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Silencing of B7-H4 induces intracellular oxidative stress and inhibits cell viability of breast cancer cells via downregulating PRDX3

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Breast cancer (BC) is the most common malignancy in women worldwide, accounting for 15.5% of total cancer deaths. B7-H4 belongs to the B7 family members and plays an important role in the development of a variety of cancers, while Peroxiredoxin III (PRDX3) is an antioxidant protein found in mitochondria. Aberrant expression of B7-H4 or PRDX3 has been implicated in the tumorigenesis of various cancers. However, the functional roles of B7-H4 and PRDX3 in BC and the underlying mechanisms remain unclear. In this research, we found that silencing of B7-H4 by siRNA could lead to not only cell viability inhibition but also the downregulation of PRDX3 in MCF-7 and T47D cells. In order to reveal the roles of PRDX3 in the B7-H4 pathway, we firstly transfected siRNA specifically targeting PRDX3 into MCF-7 and T47D cells, and the results showed that silencing of PRDX3 also inhibited the viability of MCF-7 and T47D cells significantly, accompanied by the increase of reactive oxygen species (ROS) levels. Then we overexpressed the expression of PRDX3 by transfecting PRDX3 expression plasmids into B7-H4 knocking-down cells of MCF-7 and T47D. The results showed that compared with the control groups (MCF-7 or T47D/siNC+pcDNA3.1 vector), cell viabilities were significantly inhibited in RNAi groups (MCF-7 or T47D/siB7-H4+pcDNA3.1 vector), and mildly inhibited in revertant groups (MCF-7 or T47D/siB7-H4+pcDNA3.1 PRDX3), meanwhile, ROS levels significantly elevated in RNAi groups and had no significant changes in revertant groups. All these results indicate that silencing of B7-H4 increases intracellular ROS levels and affects cell viability by modulating the expression of PRDX3 in BC cells, which may provide a potential strategy and therapeutic target for the treatment of BC.

Key words: breast cancer, B7-H4, PRDX3, ROS

As the leading cause of cancer death in women, the incidence of BC is much higher than that of lung and colon cancer in women worldwide [1]. BC has been classified into four biological types: luminal A, luminal B, HER2-like, and basal-like [2]. The pathogenesis of BC is complex and has many influencing factors. Studying the molecular mechanism of BC occurrence and development is of great significance for the early diagnosis and clinical treatment of BC.

The peroxiredoxin family is mainly involved in redox regulation of the cells and protects radical-sensitive enzymes from oxidative damage through a radical-generating system. It has been shown that proteins of this family are involved in regulating many other cells' biological phenomena through regulating downstream signaling cascades by modulating intracellular hydrogen peroxide levels, which further affects gene expression, cell proliferation, apoptosis, and differentiation [3]. PRDX3 has been reported to be highly expressed in many kinds of tumors and plays diverse roles in tumorigenesis, such as uveal melanoma, prostate cancer, ovarian cancer, acute myeloid leukemia, hepatocellular carcinoma, and renal cell carcinoma [4–9]. However, as a key enzyme regulating intracellular hydrogen peroxide levels in cancer cells, the functional role of PRDX3 in BC remains to be explored.

The co-inhibitory molecule B7-H4 is an important member of the B7 family and is aberrantly expressed in tumors, inflammatory and autoimmune diseases [10]. B7-H4 is highly expressed in a variety of tumors and can be used as a biomarker for tumor diagnosis, which is closely associated with a poor prognosis and high recurrence rates [11, 12]. Recent studies indicate that B7-H4 plays an important role in tumor initiation and progression, including cell proliferation, invasion, metastasis, and resistance to apoptosis [13–16]. Moreover, inhibiting the glycosylation of B7-H4, combined with immunogenic chemotherapy and PD-L1 blockade, could be an efficient strategy for the treatment of TNBC (triple-negative breast cancer) [17]. However, the mechanism of B7-H4 on BC development and progression still remains unclear.

In this experiment, we found that silencing of B7-H4 or PRDX3 by siRNA could both lead to cell viability inhibition in BC cells, moreover, silencing of B7-H4 could lead to downregulation of PRDX3 and upregulation of ROS levels in BC cells. Therefore, the objective of this study is to investigate the roles of PRDX3 on cell viability and ROS level changes of BC cells induced by silencing of B7-H4, which may provide a potential strategy and therapeutic target for the treatment of BC.

