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Regulatory effect of peroxiredoxin 1 (PRDX1) on doxorubicin-induced apoptosis in triple negative breast cancer cells

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Abstract

Patients with triple negative breast cancer (TNBC) lack the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2; thus, conventional hormone and targeted therapies have minimal effect on them. Therefore, clinical treatment of TNBC is still based on chemotherapy and supplemented by other methods. Doxorubicin (DOX), a common drug used in TNBC chemotherapy, has high affinity for cardiolipin, and the nematosomes are rich in cardiolipin; therefore, DOX has high mitochondria-targeting ability. DOX accumulates and plunders the electrons of nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome C in mitochondria to produce semiquinone DOX. Under the action of oxygen molecules, semiquinone DOX is reduced to DOX and reactive oxygen species (ROS) are generated. The accumulation of ROS can cause mitochondrial dysfunction and lead to mitochondrial dependent apoptosis. Bioinformatic analysis of samples from TNBC patients revealed that peroxiredoxin 1 (PRDX1) was highly expressed in TNBC tissues, and the poor prognosis of patients with high PRDX1 expression was considerably increased. Previous studies determined that DOX can upregulate the expression of the PRDX1 protein in the human TNBC cell line (MDA-MB-231). Thus, we speculate that PRDX1 plays an important role in the process of DOX-induced TNBC cell apoptosis. In this study, we aimed to explore the role of PRDX1 in the process of DOX-induced TNBC cell apoptosis. We found that PRDX1 deletion increased the sensitivity of MDA-MB-231 cells to DOX, which was mainly due to mitochondrial oxidative stress caused by intracellular ROS accumulation, leading to mitochondria-dependent apoptosis. Deletion of PRDX1 promotes the PI3K/Akt signaling pathway to mediate the expression of GSK3β. Gsk3β is an upstream signal of mitochondria-dependent apoptosis, and is also an important target of ROS. PRDX1 participates in adriamycin-induced apoptosis of TNBC cells by regulating the expression level of GSK3β. Our findings present new insights to treat breast cancer and TNBC, outlines the clinical use of DOX, and provides a basic theory to develop PRDX1 gene function.

Keywords: Doxorubicin, Reactive oxygen species, TNBC, PRDX1, Apoptosis

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Introduction

Breast cancer is a common malignant tumor in women [1]. Among the different types of breast cancer, triple negative breast cancer (TNBC) results in a high mortality rate owing to the lack of existing treatment methods [2]. Chemotherapy is the current treatment method. Doxorubicin (DOX) is the first-line drug for the clinical treatment of breast cancer. It can maintain the apoptosis of breast cancer cells by regulating the level of

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intracellular reactive oxygen species (ROS) [3]. However, the side effects of DOX, including cardiotoxicity and bone marrow suppression, restrict its use in clinical practice [4]. Therefore, the combined use of multiple chemotherapeutic drugs can help reduce the dosage of DOX and avoid the occurrence of DOX-induced side effects [5–8]. However, the clinical use of chemotherapeutic drugs has certain toxic side effects and it is important to seek a new DOX treatment method to reduce the dosage of DOX without affecting its therapeutic effect.

Redox balance plays a key role in cell physiology and pathology. ROS can activate or inhibit various receptors, proteins, ions, and other signaling molecules to affect cell growth and death. ROS include free radicals such as superoxide free radicals, hydroxyl free radicals, and singlet oxygen, as well as non-free radicals such as hydrogen peroxide. Oxygen-free radicals are highly active and have the ability to destroy cellular components such as proteins, lipids, and nucleic acids [9, 10]. Peroxiredoxin 1 (PRDX1), a member of the 2-Cys PRDXs family that primarily exists in the cytoplasmic matrix, was first reported as an antioxidant protein highly sensitive to oxidative stress; however, its role in redox equilibrium remains unclear. Later studies determined that PRDX1 was transformed from peroxidase to molecular chaperone under stress [11] and could enhance the cytotoxicity of natural killer cells. Many studies have shown that PRDX1 is abnormally expressed in a variety of human cancers and it coordinates redox signal transduction in a H_2O_2 dose-dependent manner through its oxidation state of cysteine Cys52 in breast cancer [12]. In our previous studies, we found that TNBC cells lacking PRDX1 were more sensitive to DOX than those that do not lack PRDX1.

Glycogen synthase kinase (GSK) 3 is a widely expressed serine/threonine protein kinase. GSK3β is located at the junction of many signal pathways, and its homologous substrate dominates the activities of many signal transducers and transcription factors such as RelA/p65, β-catenin, and cyclin D1 [13, 14]. In addition, GSK3β is a redox-sensitive signal molecule, performing a key role in controlling self-protective antioxidant defense. GSK3β activity is induced by ROS and participates in changes in mitochondrial permeability. GSK3β can regulate mitochondrial permeability changes through the phosphorylation of different targets, and inhibiting GSK3β can protect mitochondrial membrane permeability and prevent the occurrence of mitochondria-dependent cell apoptosis [15]. In the Wnt signaling pathway, the transcriptional co-activator β-catenin is involved in promoting cell growth and survival; however, GSK3β phosphorylates β-catenin which results in its proteasomal degradation [16, 17]. The activation of GSK3β can promote the phosphorylation and degradation of β-catenin and inhibit the expression of anti-apoptotic protein Bcl-2. Inhibiting GSK3β can promote the accumulation of β-catenin and the expression of anti-apoptotic protein Bcl-2 [18]. Although PRDX1 has been reported to perform an important role in apoptosis induced by DOX, the relevant regulatory mechanism remains unclear.

Previous studies have demonstrated that PRDX2 regulates mitochondrial damage and induces apoptosis in HT22 cells through the ROS-regulated GSK3β/β-catenin signaling pathway. PRDX1 is involved in scavenging ROS during apoptosis. The Gsk3β/β-catenin signaling pathway is a key downstream signaling pathway of ROS. We hypothesize that PRDX1 is involved in regulating the apoptosis of MDA-MB-231 cells through the GSK3β/β-catenin signaling pathway.

The aims of this study are twofold; (1) to explore the function and role of PRDX1 in DOX-induced apoptosis of TNBC cells, and (2) to investigate the role and mode of action of PRDX1 in DOX-induced apoptosis of TNBC cells, demonstrating the potential of PRDX1 to treat TNBC. These findings provide a new solution for the clinical application of DOX and a potential target for the clinical treatment of TNBC.

Materials and methods
Chemicals
Cell culture dishes (100 mm) were purchased from NEST (NEST Biotechnology, Wuxi, China). Fetal bovine serum (FBS) and penicillin/streptomycin (P/S) were purchased from Solarbio (Solarbio life sciences, Beijing, China), and TRIzol was purchased from Sigma (Sigma, St. Louis, MO, USA).

Cell culture
MDA-MB-231 cell cultures were cultured in standard Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% P/S at 37 °C under a 95% air/5% CO_2 incubator. The culture medium was changed every day when it reached 90% confluence, and cells were sub-cultured after treatment with the 0.25% trypsin–ethylenediaminetetraacetic acid mixture.

Objective gene expression and survival analysis
The Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/index.html), The Cancer Genome Atlas Program (TCGA), and Genotype-Tissue Expression (GTEx) databases were used for differential expression analysis of the genes of interest in breast cancer. On the website, the genes of interest (*PRDX1, PRDX2, PRDX3, PRDX4, PRDX5*, and *PRDX6*) were entered, the boxplot was selected in the expression DIY option, and the breast cancer ("BRCA") option was identified in the dataset selection ("cancer name")

option. The "add" option was clicked to add BRCA to the dataset option box, the plot was clicked to obtain the gene expression profile of interest in breast cancer, and a backup was saved by clicking the "download" button.

Using the UALCAN website (http://ualcan.path.uab.edu/analysis.html), the gene of interest (*PRDX1*) was entered into TCGA database, and the breast invasive carcinoma option was selected. The "explore" option was clicked, and the "survival" option was selected, resulting in high and low concomitant gene expression for the gene of interest. Breast cancer patient prognosis survival expression plots were obtained, which were saved as JPEG images by clicking "download JPEG image."

Construction of MDA-MB-231 cell line with PRDX1 knockdown

The mRNA sequence of the human PRDX1 gene was found according to the NCBI GeneBank system. The PRDX1 gene sequence commonly used in stable and heritable studies was searched via the NCBI. The PRDX1 gene knockdown sequence was ultimately determined as: 5′-GGAGGACTGGGACCCATGA-3′, and the negative control group knockdown in descending order was determined as: 5′-TTCTCCGAACGTGTCACGTTTC-3′. Then, the determined sequence was submitted to Shanghai Gima Gene Company to construct the lentiviral vector. The viral titer constructed was 108 TU/mL, and puromycin was selected as the resistant gene. After the lentiviral vector was obtained, it was stored in the refrigerator at −80 ℃ for later use.

The culture medium of the Mock and shPRDX1 groups were mixed with 40 μl lentiviral vector and 1 μl promoter polybrene, and the culture was continued. The morphology of the cells was observed via an inverted phase contrast microscope every 6 h. Next, 1 μl of promoter polybrene was directly added to the 5 mL CCM culture medium and mixed well. When the cell density exceeded 95%, puromycin was added for screening, and the final concentration of puromycin was 2 μg/mL. After 24 h, the culture medium was changed to normal CCM when the cells in the blank group completely died.

3-(4,5-Dimethyldiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were plated at a density of 8 cells/well, and 10^3 cells were seeded in 96-well plates and incubated for 24 h using various concentrations of DOX (0–10 μM). Cells were treated and cell viability was assessed using the MTT assay. The 96-well cell culture plates were placed into a microplate reader for detection, wherein the wavelength of the microplate reader was set at 490 nm and the data obtained from the microplate reader were graphed and analyzed using GraphPad Prism after calculation.

