC vials for HPLC analysis. Typically, 50 µl of sample was injected into the HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA, USA) with a C-18 reverse phase column (Supelco Nucleosil C18, 250 mm × 4.6 mm, 5 µm, 120 Å; Sigma-Aldrich, St Louis, MO, USA) equilibrated with 10% acetonitrile in 0.1% trifluoroacetic acid (TFA) solution. Hydroethidine, ethidium and 2-hydroxyethidium were separated by a linear increase in acetonitrile concentration. The elution was monitored by a variable UV detector at 210 and 350 nm and a fluorescence detector with excitation and emission at 510 and 595 nm, respectively. The peak area corresponding to 2-hydroxyethidium formation was quantified and corrected with the protein concentration of the sample. The protein content was determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.

2.8. Cell cytotoxicity assay

Cell cytotoxicity was determined using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay [31]. Briefly, cells were transfected with the indicated plasmids. After 48 h transfection, the cells were plated in 96-well plates at a density of 5 × 10³ cells per well. The cells were treated with different concentrations of PTX for 24 h. The cells were washed with PBS and then 20 µl of 5 mg/ml MTT solution was added to the cells in each well. Plates were incubated for an additional 2 h at 37 °C. The medium containing MTT was removed and 150 µl of DMSO was added to dissolve the formazan crystals formed by living cells. Absorbance was measured at 490 nm using a Labsystems iEMS microplate reader (Helsinki, Finland).

2.9. Apoptotic cell death assay

The apoptosis induced by PTX was detected using propidium iodide (PI) staining [32]. Briefly, A2780 cells were transfected with the indicated plasmids. At 36 h after transfection, the cells were treated with 25 nM PTX. After 24 h treatment, the cells were collected and washed with PBS. After fixation with 70% ethanol, cells were washed twice with PBS and stained with a solution containing 20 µg/ml PI and 50 µg/ml RNase A. Cells were incubated for 30 min at room temperature and sub-G1 cells were measured using a Cytomics™ FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA).

2.10. GST recombinant protein purification and GST-pull down assay

GST protein and GST-FOXO1 recombinant proteins were expressed in BL21 Star (DE3) *Escherichia coli* strain (Invitrogen, Carlsbad, CA, USA) and purified with glutathione-agarose beads (Sigma-Aldrich, St Louis, MO, USA). GST-pull down assay was performed as described [25]. Briefly, approximately 10 µg of GST fusion proteins was incubated with 50 µg of A2780/PTX cell lysates overnight at 4 °C. Protein complexes were collected on glutathione-agarose beads and evaluated by western blotting.

2.11. Immunoprecipitation, western blotting, immunofluorescence staining and antibodies

Protein immunoprecipitations were carried out using an immunoprecipitation kit (Roche Applied Science, Mannheim, Germany) as described [25]. Western blotting and immunofluorescence staining were performed as described [25]. The antibodies used were anti-FOXO1 and anti-PARP (Cell Signaling Technology, Danvers, MA, USA); anti-FLAG (M2) (Sigma-Aldrich, St Louis, MO, USA); anti-Trx1 and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-V5 (Invitrogen, Carlsbad, CA, USA).

2.12. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total cellular RNAs were isolated from transfected cells using Trizol (Invitrogen, Carlsbad, CA, USA), and cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was done using primers specific for catalase (forward 5'-AGAGGAAACG TCTGTGTGAGAACA-3' and reverse 5'-TGACCGCTTCTTCTGGATGA-3'), manganese superoxide dismutase (MnSOD, forward 5'-GGCTGATTA TCTAA AAGCTATTGG-3' and reverse 5'-CGATCGTGGTTACTTTTGC-3'), GADD45 (forward 5'-CCATGCAGGAAGGAAAATATG-3' and reverse 5'-CCCAAATATGGC TGCACACT-3'), and glyceraldehyde-3-phosphate dehydrogenase (forward 5'-GAAGGT GAAGTTCGGAGTC-3' and reverse 5'-GA AGATGGTGATGGGATTC-3'). Expression levels of genes examined were determined using a SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an iCycler iQ platform (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol [33]. Reactions were carried out in triplicate and gene expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase.

2.13. Chromatin immunoprecipitation (ChIP) assay

A2780 cells were transfected with the indicated plasmids. At 48 h after transfection, the cells were crosslinked with 1% formaldehyde and subjected to ChIP assay as previously described [33]. The soluble chromatin was incubated with 2 µg of anti-FLAG (M2) or control mouse IgG overnight. The ChIP DNA was extracted with a PCR purification kit (Invitrogen, Carlsbad, CA, USA) and subjected to PCR amplification using the primers specific for the FOXO1 binding region in the promoter of MnSOD (forward 5'-TCTGACGTCTGTAAACAAGCCCAG-3'

and reverse 5'-TTCTTCTCGCGCTGTCTGTAGC-3'). The PCR products were analyzed by 2% agarose gels and stained with ethidium bromide.

2.14. Statistics

Experiments were carried out with three or four replicates. Statistical analyses were performed by Student's *t* test. Values with *P* < 0.05 are considered significant.

3. Results

3.1. Trx1 and FOXO1 are involved in drug resistance in ovarian cancer cells

To explore the molecular pathways involved in drug resistance in ovarian cancer, the PTX-sensitive human ovarian cancer A2780 cells and the PTX-resistant A2780 cells (A2780/PTX) were used. As one might expect, the IC₅₀ value of PTX in A2780/PTX cells was about 570-fold higher than that in A2780 cells [34], confirming that A2780/PTX cells possessed considerably higher drug resistance to PTX compared with A2780 cells. As shown in Fig. S1, A2780/PTX cells expressed increased levels of Trx1 and FOXO1 relative to parental A2780 cells. Correspondingly, Trx1 and FOXO1 protein levels were markedly induced by PTX in a dose-dependent manner at 48 h after treatment in A2780 cells (Fig. 1A). However, PTX-stimulated expressions of Trx1 and FOXO1 were very limited in A2780/PTX cells (Fig. 1B). Furthermore, the activities of Trx1 and FOXO1 were also found to be upregulated in response to PTX treatment in A2780 cells, but not in A2780/PTX cells (Fig. 1C and D). These data indicated that the induction of Trx1 and FOXO1 might correlate with drug resistance in ovarian cancer cells.