Materials and methods

Cell lines and cell culture. Human breast cancer cell lines T47D, MCF-7, and SK-BR-3 were purchased from Procell Life Technology Co. Ltd (Wuhan, China). Cells were cultured in DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (Excell Bio, USA) at 37 °C in a humidified atmosphere consisting of 5% CO_2.

Cell transfection. B7-H4-specific siRNA (5'-GCUCUACAAUGUUACGAUCAATT-3'), PRDX3-specific siRNA (5'-GCCCGACAUGUGAGUGCCAUUTT-3'), and negative control siRNA (scrambled sequence, 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Shenggong Bioengineering Co. Ltd (Shanghai, China). The PRDX3 coding sequence was synthesized by polymerase chain reaction using the cDNA of MCF-7 and T47D cells as templates and was inserted into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA). The successfully constructed plasmids were confirmed by DNA sequencing. Both siRNA and plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 6 hours of transfection, the medium was replaced with a fresh medium.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA from cell lines was extracted using the TRIzol reagent (TaKaRa Biotechnology, Tokyo, Japan), and 1 μg of RNA from each sample was used for cDNA synthesis using PrimeScript RT Kit (TaKaRa Biotechnology) according to the manufacturer's instruction. The primer pairs used for qRT-PCR are as follows: GAPDH: sense 5'-CCACATCGCTCAGACACCAT-3' and antisense 5'-GGCAACAATATCCACTTTACCAGAGT-3'; B7-H4: sense 5'-CGCTGCTAATTGACTGCCAC-3' and antisense 5'-GCCGACTGCTCTGTTTATGC-3'; PRDX3: sense 5'-ACGGTGTGCTGTTAGAAGGT-3' and antisense 5'-TTCACCAAGCGGAGGGTTTC-3'. Expression data were normalized to the geometric mean of the GAPDH gene to control the variability in expression levels.

Western blot analysis. Cell protein lysates were prepared and subjected to western blotting as previously described [18]. Samples were obtained by cell lysis in RIPA buffer (Beyotime Biotechnology, China) and separated by SDS-PAGE before being tested by using ECL chemiluminescent (Bio-Rad Chemidoc MP, Singapore). The primary antibodies used in this experiment were antibodies against B7-H4 (66817-1-lg, ProteinTech Group, Chicago, IL, USA) and PRDX3 (ab128953, Abcam, Cambridge, UK) at 1:1000 dilution. Goat anti-mouse and goat anti-rabbit IgG coupled to HRP (ZSGB-BIO, Beijing, China) were used as secondary antibodies at 1:5000 dilution. GAPDH (ab8245, Abcam) was used as an internal standard.

Cell viability assays. Cell viability was detected by using the Cell Counting Kit (KeyGen Biotech, Nanjing, China). Cells were seeded into 96-well plates and were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere consisting of 5% CO_2. Then, 10 μl CCK-8 solution was added into each well at different time points of 0 h, 24 h, 48 h, 72 h respectively, and incubated at 37 °C for another 2 h. OD values at 450 nm for each well were detected by BioTek Epoch microplate spectrophotometer (VT, USA).

ROS measurements. Cells were collected and suspended in DCFH-DA (Beyotime Biotechnology, China), and then diluted in a serum-free medium according to the instructions. The cells were incubated for 30 min in a 37 °C cell incubator and mixed thoroughly by inverting every 5 min to allow the probe and cells to come into contact. Cells were washed three times with a serum-free cell culture medium and detected with flow cytometry.

Statistical analysis. All experiments were repeated at least three times. Results are expressed as means ± standard deviation (SD). Student's t-test and one-way analysis of variance (ANOVA) tests were used. Statistical analyses were performed using SPSS version 21.0 (IBM Company, USA) and GraphPad Prism 8.3.0 Software (GraphPad, USA). Values of $p<0.05$ were considered significant and indicated by asterisks in all the figures.