Annexin V-PE analysis

Apoptosis was assessed by fluorescence microscopy. After 12 h of DOX treatment, the six-well cell culture plates were removed, and the discarded liquid was aspirated using a peristaltic pump. Then, the cells were washed once using Hank's Balanced Salt Solution (HBSS) by adding 300 μL to each well μL of the binding solution with 1 μL of Annexin V-PE. During apoptosis, phosphatidylserine from the inner membrane of cells is everted to the outer membrane; Annexin V has a high affinity to phosphatidylserine. Therefore, Annexin V labeled with fluorescein PE can be used as a fluorescent probe to detect apoptosis. Next, the six-well cell culture plate was placed in the dark at room temperature for 15 min. After 15 min, cell apoptosis was observed using a fluorescence microscope.

MitoSOX analysis

Mitochondrial ROS was detected by fluorescence microscopy. The treated MDA-MB-231 cells were washed with phosphate buffered saline (PBS), incubated with MitoSOX at 37 ℃ for 30 min, and the ROS level in the cells was observed via fluorescence microscopy. All tests were performed and data were obtained from three independent experiments.

Detection of mitochondrial membrane potential

The mitochondrial membrane potential of the MDA-MB-231 cells was determined by a fluorescence probe JC-1. Cells were planted in six-well plates with DMEM containing 10% FBS (ensuring the same number of cells in each well), treated with DOX, and washed with PBS. Then, 300 μL of JC-1 staining solution was added to each well, and the cells were treated at 37 ℃ for 15 min under dark conditions. Afterwards, the cells were washed twice with PBS and observed by fluorescence microscopy.

Western blot

The total proteins from the MDA-MB-231 cell lysates were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using 12% SDS−polyacrylamide gel, and then electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk in Tris-buffered saline with 0.1% Tween 20 detergent (TBST), and then incubated overnight at 4 °C with anti-PRDX1, anti-PRDX2, anti-PRDX3, anti-PRDX4, anti-PRDX5, anti-PRDX6, anti-Bad, anti-cleaved-caspase7, anti-cleaved-caspase9, anti-Bcl2, anti-cleaved-caspase 3, anti-pro-caspase3, anti-β-actin, anti-pGSK3β (Ser9), anti-GSK3β, anti-β-catenin, anti-PI3K, anti-Akt, and anti-P-Akt, which were all purchased from Santa Cruz

Biotechnology (Dallas, TX, USA). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. After removing excess antibodies by washing with TBST, specific binding was detected using a chemiluminescence detection system (General Electric Company, Shanghai, China). Band intensities were quantified using the Image J software (National Institutes of Health, Bethesda, MD, USA). For all western blot bands, β-actin was used as an internal reference for quantitative analysis.

Statistical analysis

All experimental data were repeated more than three times, and the differences among the experimental groups were analyzed by repeated measures analysis of variance (ANOVA). Data differences were considered statistically significant when the *P*-value was less than 0.05 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

PRDX1 is highly expressed in TNBC

TCGA and GTEx databases were accessed via the GEPIA website (http://gepia.cancer-pku.cn/index.html) and used for the differential expression analysis of PRDXs in breast cancer. As shown in Fig. 1A, among the other genes of the PRDX family, only the *PRDX1* gene showed markedly higher expression in breast cancer tissue than in normal breast tissue, and the expression of other genes of the same family was not significantly different in both tissues.

Next, via the UALCAN website (http://ualcan.path.uab.edu/analysis.html), TCGA database was used for the analysis of the *PRDX1* gene during different stages of cancer onset, in various types of breast cancer, and for prognostic survival. The expression levels of the *PRDX1* gene were significantly higher in all four stages of breast cancer, and no significant differences were observed among the four stages (Fig. 1B).

The expression levels of the *PRDX1* gene were significantly high in different types of breast cancer, including
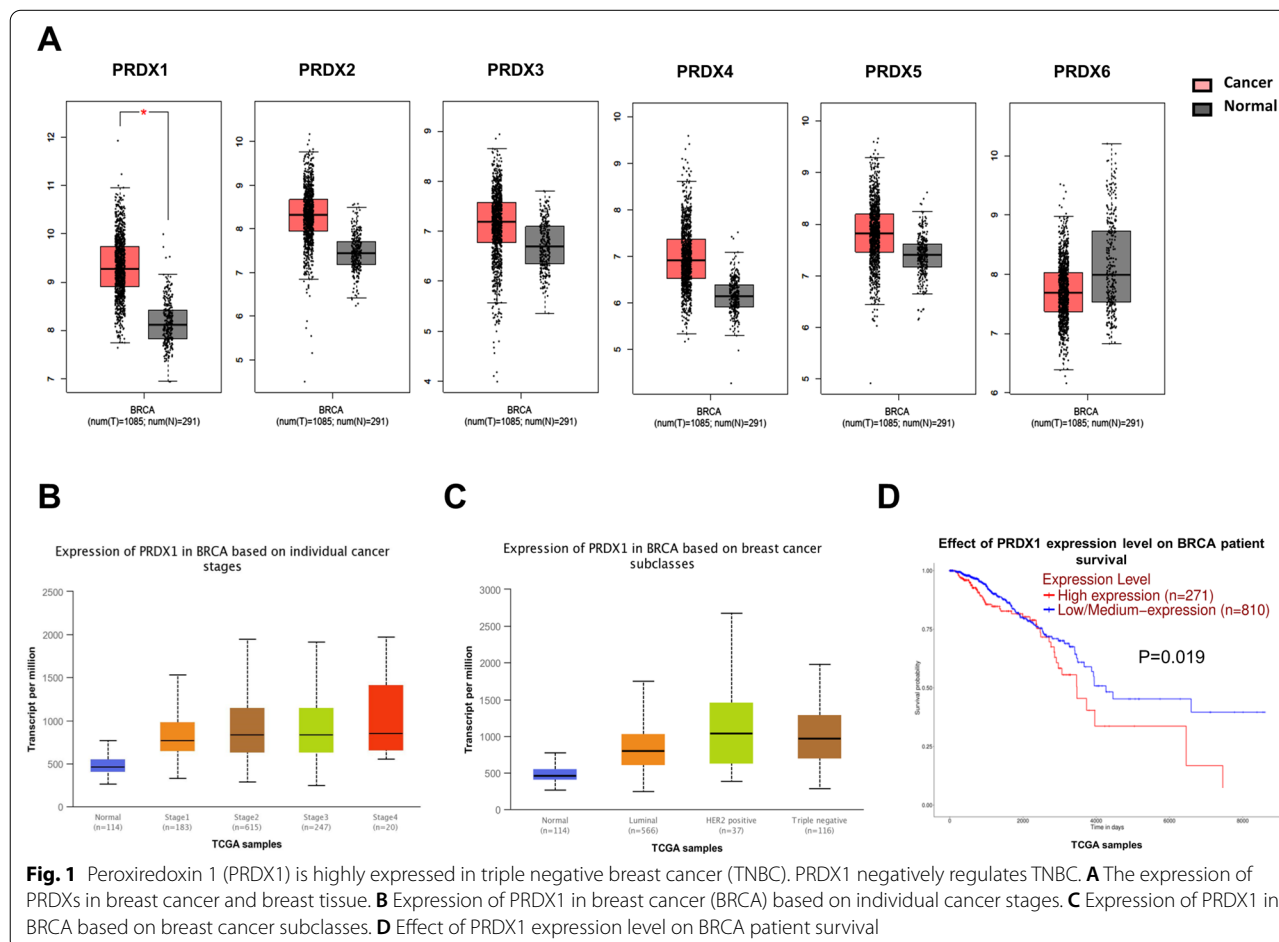

Fig. 1 Peroxiredoxin 1 (PRDX1) is highly expressed in triple negative breast cancer (TNBC). PRDX1 negatively regulates TNBC. **A** The expression of PRDXs in breast cancer and breast tissue. **B** Expression of PRDX1 in breast cancer (BRCA) based on individual cancer stages. **C** Expression of PRDX1 in BRCA based on breast cancer subclasses. **D** Effect of PRDX1 expression level on BRCA patient survival

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TNBC, HER2 positive (both positive for epidermal growth factor receptor 2 and negative for estrogen receptor and progesterone receptor), and luminal types (Fig. 1C). From this, it was found to be accompanied by the high expression of the *PRDX1* gene in therapeutically difficult breast cancer types.