To further verify whether drug resistance in ovarian cancer was mediated by Trx1 or FOXO1, the effects of Trx1 shRNA and FOXO1 shRNA on the drug response of A2780/PTX cells were evaluated. As shown in Fig. 1E and F, silencing of Trx1 or FOXO1 significantly decreased the cell viability of A2780/PTX cells in response to PTX treatment compared with control shRNA, confirming that FOXO1 and Trx1 were important mediators in drug resistance in ovarian cancer cells.

3.2. ROS are involved in PTX-induced cytotoxicity in ovarian cancer cells

Low levels of ROS have been implicated as intracellular signaling molecules in cellular processes such as proliferation, apoptosis and senescence [35]. As shown in Fig. 2A, PTX treatment significantly increased total intracellular ROS detected by H₂DCF-DA in A2780 cells. Accordingly, there was a marked increase in the generation of extracellular H₂O₂ and intracellular superoxide in PTX-treated A2780 cells (Fig. 2B and C). However, no significant difference in total intracellular ROS, extracellular H₂O₂ and intracellular superoxide was observed when A2780/PTX cells were treated with PTX, suggesting that ROS might be involved in PTX-induced cell death in ovarian cancer cells. To further confirm the role of ROS in PTX-induced cytotoxicity in A2780 cells, the effects of N-acetyl-cysteine (NAC), a powerful antioxidant and free radical scavenger, on PTX-induced ROS generation and cytotoxicity were examined. As shown in Fig. 2D and E, co-treatment with NAC fully reversed PTX-induced increase in ROS and cytotoxicity in A2780 cells. These data indicated that ROS played an important role in PTX-induced cytotoxicity in A2780 cells.

3.3. PTX-induced ROS cause the activation and nuclear translocation of Trx1 and FOXO1 in ovarian cancer cells

To explore the role of ROS in Trx1- or FOXO1-mediated drug resistance in ovarian cancer, the effects of PTX-induced ROS on the activities of Trx1 and FOXO1 were firstly examined in A2780 cells. As shown in Fig. 3A and B, PTX increased the activities of Trx1 and FOXO1 in A2780 cells, while co-treatment with NAC abolished the upregulation of Trx1 and FOXO1 activities induced by PTX. Furthermore the effects of PTX-

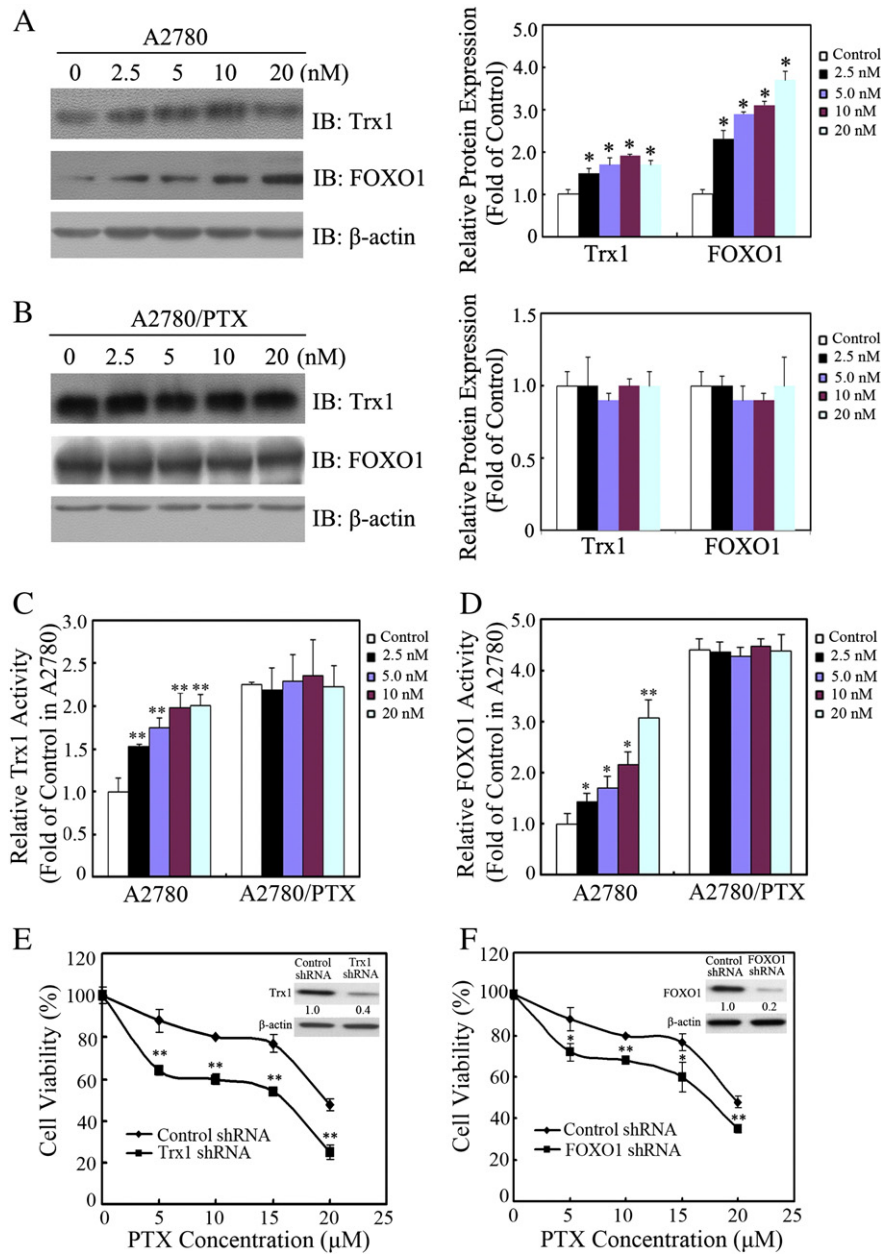


Fig. 1. Trx1 and FOXO1 confer PTX resistance in ovarian cancer cells. (A, B) Induction of Trx1 and FOXO1 protein expressions by PTX treatment in A2780 and A2780/PTX cells. A2780 cells (A) and A2780/PTX cells (B) were treated with different concentrations of PTX for 24 h. The cells were harvested and subjected to western blotting analysis for expression of Trx1 and FOXO1 proteins. β -actin was used as a loading control. Results from one representative experiment were shown on the left. Quantitative results were shown on the right. Data as mean values \pm S.D. ($n = 3$). * $P < 0.01$. (C, D) Induction of Trx1 and FOXO1 activities by PTX treatment in A2780 and A2780/PTX cells. A2780 cells and A2780/PTX cells were treated with different concentrations of PTX for 24 h. The cells were harvested and the activities of Trx1 (C) and FOXO1 (D) were determined by insulin assay and luciferase reporter assay, respectively. Error bars indicated S.D. among three individual experiments. * $P < 0.05$, ** $P < 0.01$ compared with control group. (E, F) Effects of knocking down Trx1 or FOXO1 on the viability of A2780/PTX cells in response to PTX treatment. A2780/PTX cells were transfected with control shRNA, Trx1 shRNA (E) or FOXO1 shRNA (F). At 48 h after transfection, the cells were treated with different concentrations of PTX for 24 h and the cell viability was measured using MTT assay. Error bars indicated S.D. among three individual experiments. * $P < 0.05$, ** $P < 0.01$ compared with control shRNA group at the corresponding PTX concentration. The inserts showed the protein expression of Trx1 or FOXO1 after transfection with Trx1 shRNA or FOXO1 shRNA.