Results

Expression of B7-H4 in various molecular subtypes and cell lines of BC. To understand how B7-H4 is expressed in BC, we analyzed RNA-seq data from the GEPIA2 public database (http://gepia2.cancer-pku.cn/#index). As shown in Figure 1A, B7-H4 expression was significantly elevated in basal-like subtype BC tissues. Survival analyses showed that high levels of B7-H4 were significantly associated with poorer overall survival (OS) in luminal B subtype BC patients (Figure 1B). Following, we assessed the B7-H4 expression in three BC cell lines from our laboratory at both RNA and protein levels. Results showed that B7-H4 was highly expressed in T47D and MCF-7 cell lines, and these two cell lines were selected for subsequent experiments (Figures 1C, 1D).

Silencing of B7-H4 downregulated the expression of PRDX3 and decreased cell viability of BC cells accompanied by increased ROS levels. siRNA specifically targeting B7-H4 was transfected into MCF-7 and T47D cells to inhibit B7-H4 expression. RT-qPCR and western blotting results showed significant reductions of B7-H4 expression

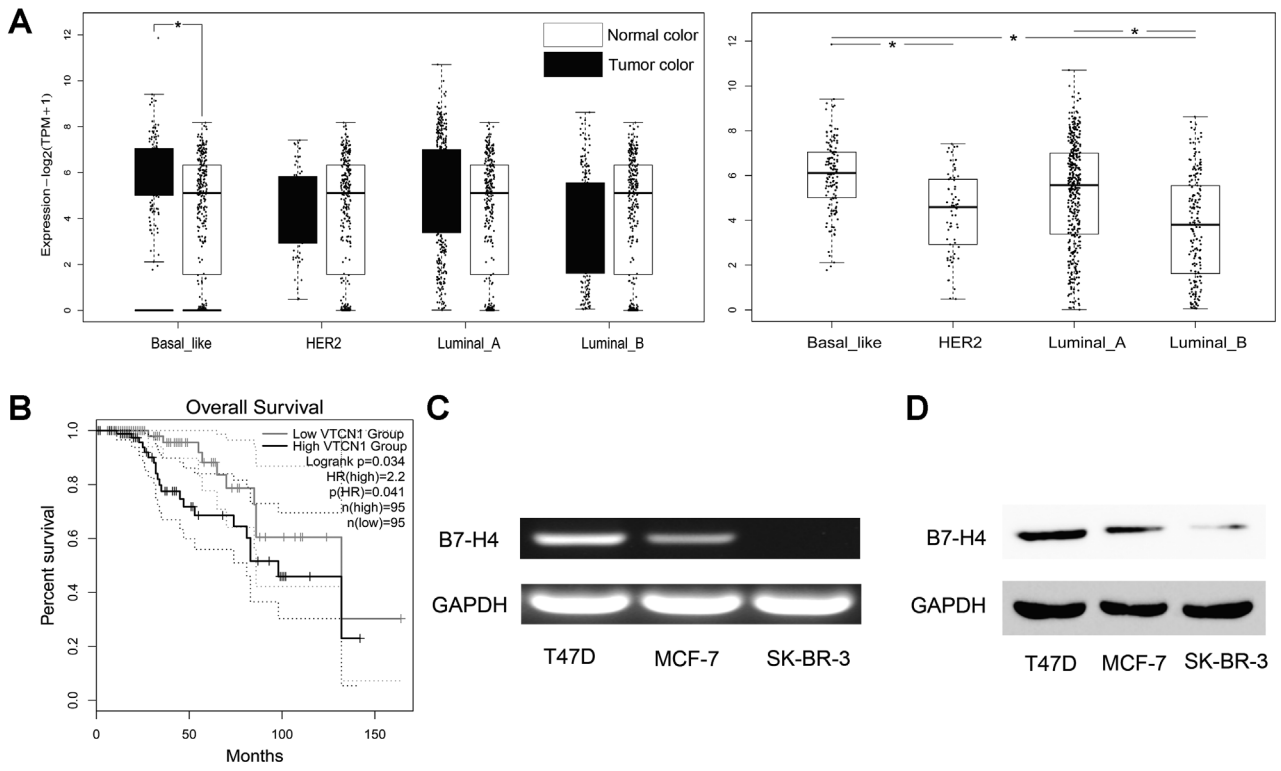


Figure 1. B7-H4 is highly expressed in basal-like subtype BC tissues and breast cancer cell lines. A) and B) RNA-seq data and survival analysis result from the GEPIA 2.0 public databases; C) Electrophoresis showed the RNA levels of B7-H4 in BC cell lines; D) Western blot result showed the protein levels of B7-H4 in BC cell lines. (*p<0.05)