As shown in Fig. 1D, breast cancer patients with high expression of the *PRDX1* gene exhibited significantly poorer prognostic survival than those with low expression of the *PRDX1* gene. The above experimental results verified that PRDX1 was highly expressed in breast cancer and TNBC, and patients with a high expression of
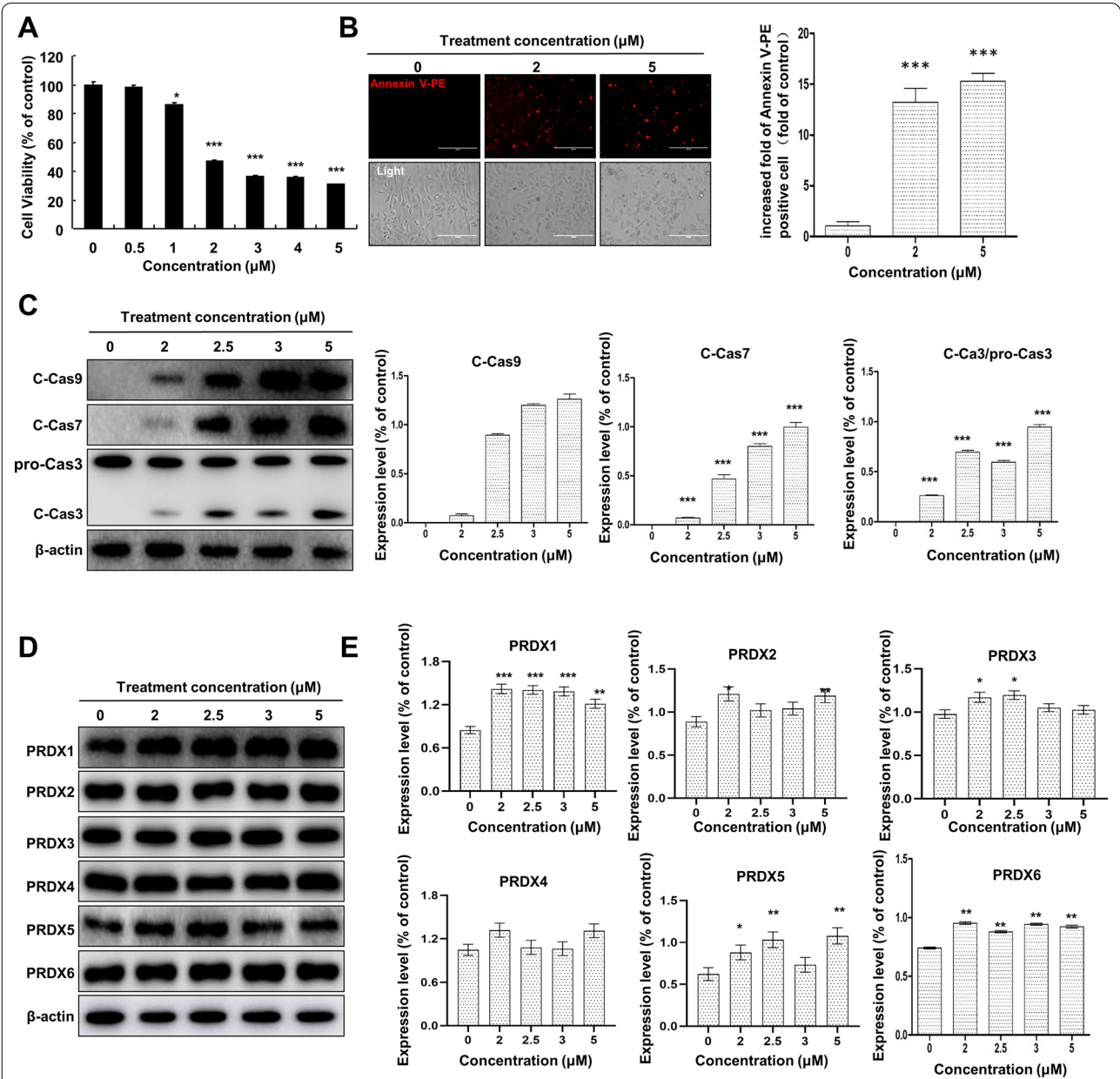

Fig. 2 The anticancer effect of doxorubicin (DOX) on MDA-MB-231 cells. **A** A 3-(4,5-dimethyldiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect cell survival rate. **B** MDA-MB-231 cells were treated with DOX at concentrations of 2 μM and 5 μM, and apoptosis was detected by immunofluorescence detection of Annexin V-PE. **C** Western blot analysis was used to detect the expression of mitochondrial-dependent apoptosis protein. **D** The protein expression of peroxiredoxins (PRDXs) was detected by western blot analysis. **E** The expression levels of PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, and PRDX6 proteins were statistically analyzed

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PRDX1 exhibited a considerably poorer prognosis than those with low expression of PRDX1.

Anticancer effect of DOX in MDA-MB-231 cells

An MTT assay was used to detect the cytotoxicity of DOX to MDA-MB-231 cells. As shown in Fig. 2A, DOX exhibited a good lethal effect on MDA-MB-231 cells, and the survival rate of MDA-MB-231 cells significantly decreased as DOX concentration increased. The difference was significant when the concentration of DOX was 1 μM and extremely significant when the concentration of DOX exceeded 2 μM. We determined that DOX was cytotoxic to MDA-MB-231 cells; therefore, the concentration of DOX was set to 2 μM and 5 μM in subsequent experiments. To further verify the association between DOX and MDA-MB-231 cell death, an annexin V-PE kit was used for staining, and fluorescence microscopy was used for detection. As shown in Fig. 2B, the proportion of apoptotic cells increased with elevated DOX concentration.

The protein expression of mitochondria-dependent apoptosis proteins C-caspase 9, C-Caspase 7, pro-Caspase 3, and C-Caspase 3 was detected by western blot analysis. With increasing DOX concentration, the expression levels of C-caspase 9, C-Caspase 7, and C-Caspase 3 in MDA-MB-231 cells increased, while the expression level of pro-Caspase 3 in MDA-MB-231 cells decreased (Fig. 2C). These results indicate that DOX-targeted mitochondria continuously produced ROS through DOX semiquinone circulation in mitochondria, promoting the expression of mitochondria-dependent apoptotic proteins C-caspase 9, C-Caspase 7, and C-Caspase 3 and triggering the occurrence of mitochondria-dependent apoptosis in MDA-MB-231 cells.

To further explore the relationship between PRDXs and TNBC and to clarify the changes of PRDXs in the apoptosis of TNBC cancer cells induced by DOX, TNBC cells were treated with DOX, and the expression of the PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, and PRDX6 proteins were detected by western blotting. As shown in Fig. 2D, DOX significantly increased the expression of PRDX1 in TNBC cells, which increased with elevated DOX concentration. Figure 2E is the quantitative analysis of Fig. 2D. According to the bioinformatic analysis, PRDX1 expression is often associated with poor prognosis; therefore, PRDX1 may be involved in the DOX-mediated killing of MDA-231 cells. Next, we explored the role of PRDX1 in the killing of TNBC cells by DOX.

Deletion of PRDX1 increased the apoptosis of MDA-MB-231 cells

To further investigate the role and function of the *PRDX1* gene in the action of DOX on MDA-MB-231 cells, the *PRDX1* gene was knocked down in the MDA-MB-231 cells by using lentivirus transfection technology. The knockdown effect of the *PRDX1* gene was tested by immunofluorescence, flow cytometry, and western blotting. MDA-MB-231 cells that were not transfected with lentivirus were named "Blank MDA-MB-231 cells (blank)," and MDA-MB-231 cells transfected with lentivirus were named "Mock MDA-MB-231 cells." MDA-MB-231 cells transfected with PRDX1 siRNA by lentivirus were named "shPRDX1 MDA-MB-231 cells." To determine the transfection effect, the expression level of green fluorescent proteins (GFP) were observed by fluorescence photography. As shown in Fig. 3A, both the mock and shPRDX1 groups expressed the GFP, while the blank group did not, indicating that lentivirus had successfully entered the cells. Flow cytometry was used to further determine the cell transfection rate. As shown in Fig. 3B, the expression of GFP in both the mock and shPRDX1 groups exceeded 90%. Finally, the expression of the PRDX1 protein was detected by western blotting. As shown in Fig. 3C, the expression level of the PRDX1 protein in the shPRDX1 group was significantly lower than that in the blank and mock groups, indicating that the *PRDX1* gene in the shPRDX1 group had been successfully knocked down. These results demonstrated that MDA-MB-231 cell lines with the *PRDX1* knockdown gene were successfully constructed and were therefore used in subsequent experiments.

Through bioinformatic analysis, PRDX1 was determined to play an important role in the occurrence, development, and prognosis of TNBC; however, the role of PRDX1 in the treatment of TNBC with DOX remained unclear. Therefore, the mock and shPRDX1 MDA-MB-231 cells treated with DOX were analyzed by MTT assay. As shown in Fig. 3D, the survival rate of the mock and shPRDX1 MDA-MB-231 cells decreased significantly with increasing DOX concentration, and the survival rate of the shPRDX1 MDA-MB-231 cells was significantly lower than that of the mock MDA-MB-231 cells at the same concentration. These results indicate that MDA-MB-231 cells with *PRDX1* gene deletion were more sensitive to DOX, and 2 μM and 5 μM of DOX were determined to be cytotoxic. To further determine the differences in the extent of DOX-induced apoptosis between mock and shPRDX1 MDA-MB-231 cells, an annexin V-PE immunofluorescence assay was used. DOX treatment increased apoptosis, and apoptosis was more evident in the shPRDX1 MDA-MB-231 group than in the other groups (Fig. 3E).