induced ROS on the subcellular localization of Trx1 and FOXO1 were determined by immunofluorescence staining considering that nuclear translocation was an indicator of the activation of FOXO1 and Trx1 proteins [19,36]. As shown in Fig. 3C and D, endogenous Trx1 and FOXO1 were localized in both the nucleus and the cytosol in A2780 cells. However, more Trx1 and FOXO1 were localized in nucleus when A2780 cells were treated with PTX. Moreover, co-treatment with NAC inhibited the nuclear translocation of Trx1 and FOXO1 induced by PTX. These data suggested that PTX-induced ROS resulted in the activation and nuclear translocation of Trx1 and FOXO1 in ovarian cancer cells.

3.4. Trx1 increases FOXO1 transcriptional activity in ovarian cancer cells

Given that both Trx1 and FOXO1 were responsible for drug resistance in ovarian cancer cells, we attempted to explore whether Trx1 and FOXO1 were likely to be involved in the same signaling pathway leading to drug resistance. To test this notion, the effects of Trx1 on FOXO1 transcriptional activity were determined using a FOXO1 luciferase reporter gene that contains three copies of forkhead response elements. As shown in Fig. 4A, forced expression of Trx1 enhanced the transcriptional activity of both endogenous FOXO1 and exogenous FOXO1 in a

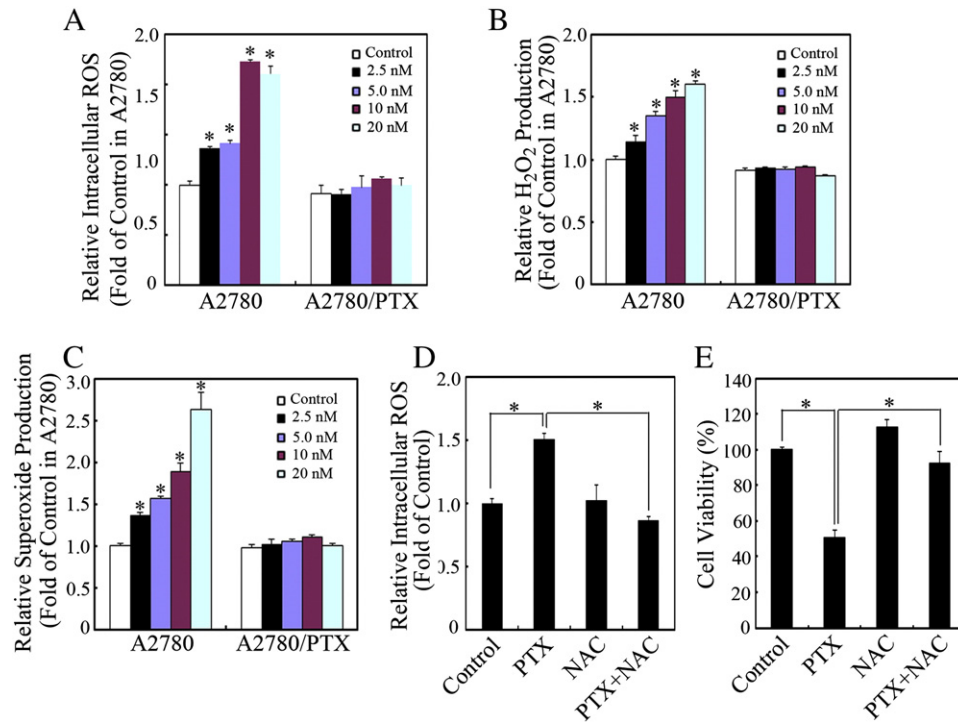


Fig. 2. ROS are involved in PTX-induced cytotoxicity in ovarian cancer cells. (A–C) Effects of PTX on the production of total intracellular ROS, extracellular H₂O₂ and intracellular superoxide in A2780 and A2780/PTX cells. The cells were treated with different concentrations of PTX for 24 h. The cells were harvested, and total intracellular ROS (A), extracellular H₂O₂ (B) and intracellular superoxide (C) were determined using H₂DCF-DA staining, Amplex Red Hydrogen Peroxide/Peroxidase assay and HPLC, respectively. Error bars indicated S.D. among three individual experiments. **P* < 0.01 compared with control group. (D, E) Effects of NAC on the PTX-induced intracellular ROS and cytotoxicity in A2780 cells. The cells were treated with 10 nM PTX in the presence or absence of 3.2 mM NAC for 24 h. Intracellular ROS (D) and cytotoxicity (E) were determined using H₂DCF-DA staining and MTT assay, respectively. Error bars indicate S.D. among three individual experiments. **P* < 0.01.

dose-dependent manner. In contrast, knocking down endogenous Trx1 by Trx1 shRNA inhibited the endogenous and exogenous FOXO1 transcriptional activities (Fig. S2). However overexpression of Trx1 or knocking down Trx1 did not affect FOXO1 protein expression (Fig. 4A and S2), indicating that Trx1 increased FOXO1 transcriptional activity independent of its protein expression change.

Trx1 was known to act as an important redox regulatory factor. To determine whether the redox status of Trx1 was important for regulating FOXO1 transcriptional activity, the effects of overexpression of Trx1 C32/35S mutant, in which the redox regulatory domain (cysteine 32 and 35) was mutated to serine, on the transcriptional activity of FOXO1 were determined. As shown in Fig. S3, overexpression of Trx1 C32/35S mutant did not affect FOXO1 transcriptional activity, suggesting that Trx1-induced FOXO1 transcriptional activation was not dependent on Trx1 redox activity. Interestingly, overexpression of Trx1 C69S mutant, in which cysteine 69 was mutated to serine, was found to significantly abrogate the upregulation of FOXO1 transcriptional activity (Fig. 4B). It was reported that cysteine residue at position 69 of Trx1 had two major functions: (1) Cysteine 69 contributed to the redox regulatory functions of Trx1 [37]; (2) Trx1 was S-nitrosylated on cysteine 69 and Trx1 C69S mutant was barely detected in the nucleus [38]. Considering that Trx1 redox activity was irrelevant to FOXO1 transcriptional activation, Trx1-mediated FOXO1 transcriptional activation might depend on the nuclear import of Trx1. Indeed, very few Trx1 C69S mutant was detected in the nucleus when A2780 cells were incubated with or without PTX treatment (Fig. S4).