in both MCF-7 and T47D cells compared to the corresponding control cells (Figures 2A, 2B). In the meantime, PRDX3 expression was also observed to be downregulated in parallel with decreased B7-H4 expression (Figures 2A, 2B). The results of the CCK-8 assay (Figure 2C) showed that, compared with MCF-7/T47D-siNC, the viability of MCF-7/T47D-siB7-H4 cells was significantly inhibited. Moreover, we observed increased intracellular ROS levels in both B7-H4 knocked down MCF-7 and T47D cells (Figures 2D, 2E). These findings suggested that silencing of B7-H4 downregulated the expression of PRDX3 and decreased cell viability in BC cells accompanied by increased intracellular ROS levels.

Silencing of PRDX3 induced intracellular oxidative stress and cell viability inhibition in BC cells. To investigate the effect of reduced PRDX3 expression on BC cell lines, we transfected siRNA targeting PRDX3 and non-coding siRNA into both MCF-7 and T47D cells, respectively. RT-qPCR and western blotting results (Figures 3A, 3B) showed that, compared with the corresponding control cells, the mRNA and protein expression levels of PRDX3 were significantly decreased in PRDX3-siRNA cells, indicating the successful inhibition of PRDX3 expression in T47D and MCF-7 cells. Using these two PRDX3 downregulated cell models, we tested changes in cell viability and intracellular ROS levels. The results of the CCK-8 assay (Figure 3C) showed that, compared with MCF-7/T47D-siNC, the viability of MCF-7/T47D-siPRDX3 cells was significantly inhibited. Furthermore, the silencing of PRDX3 in both MCF-7 and T47D cells resulted in elevated intracellular ROS levels significantly (Figures 3D, 3E). The above results indicated that silencing of PRDX3 in MCF-7 and T47D cells induced intracellular oxidative stress and inhibited cell viability.

Overexpression of PRDX3 partially attenuated B7-H4 silencing-induced intracellular oxidative stress and cell viability inhibition of BC cells. To further explore whether PRDX3 mediated the B7-H4 function of regulating BC intracellular ROS levels and cell viability, we examined the rescue effect by overexpressing PRDX3 in B7-H4-knockdown cells. The cells were divided into three groups: control groups (MCF-7 or T47D/siNC+pcDNA3.1 vector), RNAi groups (MCF-7 or T47D/siB7-H4+pcDNA3.1 vector), and revertant groups (MCF-7 or T47D/siB7-H4+pcDNA3.1 PRDX3). We found that, in the RNAi groups, silencing of the B7-H4 expression led to the downregulation of PRDX3 accompanied by cell viability inhibition and upregulation of ROS levels (Figures A–E), While, in the revertant groups, the PRDX3 expression level was successfully reversed in