PRDX1 knockout promotes DOX-induced mitochondrial damage in MDA-MB-231 cells

Many previous studies have shown that DOX enters the cells and generates a vast amount of ROS through cycling DOX semiquinone to induce cell apoptosis. Therefore,
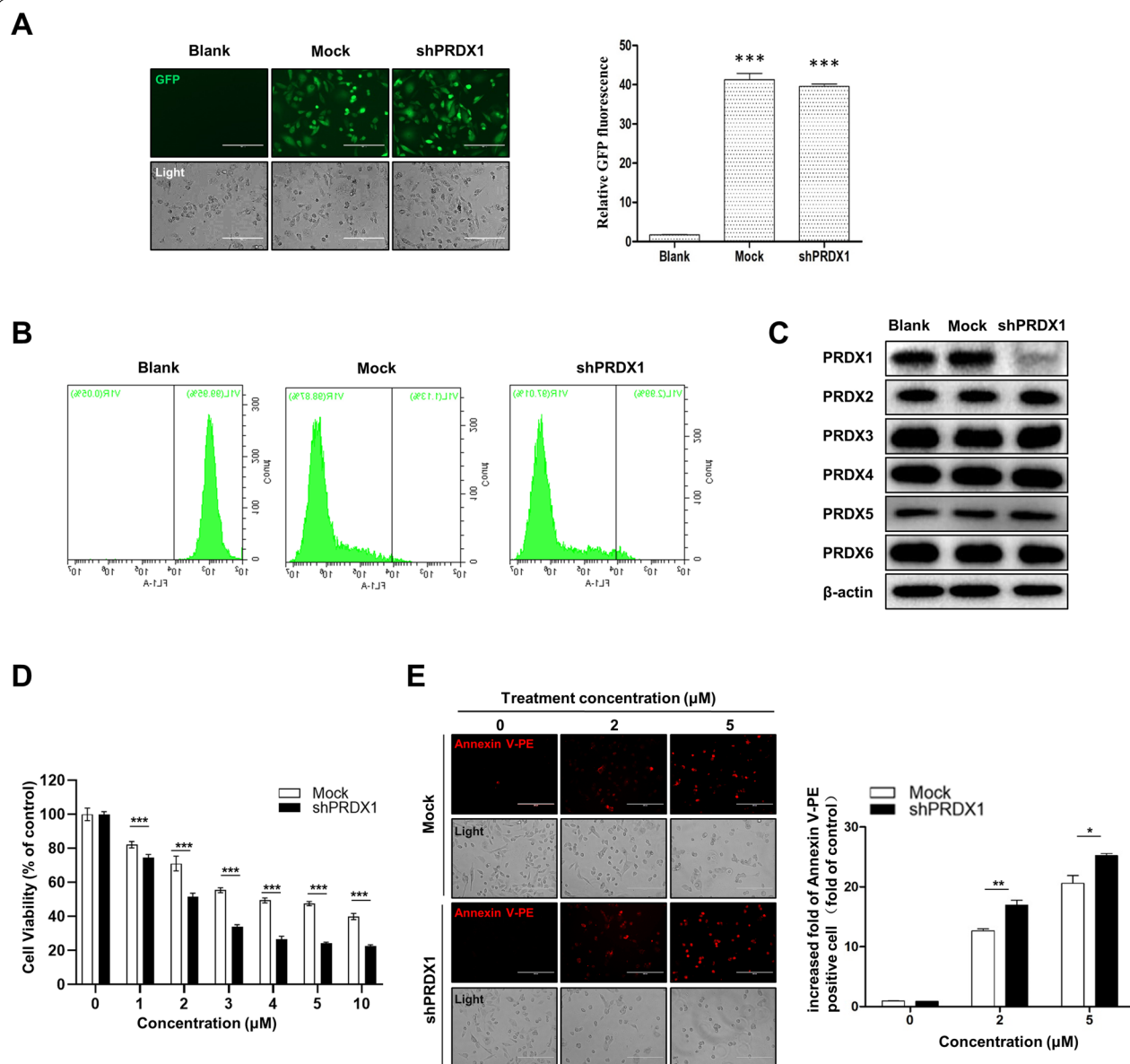
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Fig. 3 Deletion of peroxiredoxin 1 (PRDX1) increased the apoptosis of MDA-MB-231 cells. **A** Green fluorescent protein (GFP) expression was detected by fluorescence microscopy. **B** The transfection rate of lentivirus was observed by flow cytometry. **C** The expression of peroxiredoxins (PRDXs) was detected by western blot analysis. **D** A 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect the cell survival rate after 12 h of DOX treatment. **E** Mock MDA-MB-231 cells and shPRDX1 MDA-MB-231 cells were treated with DOX at concentrations of 2 μM and 5 μM, and apoptosis was detected by immunofluorescence detection of Annexin V-PE

the level of ROS in cells was detected by flow cytometry. DOX treatment significantly increased the intracellular ROS levels in the mock and shPRDX1 MDA-MB-231 cells, and the ROS level in the shPRDX1 group was significantly higher than that in the mock group (Fig. 4A). The expression of mitochondrial ROS (MitoSox) was detected by fluorescence microscopy, and DOX increased the ROS level in mitochondria. Further, the ROS level in shPRDX1 group was higher than that in the mock group (Fig. 4B).

To further investigate whether ROS can cause mitochondrial dysfunction, mitochondrial membrane potential (JC-1) was observed by fluorescence microscopy. As shown in Fig. 4C, DOX could change the mitochondrial membrane potential, and this change in the shPRDX1 group was higher than that in the mock group. The expression level of the mitochondrial-dependent apoptosis-related proteins was detected by western blotting. As shown in Fig. 4D and E, DOX significantly increased the
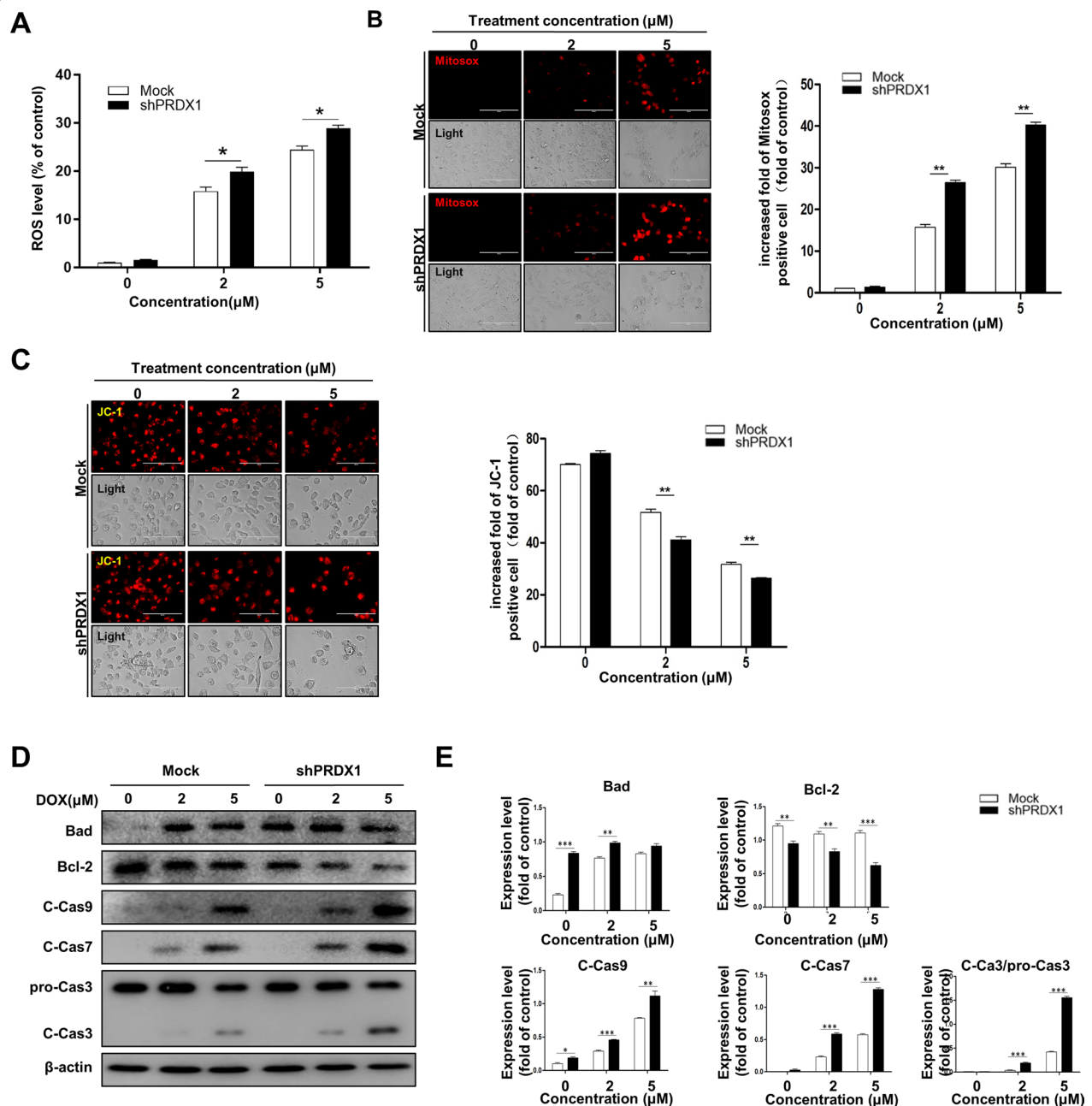
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Fig. 4 Peroxiredoxin 1 (PRDX1) knockout promoted doxorubicin (DOX)-induced mitochondrial damage in MDA-MB-231 cells. **A** Flow cytometry was used to detect intracellular reactive oxygen species (ROS) level after DOX treatment. **B** The level of ROS in the mitochondria was detected by immunofluorescence microscopy. **C** Mock MDA-MB-231 cells and shPRDX1 MDA-MB-231 cells were treated with DOX at concentrations of 2 μM and 5 μM, and the mitochondrial membrane potential was detected by immunofluorescence detection of JC-1. **D** The expression levels of Bad, Bcl-2, C-Cas9, C-Cas7, pro-Cas3, and C-Cas3 proteins were detected by western blot analysis. **E** The expression levels of Bad, Bcl-2, C-Cas9, C-Cas7, and C-Cas3/pro-Cas3 proteins were statistically analyzed

expression of the pro-apoptotic protein Bad, inhibited the expression of the anti-apoptotic protein Bcl-2, and promoted the cascade of C-Caspase 9, C-Caspase 7, and C-Caspase 3, thus, promoting the occurrence of apoptosis. In addition, the expression levels of pro-apoptotic

proteins Bad, C-caspase 9, C-Caspase 7, and C-Caspase 3 in the shPRDX1 group were significantly higher than those in the mock group following DOX treatment, while the expression level of anti-apoptotic protein Bcl-2 was significantly lower than that in the mock group. These

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results indicate that PRDX1 knockout promotes DOX-induced mitochondrial damage in MDA-MB-231 cells.