3.5. Trx1 interacts with FOXO1

To examine the molecular mechanism responsible for Trx1-mediated upregulation of FOXO1 transcriptional activity, we sought to determine whether Trx1 physically interacted with FOXO1. Co-

immunoprecipitation assays showed that endogenous Trx1 formed a protein complex with endogenous FOXO1 in A2780 cells. This interaction increased in response to PTX treatment while co-treatment with NAC decreased the interaction between Trx1 and FOXO1 (Fig. 5A), which suggested that Trx1 interacted with FOXO1 in A2780 cells and this interaction might be regulated by PTX-induced ROS. Furthermore, ectopically expressed FLAG-tagged Trx1 formed a complex with V5-tagged FOXO1 in A2780 cells (Fig. 5B). Consistent with our findings that Trx1 C69S mutant abolished the upregulation of FOXO1 transcriptional activity, the interaction between Trx1 C69S mutant (FLAG-Trx1 C69S) and V5-FOXO1 decreased compared with the complex of FLAG-Trx1/V5-FOXO1 (Fig. 5B). To examine whether Trx1 interacted directly with FOXO1, five GST-FOXO1 fusion proteins were purified (Fig. 5C, left and bottom right) and incubated with lysates of A2780/PTX cells. As shown in Fig. 5C (top right), Trx1 were specially pulled down by GST-FOXO1 fusion proteins FO1-3 and FO1-4, which encompassed amino acids 211–419 and 354–503 in FOXO1, respectively. As a negative control, no interaction between Trx1 and GST proteins was observed. Considering that PTX-induced ROS induced Trx1 nuclear translocation and Trx1 C69S mutant was barely detected in the nucleus, the results indicated that Trx1 interacted directly with FOXO1 and the interaction might be associated with Trx1 nuclear translocation.

3.6. Trx1 increases expression of endogenous genes regulated by FOXO1

FOXO transcription factors are critical for the regulation of cellular response to stress stimuli by transactivating a series of target genes. For example, FOXOs activate GADD45 involved in DNA damage repair, MnSOD and catalase in ROS detoxification [19]. To confirm the upregulation of FOXO1 activity mediated by Trx1, the effects of Trx1 on the expression of FOXO1 target genes were determined by qRT-PCR. As expected, overexpression of FOXO1 resulted in the increase of MnSOD,

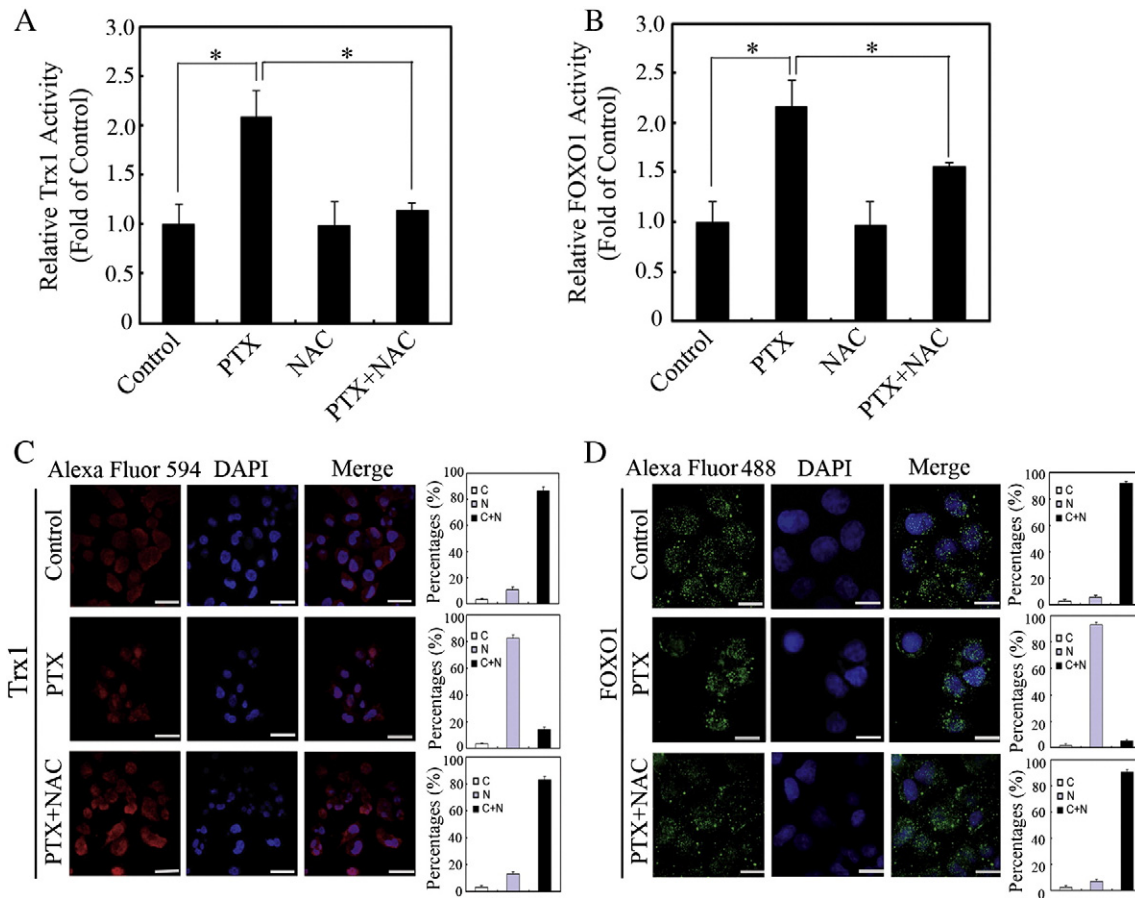


Fig. 3. PTX-induced ROS promote the activation and nuclear translocation of Trx1 and FOXO1 in A2780 cells. (A, B) Effects of NAC on the activities of Trx1 and FOXO1 in A2780 cells. The cells were treated with 10 nM PTX in the presence or absence of 3.2 mM NAC for 24 h. The cells were harvested, and the activities of Trx1 (A) and FOXO1 (B) were determined by insulin assay and luciferase reporter assay, respectively. Error bars indicated S.D. among three individual experiments. * $P < 0.01$. (C, D) Effects of NAC on the nuclear translocation of Trx1 and FOXO1 induced by PTX in A2780 cells. A2780 cells were treated with 10 nM PTX in the presence or absence of 3.2 mM NAC. After 24 h treatment, immunofluorescence chemistry was performed using anti-Trx1 (red) to detect endogenous Trx1 expression (C) and anti-FOXO1 antibody (green) to detect endogenous FOXO1 expression (D). DAPI was used to stain nuclei. Representative confocal microscopy images of intracellular distribution of Trx1 and FOXO1 were shown on the left. The scale bars represented 25 μm for C and 10 μm for D. The quantification of cell fractionation was shown in bar graphs on the right. Error bars indicated S.D. among three individual experiments. C, cells in which Trx1 or FOXO1 was only located in cytoplasm; N, cells in which Trx1 or FOXO1 was only located in nucleus; C + N, cells in which Trx1 or FOXO1 was located in both cytoplasm and nucleus.

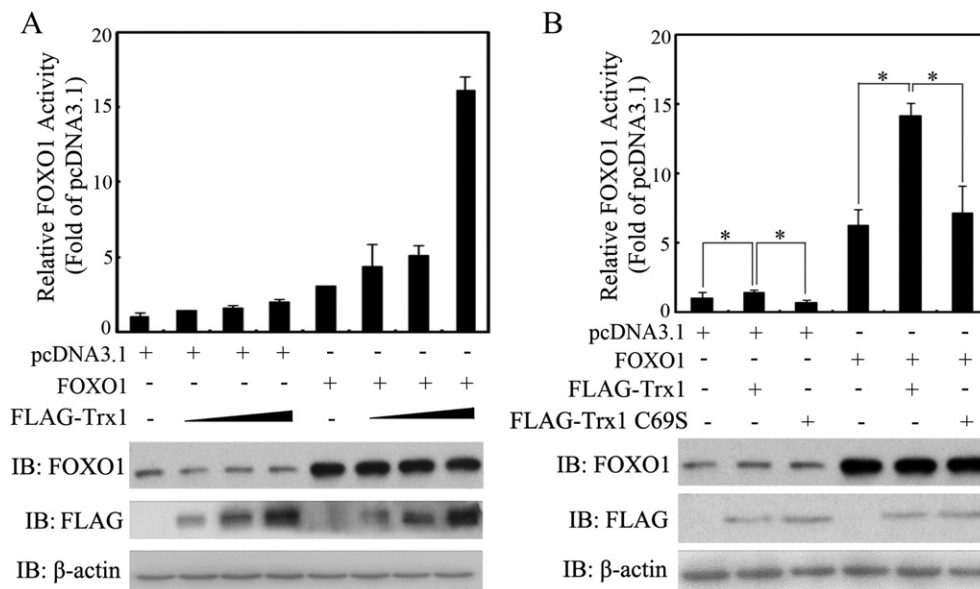


Fig. 4. Trx1 upregulates FOXO1 transcriptional activity. (A) Effects of Trx1 overexpression on the transcriptional activity of FOXO1. (B) Effects of Trx1 C69S mutant on the transcriptional activity of FOXO1. A2780 cells were transfected with firefly and *Renilla* luciferase reporter constructs in combination with the plasmids as indicated. At 36 h after transfection, cells were subjected to luciferase activity measurement (upper panel) or western blotting analysis (lower panel). Error bars indicate S.D. among three individual experiments. * $P < 0.05$.