Inhibition of ROS reversed PRDX1-mediated apoptosis in MDA-MB-231 cells

ROS are the main cause of mitochondrial dysfunction and mitochondrial-dependent apoptosis. Compared with

the mock group, the shPRDX1 group had a higher ROS level and more evident apoptosis. Therefore, PRDX1 may regulate MDA-MB-231 cell apoptosis through ROS. Therefore, the cells were pretreated with the reactive oxygen scavenger N-acetyl-L-cysteine (NAC) for 30 min, and DOX was added after NAC pretreatment. The intracellular ROS level was detected by flow cytometry. As shown
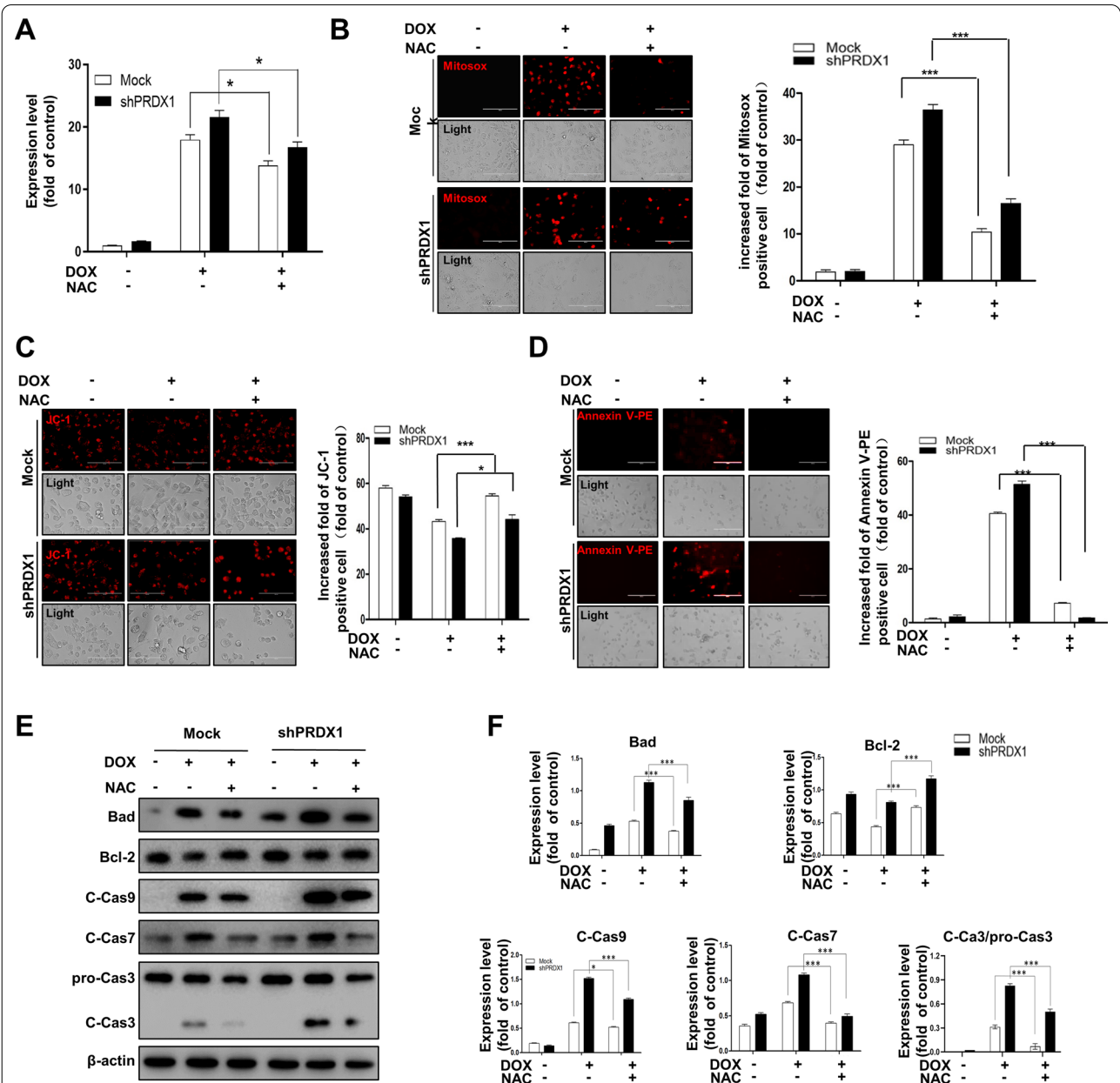

Fig. 5 Inhibition of reactive oxygen species (ROS) reversed peroxiredoxin 1 (PRDX1)-mediated apoptosis in MDA-MB-231 cells. **A** The level of reactive oxygen species (ROS) species was detected by flow cytometry. **B** ROS in the mitochondria was detected by fluorescence microscopy. **C** The mitochondrial membrane potential was observed by fluorescence microscopy. **D** Apoptosis was observed by fluorescence microscopy. **E** The expression level of Bad, Bcl-2, C-Cas9, C-Cas7, pro-Cas3, and C-Cas3 proteins were detected by western blot analysis. **F** The expression levels of Bad, Bcl-2, C-Cas7, pro-Cas3, and C-Cas3 proteins were statistically analyzed

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in Fig. 5A, the ROS levels in the mock and shPRDX1 groups were significantly reduced after the addition of NAC. The expression of mitochondrial ROS was detected by fluorescence microscopy (Fig. 5B). After the addition of NAC, the ROS water in the mock and shPRDX1 groups declined. Mitochondrial membrane potential was observed by fluorescence microscopy (Fig. 5C) and it was restored following the addition of NAC. Apoptosis was observed under fluorescence microscopy, and we observed that NAC could restore the DOX-induced apoptosis of MDA-MB-231 cells (Fig. 5D). The expression of the pro-apoptotic protein Bad/C-Caspase 9/C-Caspase 7/C-Caspase 3 induced by DOX was inhibited, the caspase cascade was inhibited, and the expression of the anti-apoptotic protein Bcl-2 induced by DOX was restored. As shown in Fig. 5E and F, these results suggest that NAC inhibits intracellular ROS levels, mitochondrial ROS levels, mitochondrial damage, and mitochondria-dependent apoptosis. These findings indicate that ROS is a key factor in DOX-induced apoptosis, and PRDX1 regulates DOX-induced apoptosis through ROS.

PRDX1 regulates DOX-induced apoptosis of MDA-MB-231 cells through the GSK3β/β-catenin signaling pathway

GSK3β plays an important role in the occurrence and development of various cancers by managing the process of cell apoptosis by regulating the mitochondrial permeability transition pore. Meanwhile, GSK3β can promote the phosphorylation and degradation of β-catenin, thereby inhibiting the entry of β-catenin into the nucleus and preventing cell proliferation. When phosphorylation of GSK3β (Ser9) occurs, the activity of GSK3β is inhibited to promote cell survival, and ROS are an important activation component of GSK3β. Previous studies have demonstrated that PRDX2 regulates mitochondrial damage and induces apoptosis in HT22 cells through the ROS-regulated GSK3β/β-catenin signaling pathway. Therefore, we hypothesized that PRDX1 may regulate apoptosis of MDA-MB-231 cells through the ROS-mediated GSK3β/β-catenin signaling pathway. The expression level of the GSK3β/β-catenin signal in Mock/shPRDX1 MDA-MB-231 cell apoptosis induced by DOX was detected by western blot analysis. As shown in Fig. 6A

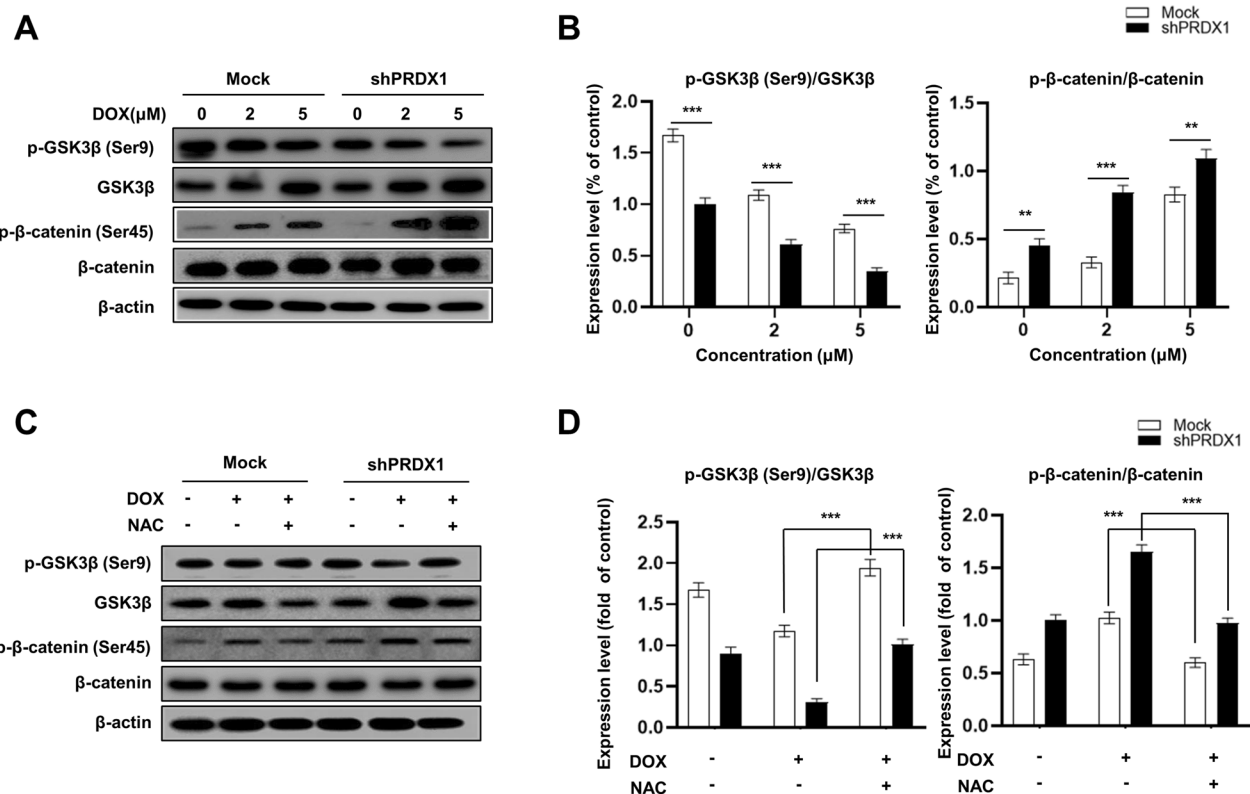**Fig. 6** Peroxiredoxin 1 (PRDX1) regulates doxorubicin (DOX)-induced apoptosis of MDA-MB-231 cells through glycogen synthase kinase 3β (GSK3β)/β-catenin signaling pathway. **A** Western blot was used to detect the expression of GSK3β, p-GSK3β, β-catenin, and p-β-catenin related proteins. **B** The expression levels of p-GSK3β/GSK3β and p-β-catenin/β-catenin-related proteins were statistically analyzed. **C** Western blot analysis was used to detect the expression of GSK3β, p-GSK3β, β-catenin, and p-β-catenin-related proteins. **D** The expression levels of p-GSK3β/GSK3β and p-β-catenin/β-catenin-related proteins were statistically analyzed