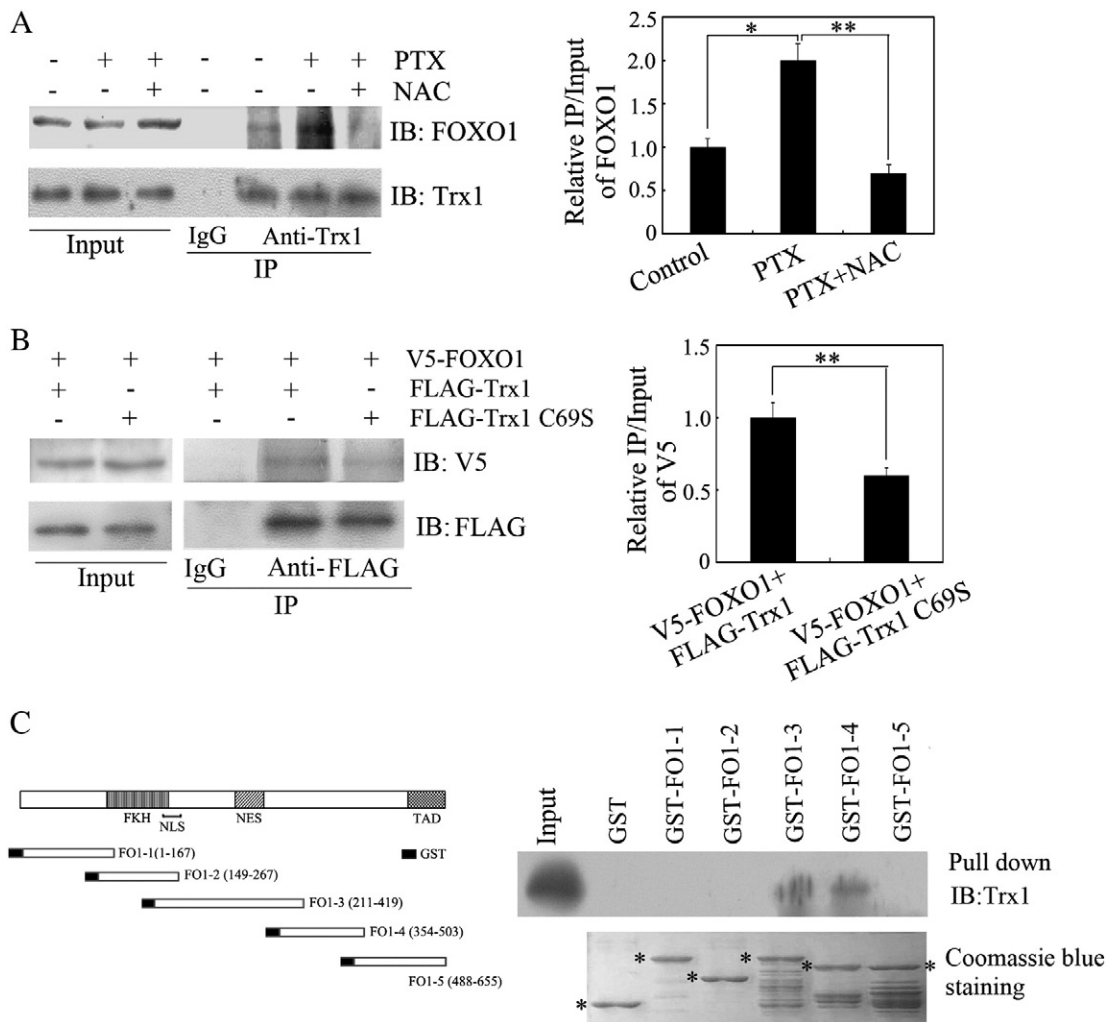


Fig. 5. Trx1 interacts with FOXO1. (A) Interaction of endogenous Trx1 and FOXO1 proteins in A2780 cells. A2780 cells were treated with 10 nM PTX in the presence or absence of 3.2 mM NAC. After 24 h treatment, cells were subjected to co-immunoprecipitation using antibody against Trx1. Results from one representative experiment were shown on the left. Quantitative results of the interaction between Trx1 and FOXO1 were shown on the right. Data as mean values \pm S.D. ($n = 3$). * $P < 0.05$, ** $P < 0.01$. (B) Interaction of exogenous FOXO1 and Trx1 proteins. A2780 cells were transfected with the plasmids as indicated. At 36 h after transfection, cells were subjected to immunoprecipitation and western blotting analysis. Results from one representative experiment were shown on the left. Quantitative results of the interaction between FLAG-Trx1 and V5-FOXO1 were shown on the right. Data as mean values \pm S.D. ($n = 3$). ** $P < 0.01$. (C) *In vitro* interaction between Trx1 and FOXO1 proteins. Left: a diagram showing five GST-FOXO1 recombinant proteins. Upper right: A2780/PTX cell lysates were subjected to GST pull-down assay. Bottom right: GST and GST-FOXO1 recombinant proteins were analyzed by coomassie blue staining. GST and GST-FOXO1 recombinant proteins at correct molecular mass were indicated by arrowheads.

catalase and GADD45 expressions. The increased expression of these target genes was further enhanced by overexpression of Trx1. However, this effect was significantly compromised by the expression of Trx1 C69S mutant (Fig. 6A). These data suggested that Trx1 upregulated the expressions of FOXO1 target genes and Trx1 cysteine 69 was important for the upregulation of FOXO1 target genes.

To further explore the mechanism underlying the regulation of FOXO transcriptional activity by Trx1, we examined whether forced expression of Trx1 affected the binding of FOXO1 to the promoter of MnSOD using ChIP assay. As shown in Fig. 6B, the binding of ectopically expressed FOXO1 to the promoter of MnSOD was increased in A2780 cells transfected with wild type Trx1. However, Trx1 C69S mutant abolished the upregulation of FOXO1 binding to MnSOD promoter. These data suggested that Trx1-FOXO1 interaction might increase the binding of FOXO1 to the promoter of its target genes.

3.7. Trx1/FOXO1 signaling pathway is involved in drug resistance in ovarian cancer cells

To evaluate the biological significance of Trx1/FOXO1 signaling pathway in drug resistance in ovarian cancer cells, we assessed whether

Trx1-mediated drug resistance could be reversed by knocking down FOXO1 in A2780 cells. As shown in Fig. 7A, overexpression of Trx1 protected A2780 cells from PTX-induced apoptosis by flow cytometric analysis using PI staining. This effect of Trx1 was abrogated by cotransfection with FOXO1 shRNA. To further examine the importance of the regulation of FOXO1 by Trx1 at cysteine 69 in drug resistance, the effects of wild type Trx1 or Trx1 C69S mutant on FOXO1-mediated drug resistance in A2780 cells were investigated. As expected, Trx1 substantially enhanced FOXO1-regulated drug resistance in A2780 cells. However, Trx1 C69S mutant almost completely abolished the regulation of FOXO1-mediated drug resistance by Trx1 (Fig. 7C). These results were further confirmed by cleavage of poly(ADP-ribose) polymerase (PARP), an apoptotic marker (Fig. 7B and D). Therefore, Trx1/FOXO1 signaling pathway might be involved in drug resistance in ovarian cancer cells.

4. Discussion

Although most ovarian cancers are responsive to PTX-based chemotherapy, cancer cells often develop drug resistance, which leads to treatment failure and disease relapse. Thus, identification of molecular