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and B, the expression level of GSK3β increased, and the expression level of GSK3β (Ser9) decreased in the mock/shPRDX1 MDA-MB-231 cells induced by DOX, indicating that GSK3β was successfully activated. In addition, the GSK3β activation level of shPRDX1 MDA-MB-231 cells significantly differed from that of the mock MDA-MB-231 cells. As shown in Fig. 6C and D, the expression level of β-catenin was significantly altered after GSK3β activation; however, the expression level of β-catenin significantly increased after GSK3β activation. β-catenin was degraded by proteasome after phosphorylation, thus inhibiting the uptake of β-catenin into the nucleus and promoting cell proliferation. The expression level of P-β-catenin significantly increased after *PRDX1* gene knockout, which was consistent with the results of apoptosis induced by DOX and apoptosis protein expression. To further demonstrate that PRDX1 regulates apoptosis by regulating intracellular ROS levels, the addition of NAC inhibited the activation of GSK3β induced by DOX and the phosphorylation of β-catenin were increased, which corresponded with the extent of apoptosis and expression levels of apoptosis proteins after the addition of NAC. Therefore, ROS is a key factor in DOX-induced apoptosis, and PRDX1 regulates this apoptosis through ROS-dependent GSK3β signaling.

GSK3β inhibitors inhibit MDA-MB-231 cell apoptosis

To further prove the critical role of GSK3β, we pretreated the cells with LiCl, a GSK3β inhibitor, for 1 h and then added DOX. Western blot analysis was used to detect GSK3β/β-catenin expression in the mock/shPRDX1 MDA-MB-231 cells pretreated with DOX. As shown in Fig. 7A and B, DOX inhibited the upregulated expression of GSK3β and P-β-catenin, while β-catenin and P-GSK3β expression did not significantly change after LiCl treatment. Mitochondrial membrane potential was observed by JC-1 staining (Fig. 7C). The mitochondrial membrane potential was recovered after the addition of LiCl, a GSK3β inhibitor. Apoptosis was observed by Annexin V-PE staining, and we found that LiCl could restore DOX-induced apoptosis of MDA-MB-231 cells (Fig. 7D). After adding LiCl, the DOX-induced expression of pro-apoptotic protein Bad/C-caspase 9/C-caspase 7/C-caspase 3 was inhibited, and the cascade reaction of caspase was inhibited. However, DOX-induced anti-apoptotic protein Bcl-2 did not change significantly (Fig. 7E and F), indicating LiCl mitochondria damage and mitochondria-dependent cell apoptosis.

PRDX1 regulates GSK3β activity through the PI3K/Akt pathway

GSK3β is a serine/threonine kinase consisting of Axis inhibition protein Axin, β-catenin, and adenomatous colonic polyposis protein. In addition to GSK-3α and GSK-3β, the amino acid sequences of the catalytic active regions of GSK-3α and GSK-3β were 97% homologous and widely expressed in cells and tissues, exhibiting similar biological characteristics. GSK3β is an important substrate of Akt, negatively regulated by Akt and plays an important role in cell proliferation, apoptosis, and other biological processes. In addition, PRDX1 has been reported to maintain the apoptosis of cancer cells by regulating the PI3K/Akt signaling pathway. Thus, we speculate that the PI3K/Akt signaling pathway was the key for PRDX1 to regulate the GSK3β/β-catenin signaling pathway in this experiment. The expression level of the PI3K/Akt protein in the mock/shPRDX1 MDA-MB-231 cells was detected by western blot analysis. The expression level of PI3K and P-Akt decreased in the mock/shPRDX1 MDA-MB-231 cells treated with DOX. Moreover, the p-Akt expression level of the shPRDX1 MDA-MB-231 cells significantly differed from that of the mock MDA-MB-231 cells, suggesting that *PRDX1* regulates GSK3β activation through the PI3K/Akt signaling pathway (Fig. 8A and B). To further verify the role of the PI3K/Akt signaling pathway, the MDA-MB-231 cells were treated with Akt activator SC79 and DOX, and apoptosis levels were measured by fluorescence photography. Annexin V-PE indicated that SC79 inhibited DOX-induced apoptosis (Fig. 8C). The western blotting results showed that the expression level of P-Akt increased. Further, DOX-induced expression of pro-apoptotic protein Bad/C-caspase 9/C-caspase 7/C-caspase 3 and the cascade reaction of caspase were inhibited. DOX treatment restored anti-apoptotic protein Bcl-2 expression (Fig. 8D and E).

Discussion

The MDA-MB-231 cell line was isolated from the pleural effusion of a 51-year-old Caucasian woman with advanced ductal infiltrating carcinoma of the breast by the MD Anderson Cancer Center (MDACC). The main manifestations were negative ER, PR, and HER-2, with high metastasis, high recurrence, and poor prognosis. Therefore, MDA-MB-231 cells represent a good tumor model to study drug therapy for TNBC [19]. DOX, an anthracycline antibiotic, has exhibited good efficacy for chemotherapy of breast cancer and leukemia since its clinical use. DOX has become a first-line clinical drug; however, it has strong toxic side effects, and its cardiotoxicity is significant; for example, a large amount of accumulation in the body can lead to cardiac shock and death [20]. Moreover, long-term use of DOX can lead to cancer cells as well as their resistance to other chemotherapeutic drugs with different structures. Controversies currently exist regarding the clinical use of DOX; however, the advantages still outweigh the disadvantages
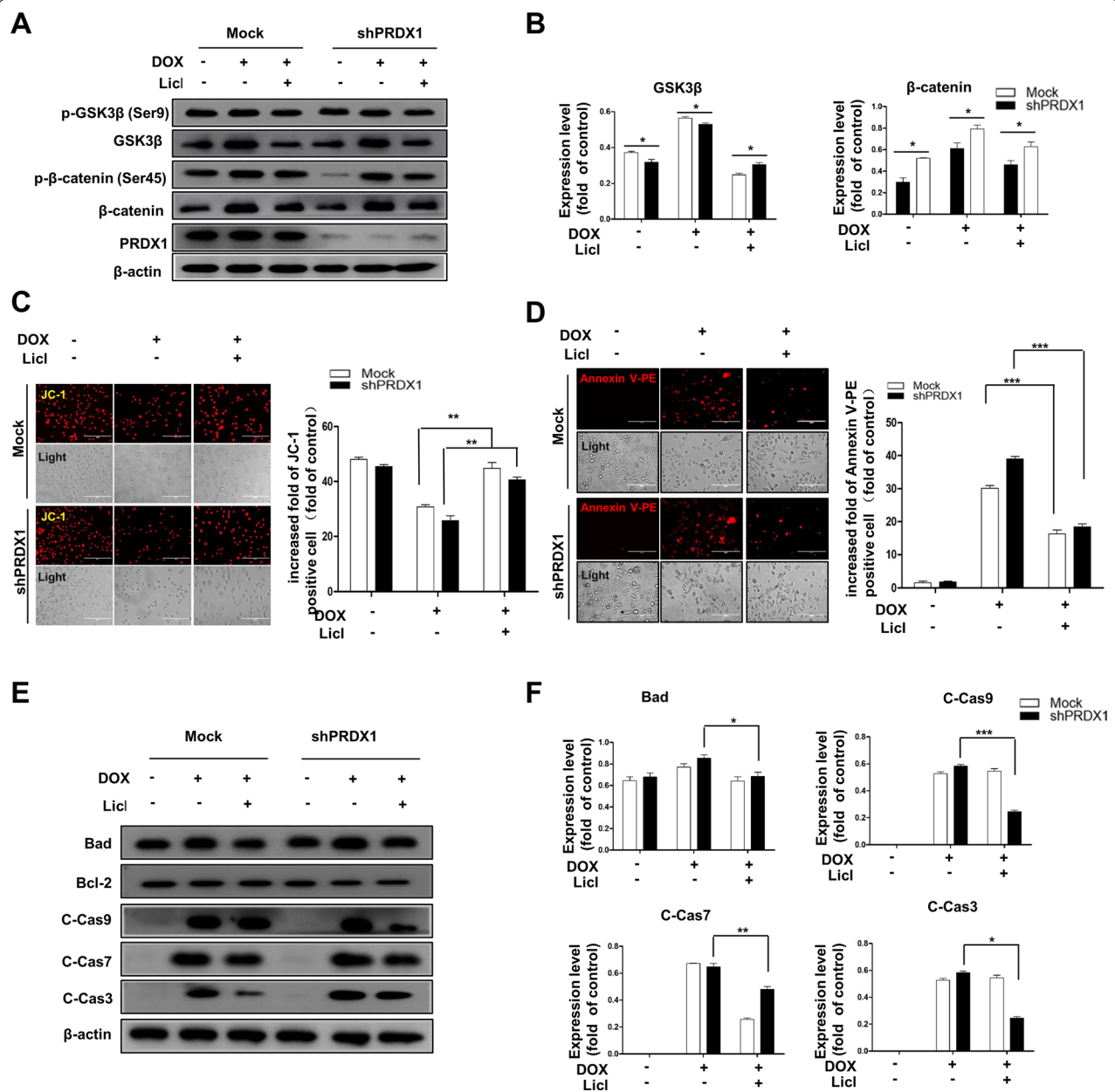
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Fig. 7 Glycogen synthase kinase 3β (GSK3β) inhibitors inhibit apoptosis of MDA-MB-231 cells. **A** Western blot analysis was used to detect the expression of GSK3β, p-GSK3β, β-catenin, and p-β-catenin-related proteins. **B** The expression levels of GSK3β and β-catenin-related proteins were statistically analyzed. **C** The mitochondrial membrane potential was observed by fluorescence microscopy. **D** Apoptosis was observed by fluorescence microscopy. **E** The expression levels of Bad, Bcl-2, C-Cas9, C-Cas7, and C-Cas3 proteins were detected by western blot analysis. **F** The expression levels of Bad, C-Cas9, C-Cas7, and C-Cas3-related proteins were statistically analyzed