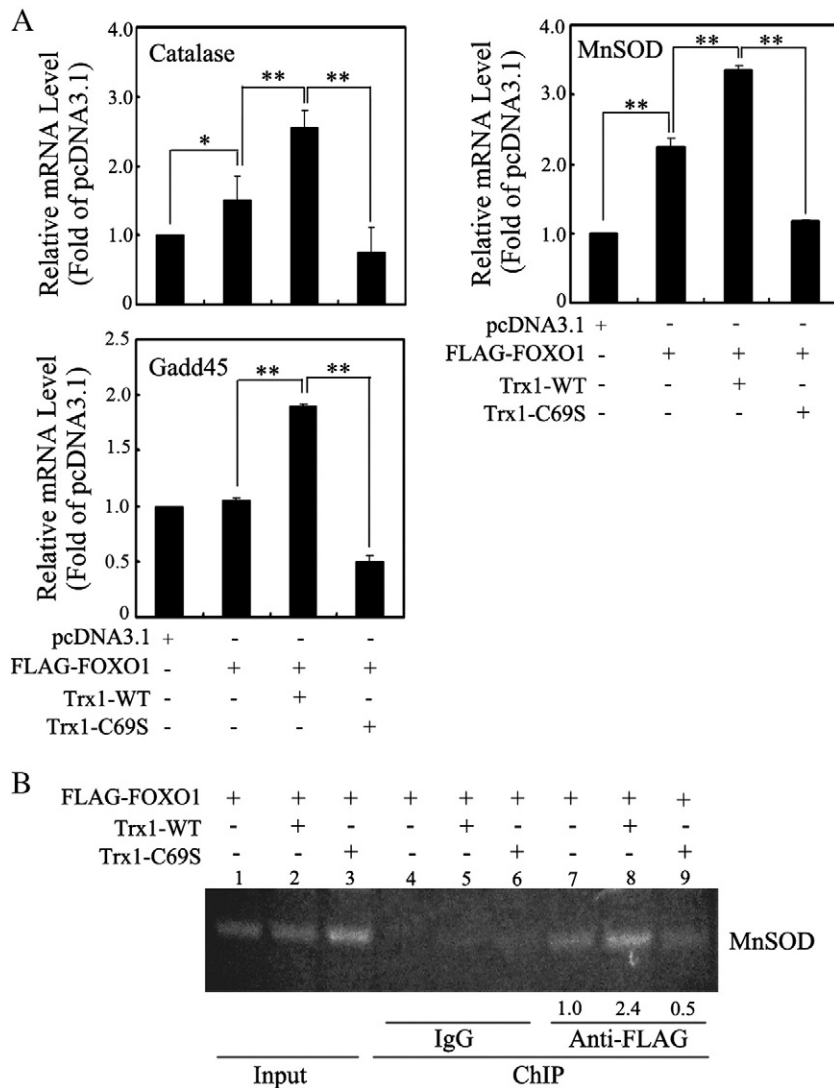


Fig. 6. Trx1 increases the expression of FOXO1 target genes. (A) Effects of Trx1 and Trx1 C69S mutant on the expression of FOXO1 target genes. A2780 cells were transfected with the plasmids as indicated. After 36 h transfection, cells were harvested for expression of catalase, MnSOD, and Gadd45 using qRT-PCR. * $P < 0.05$, ** $P < 0.01$. (B) Effects of Trx1 or Trx1 C69S mutant on the binding of FOXO1 to the promoter of MnSOD gene. A2780 cells were transfected with plasmids as indicated for 36 h and harvested for ChIP assay. The density of PCR bands from ChIP samples was normalized by that of the bands from the respective input samples. The relative ratio was determined by dividing the normalized values in lane 7, 8, and 9 by the value in lane 7.

events that mediate drug resistance in ovarian cancer cells not only enhances our understanding of the biology of chemoresistance, but also has significant implications in designing more effective therapeutic strategies which circumvent drug resistance. In the present study, we revealed a key role of Trx1/FOXO1 signaling pathway in PTX-induced chemoresistance in ovarian cancer cells. First, we showed that Trx1 and FOXO1 were overexpressed in PTX-resistant ovarian cancer A2780 cells (A2780/PTX) compared with parental A2780 cells. The activities and protein expression levels of Trx1 and FOXO1 proteins were simultaneously induced by PTX in A2780 cells, but not in A2780/PTX cells. Second, silencing of Trx1 or FOXO1 by shRNA increased sensitivity to PTX in A2780/PTX cells. Third, we found that Trx1 bound to and increased FOXO1 transcriptional activity. Trx1 increased FOXO1-induced chemoresistance, while Trx1-induced chemoresistance was significantly inhibited by FOXO1 shRNA. Therefore, our study raised the possibility that the regulation of FOXO1 by Trx1 was involved in PTX resistance in ovarian cancer cells. FOXOs were reported to not only activate the apoptotic machinery but also enhance cellular defenses and promote cell survival according to different cell lines and various types or intensities

of cellular stress response [21]. Here we provided evidence that FOXO1 acted as pro-survival effector molecules in PTX-induced cytotoxicity in ovarian cancer cells.

Oxidative stress has been demonstrated to be involved in various physiological and pathological processes, such as cancer [39]. The cell growth and cell death could be linked to an intracellular ROS level which might be fluctuated in response to intracellular as well as extracellular signals [40]. Several anticancer drugs resulted in a burst of ROS to attack tumor cells, which was the potential mechanism of action of the anticancer drugs [41,42]. However, the upregulation of antioxidants and activation of redox-sensitive transcription factors mediating survival signals were usually induced to counteract oxidative stress as an adaptation response in cancer chemotherapy [43]. The balance between these two antagonizing signals ultimately determined when and whether the stimulated cells would die. Our data showed that ROS played an important role in the PTX-induced cytotoxicity in PTX-sensitive A2780 cells, and correspondingly the activities of Trx1 and FOXO1 proteins were upregulated by PTX-induced ROS. The upregulation of Trx1 and FOXO1 might account for antagonizing PTX-induced

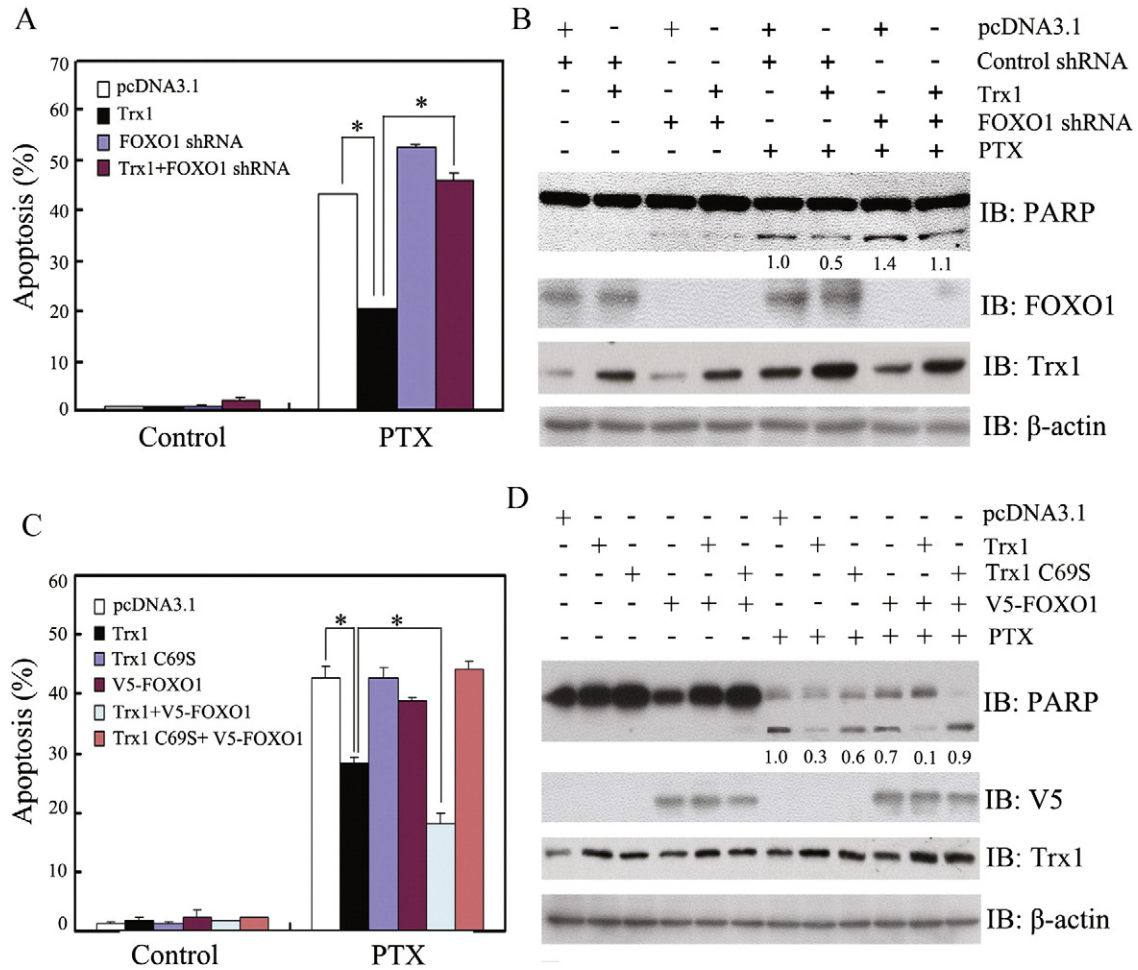


Fig. 7. Trx1/FOXO1 signaling pathway might be involved in PTX resistance in ovarian cancer cells. (A, B) Effects of knocking down FOXO1 on the anti-apoptotic protection of Trx1 in A2780 cells. (C, D) Effects of Trx1 and Trx1 C69S mutant on the FOXO1-mediated PTX resistance in A2780 cells. A2780 cells were transfected with the plasmids as indicated. After 36 h transfection, the cells were treated with or without 25 nM PTX. After 24 h treatment, the cells were harvested and subjected to apoptosis analysis (A, C) and western blotting analysis (B, D). The number underneath each band in the immunoblot indicates the relative intensity of the corresponding band. Error bars indicate S.D. among three individual experiments. **P* < 0.01.

oxidative damage in A2780 cells. In the meantime we provided evidence that Trx1 upregulated expression of endogenous genes regulated by FOXO1, including genes involved in antioxidant defense such as MnSOD and catalase, and DNA damage repair such as Gadd45, which

promoted us to speculate that Trx1/FOXO1 signaling attenuated PTX sensitivity through regulation of these target genes.

Trx1 and FOXO1 are located in both cytoplasm and nucleus. In this paper, we found that PTX-induced ROS resulted in Trx1 and FOXO1

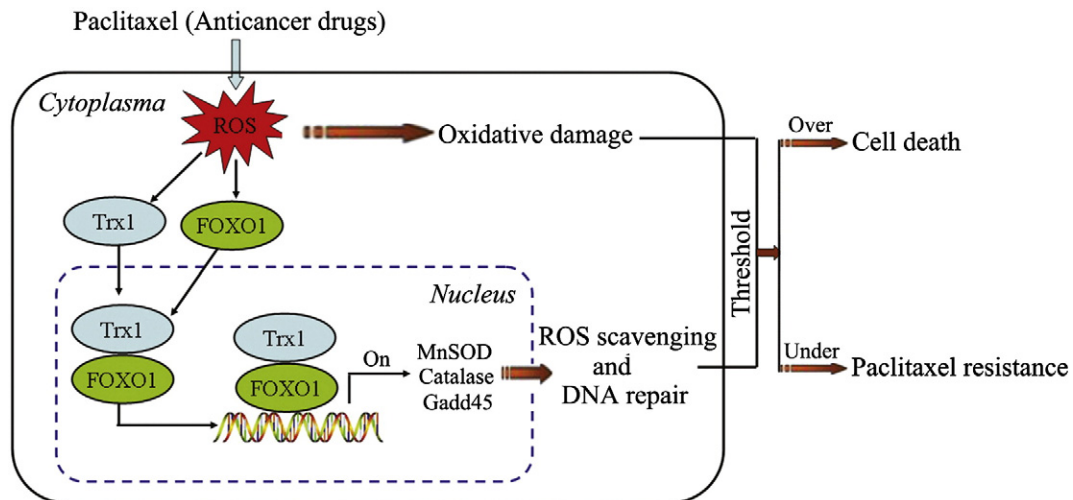


Fig. 8. A diagram depicts a hypothetical model wherein Trx1/FOXO1 signaling pathway is involved in PTX resistance in ovarian cancer cells.

nuclear translocation, and further enhanced the interaction between Trx1 and FOXO1. Moreover, we found that the regulation of FOXO1 transcriptional activity by Trx1 depended on the cysteine residue at position 69 of Trx1 since Trx1 C69S mutant attenuated FOXO1 transcriptional activity and the interaction between Trx1 and FOXO1. In addition, Trx1 C69S mutant almost completely abolished the regulation of FOXO1-mediated drug resistance by Trx1. Having excluded that Trx1 redox status was responsible for Trx1 transcriptional activation, the regulation of FOXO1 transcriptional activity might be associated with the nuclear import of Trx1, as Trx1 C69S mutant was barely detected in the nucleus. On the basis of these findings, we envisage a model wherein PTX-resistant ovarian cancer cells are not sensitive to PTX treatment since Trx1 and FOXO1 are overexpressed in these cells. However in PTX-sensitive ovarian cancer cells, PTX treatment results in ROS production to attack cancer cells, which induces Trx1 and FOXO1 expressions and nuclear translocation to antagonize oxidative stress. The final outcome is that ROS levels might probably ascend above a threshold to initiate PTX-induced tumor cell death (Fig. 8). Whether Trx1 was S-nitrosylated in PTX-treated A2780 cells and the role of Trx1 nuclear translocation in the regulation of FOXO1 transcriptional activity need to be further elucidated.

In summary, this study showed a functional interaction between Trx1 and FOXO1 that regulated drug resistance in ovarian cancer. Trx1 upregulated FOXO1 transcriptional activity and FOXO1-mediated drug resistance and the effects might depend on Trx1 nuclear translocation which was mediated by PTX-induced ROS. Thus, Trx1/FOXO1 pathway might play an important role in the development of PTX resistance in ovarian cancer.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

We thank Dr. Haojie Huang for the plasmids and Dr. Ding Ma for the cells. We also thank the Analytical and Testing Center of Huazhong University of Science and Technology for related analysis. This work was supported by the National Basic Research Program of China (973 Programs, 2012CB932500, 2011CB933100 and 2015CB931800), the National Natural Science Foundation of China (31070689 and 81372400) and the Fundamental Research Funds for the Center Universities (HUST: 2012TS013).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbadis.2014.12.002>.

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