[21]. To reduce the clinical use of DOX and alleviate its cardiotoxicity, the majority of research has focused either on vector targeting of DOX to achieve a better effect or on other methods to increase the sensitivity of cancer cells to DOX [22, 23]. DOX has a high affinity for cardiolipin and the inner membrane of mitochondria is rich in cardiolipin; therefore, DOX has a high affinity for

mitochondria [24, 25]. After entering cells, DOX can produce a large amount of ROS through the DOX semiquinone cycle to cause mitochondrial damage and induce mitochondria-dependent apoptosis.

Peroxiredoxins are intracellular antioxidant enzymes that are extremely sensitive to ROS. PRDX1 is closely related to the occurrence and development of various
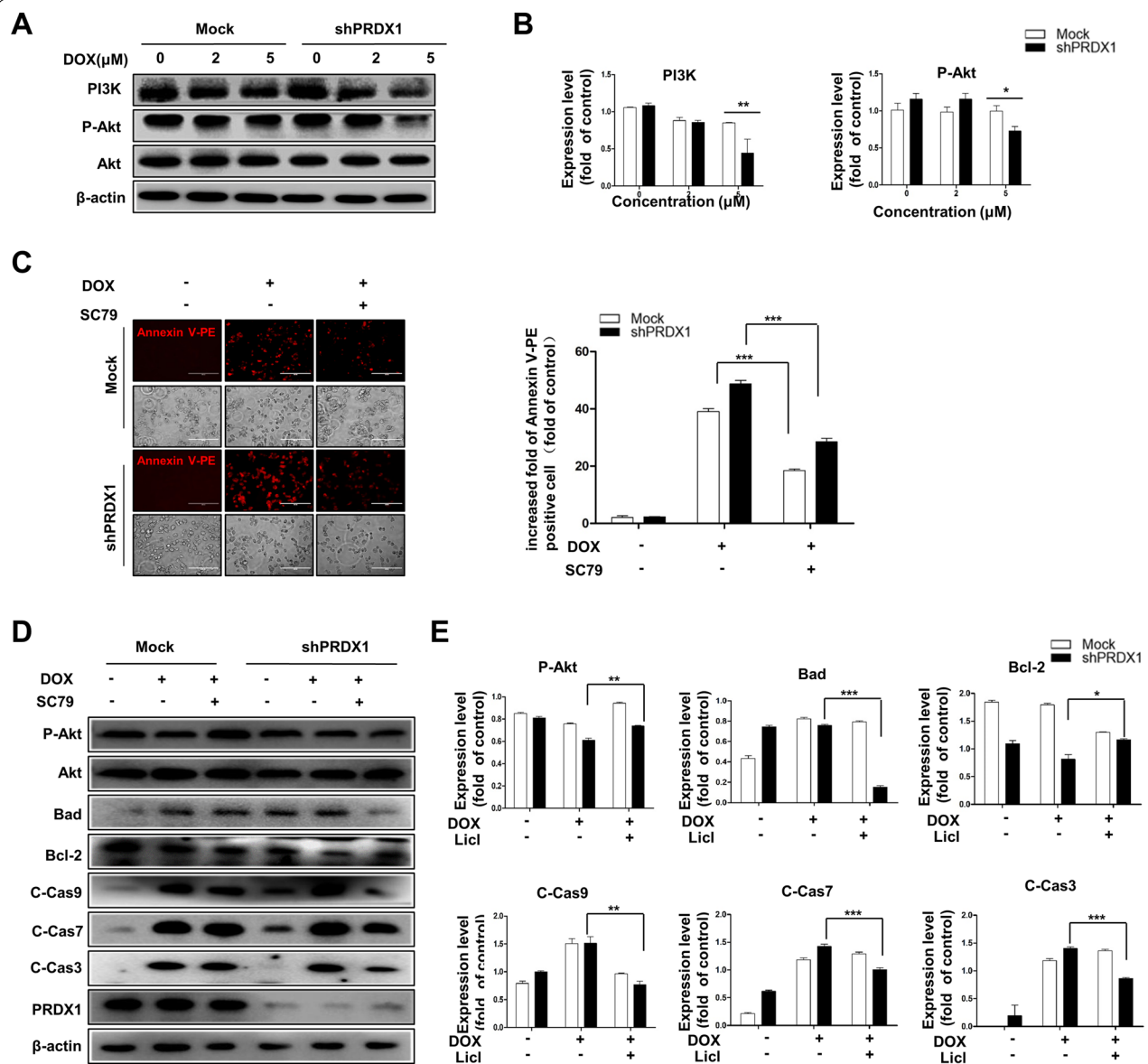
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Fig. 8 Peroxiredoxin 1 (PRDX1) regulates doxorubicin (DOX)-induced apoptosis of MDA-MB-231 cells through phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt)/glycogen synthase kinase 3β (GSK3β) signaling. **A** Western blot was used to detect the expression of PI3K, Akt, and P-Akt proteins. **B** The expression levels of PI3K and P-Akt-related proteins were statistically analyzed. **C** Apoptosis was observed by fluorescence microscopy. **D** The expression levels of P-Akt, Akt, Bad, Bcl-2, C-Cas9, C-Cas7, C-Cas3, and C-Cas3 were detected by western blot analysis. **E** The expression levels of P-Akt, bad, Bcl-2, C-Cas9, C-Cas7, and C-Cas3-related proteins were statistically analyzed

cancers and plays an important role in the pathogenesis, treatment, and prognosis of various cancers. PRDX1, as a member of the peroxidase reductase family, participates in various life activities, regulates different ROS-dependent signaling pathways, and is considered to be a key regulatory molecule that balances cell survival and apoptosis in the cell [26]. Past studies have shown that PRDX1 expression level is high in pancreatic cancer, and the upregulation of PRDX1 expression is closely related to tumor

angiogenesis. Therefore, PRDX1 is expected to be used as a tumor marker for the diagnosis and prognosis of pancreatic cancer [27]. PRDX1 also plays an important role in colorectal cancer. Compared with that in a PRDX1-deficient cell medium, the tubule formation ability of human umbilicus vein endothelial cells cultured in a PRDX1 overexpressed cell medium increased and the protein expression of matrix metalloproteinase 2 (MMP) 2, MMP9, and vascular endothelial growth factor A (VEGFA) increased

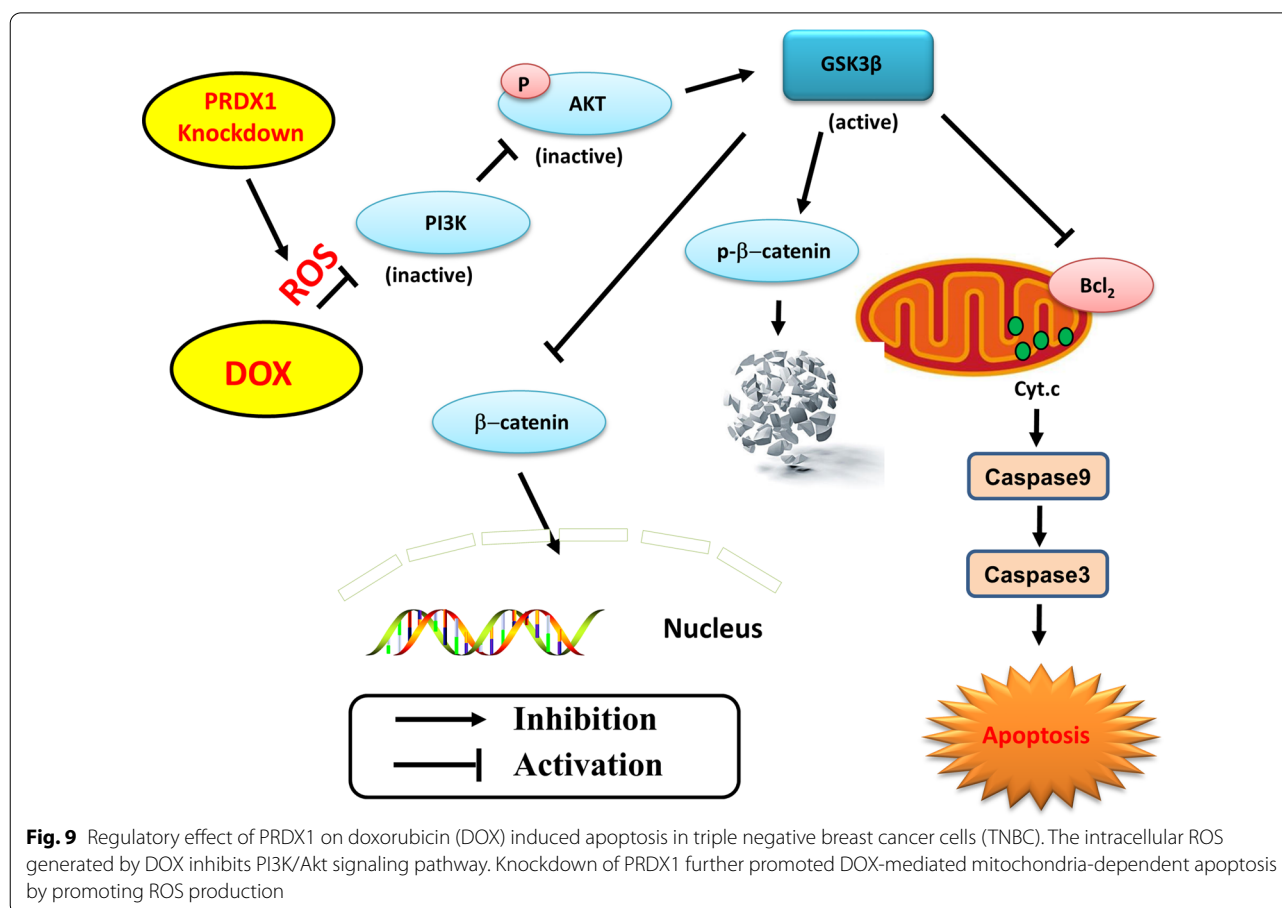Han *et al. Applied Biological Chemistry*   (2022) 65:63

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after the upregulation of PRDX1. This phenomenon suggests that PRDX1 expression can regulate tumor metastasis and angiogenesis in rectal cancer [28]. PRDX1 was highly expressed in 20% of patients with ovarian cancer, and the chemotherapy effect of these patients was worse than other patients. Further, the number of patients with high PRDX1 expression in a 5-year poor prognosis were much higher than in patients, indicating that PRDX1 is closely related to chemotherapy effect and poor prognosis in ovarian cancer [29]. PRDX1 significantly inhibited the growth rate of McF-7 and ZR-75–1 breast cancer cells, and xenografted PRDX1-deficient McF-7 cells showed inhibition of tumor growth [30].

The *PRDX1* gene was negatively correlated with the incidence and prognosis of breast cancer and TNBC. In the present study, the expression level of the PRDX1 protein in the MDA-MB-231 cells significantly increased after DOX treatment. Although the causes of the increase were not further explored in this study, this phenomenon will be investigated in future studies. The increased expression of the PRDX1 protein may resist the apoptosis of MDA-MB-231 cell line induced by DOX. To further investigate the relationship among *PRDX1*, DOX, and MDA-MB-231 cells, *PRDX1* gene knockdown was performed in the MDA-MB-231 cells. The deletion of PRDX1 increased the sensitivity of DOX to MDA-MB-231 cells. ROS were identified as a key factor after comparing the function of PRDX1 with the mechanism of DOX. Experiments showed that MDA-MB-231 cells with PRDX1 deletion exhibited higher ROS and mitochondrial activity levels than cells without PRDX1 deletion. However, a higher ROS level would destroy mitochondrial membrane potential and cause mitochondria-dependent apoptosis. We found that the changes in mitochondria-dependent apoptosis signal in the MDA-MB-231 cells were clearer following PRDX1 deletion. This confirmed that ROS is the main factor causing MDA-MB-231 cells to be more sensitive after PRDX1 deletion. However, the therapeutic effect in vivo requires further investigation.

The role and function of PRDX1 is becoming clearer as the extent of research increases. PRDX1 regulates cell-related processes by regulating intracellular ROS level [31, 32]. ROS-dependent signaling pathways regulated by PRDX1 play an important role in the progression and metastasis of human tumors, especially in breast, esophageal, and lung cancers [26]. GSK3β is a type of serine/threonine kinase commonly expressed in cells. ROS are important to activate GSK3, and the activated GSK3 is closely related to the growth and apoptosis of various cancers [33]. GSK3 is essential for the growth and survival of human mutant KRAS-dependent tumors in vitro and in vivo, and the inhibition of GSK3 upregulated the expression of β-catenin, and C-myc can resist KRAS-dependent tumors [34]. Regulation of β-catenin by GSK3β plays a key role in hepatocellular carcinoma [35]. Downregulation of GSK3β inhibits the growth, angiogenesis, and expression of VEGF in pancreatic cancer [36–39]. In addition, ROS is one of the important pathways of GSK3β activation. Previous studies have determined that PRDX2 knockdown can exacerbate alcohol damage to HT22, in which ROS mediated GSK3β/β-catenin signaling plays an important role [40]. Therefore, GSK3β/β-catenin may play an important role in DOX-induced apoptosis of mock and shPRDX1 MDA-MB-231 cells. By detection of the GSK3β/β-catenin signal expression level, DOX-induced GSK3β (Ser9) phosphorylation significantly decreased in shPRDX1 MDA-MB-231 cells and promoted the activation of GSK3β. The activated GSK3β can promote the expression of Bax and inhibit Bcl-2. Mitochondrial membrane potential changes promote the release of cytochrome C and caspase cascade to encourage cell apoptosis. These results were consistent with the results of apoptosis, suggesting that GSK3β/β-catenin is a critical point at which PRDX1 regulates the DOX-induced apoptosis of MDA-MB-231 cells. DOX was demonstrated to inhibit the activation of GSK3β following the addition of reactive oxygen scavenger NAC, which was consistent with the extent of apoptosis and expression of apoptotic proteins after the addition of NAC and further confirms that PRDX1 is regulated by ROS. Therefore, PRDX1 regulates the DOX-induced apoptosis of MDA-MB-231 cells through ROS-dependent GSK3β signaling.

However, how PRDX1 regulates GSK3β activation through ROS is still unknown. Previous studies have shown that PRDX1 is highly expressed in esophageal cancer cells, and silencing PRDX1 can inhibit the proliferation of esophageal cancer cells and promote cell apoptosis. This mechanism may be related to the inhibition of the PI3K/Akt signaling pathway [41]. Although the current study proved that PRDX1 was involved in DOX-induced apoptosis of MDA-MB-231 cells by regulating intracellular ROS level and mediating the activation of GSK3β signal, whether PRDX1 has a more involved role requires further investigation. PRDX1 is a tumor suppressor for human nasopharyngeal carcinoma and may be a prognostic marker for patients with nasopharyngeal carcinoma. PRDX1 inhibits nasopharyngeal carcinoma by inhibiting the activation of the PI3K/Akt/TRAF1 signaling pathway [42]. Therefore, PRDX1 may be a key regulator of PI3K/Akt signaling pathway. GSK3β is an important substrate of Akt, and its activity is negatively regulated by Akt [43]. Protein kinase B regulates cell growth and development by inhibiting GSK3β. Therefore, the PI3K/Akt signaling pathway may be the key to PRDX1's regulation of GSK3β activation. By detecting the

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Fig. 9 Regulatory effect of PRDX1 on doxorubicin (DOX) induced apoptosis in triple negative breast cancer cells (TNBC). The intracellular ROS generated by DOX inhibits PI3K/Akt signaling pathway. Knockdown of PRDX1 further promoted DOX-mediated mitochondria-dependent apoptosis by promoting ROS production

expression level of the PI3K/Akt signal, DOX could significantly reduce the expression level of PI3K and P-Akt in shPRDX1 MDA-MB-231 cells, promote the activation of GSK3β, and enhance cell apoptosis. Further, the apoptosis level was inhibited by the addition of Akt activator. We demonstrated that deletion of PRDX1 inhibited Akt phosphorylation and promoted GSK3β expression. GSK3β is an upstream signal of mitochondria-dependent apoptosis, and is also an important target of ROS. PRDX1 is involved in the DOX-induced apoptosis of TNBC cells by regulating the GSK3β expression level.

Conclusion

In conclusion, PRDX1 mediates the activation of PI3K/Akt signaling through the regulation of ROS levels, which then activates the GSK3β protein, inhibits the activation of β-catenin, and promotes the expression of mitochondria-dependent apoptotic proteins, thereby modulating DOX-induced apoptosis in MDA-MB-231 cells (Fig. 9). This study demonstrated that PRDX1 could regulate DOX-induced apoptosis in TNBC cells. These findings may provide a theoretical basis and potential drug target for the clinical treatment of TNBC.

Author contributions
YHH, XDL, and SJL contributed to the conception of the study, writing of the manuscript, and performing the literature search. XDL, WLL and SJL performed data analysis. YHH, MHJ, and HNS performed analysis and assessed the quality of the study. YHH, XDL, SJL and TK confirm the authenticity of all the raw. YHH and TK conceived and designed the project. All authors read and approved the final manuscript.

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