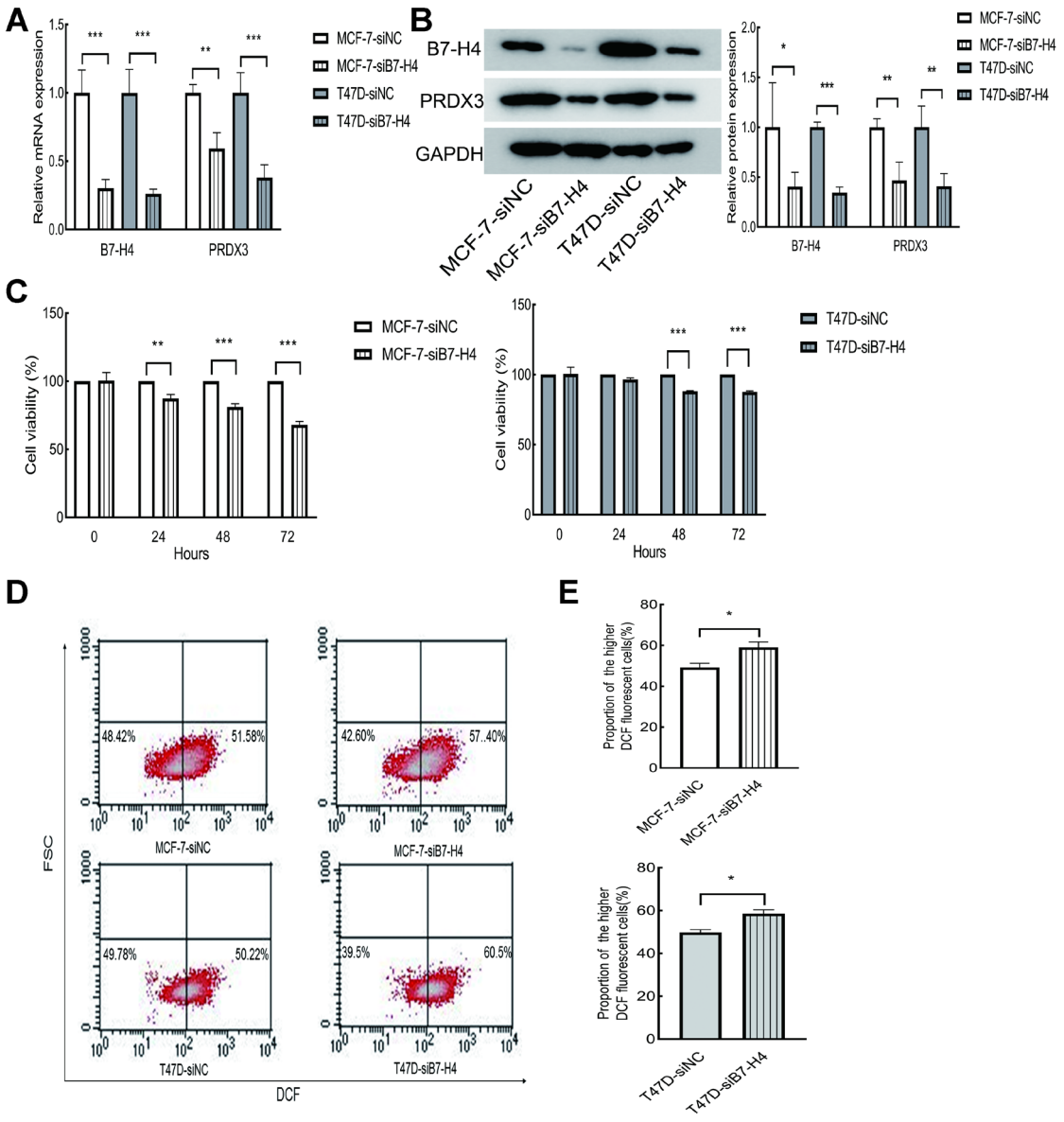


Figure 2. Silencing of B7-H4 downregulated the expression of PRDX3 in BC cells. A) The RNA expressions of B7-H4 and PRDX3 reduced in both MCF-7 cells and T47D cells after silencing of B7-H4; B) The protein expressions of B7-H4 and PRDX3 reduced in both MCF-7 cells and T47D cells after silencing of B7-H4; C) Silencing of B7-H4 led to cell viability inhibition in MCF-7 and T47D cells; D, E) Silencing of B7-H4 resulted in ROS levels elevating in MCF-7 and T47D cells. Error bars represent the means ± SEM, *p<0.05, **p<0.01, ***p<0.001

B7-H4 knocking-down cells as indicated by RT-qPCR and western blotting (Figures 4A, 4B). Meanwhile, as indicated by the CCK8 assays (Figure 4C), the viability inhibition by silencing of B7-H4 was partially reversed in revertant groups. Moreover, overexpression of PRDX3 reversed intracellular ROS levels in revertant groups compared with RNAi groups (Figures 4D, 4E). These results suggested that PRDX3 played an important role in mediating the B7-H4 function of inducing intracellular oxidative stress and viability inhibition in BC cells.

Discussion

BC is the most commonly diagnosed cancer and the leading cause of cancer deaths among women. It accounts for one in four cancer cases and one in six cancer deaths, ranking first for incidence in the vast majority of countries in 2020 [1]. New means of early clinical diagnosis and treatment of BC are urgently needed.

B7-H4, which belongs to the B7 family with PD-L1, has been found in multiple solid tumors and is inversely corre-

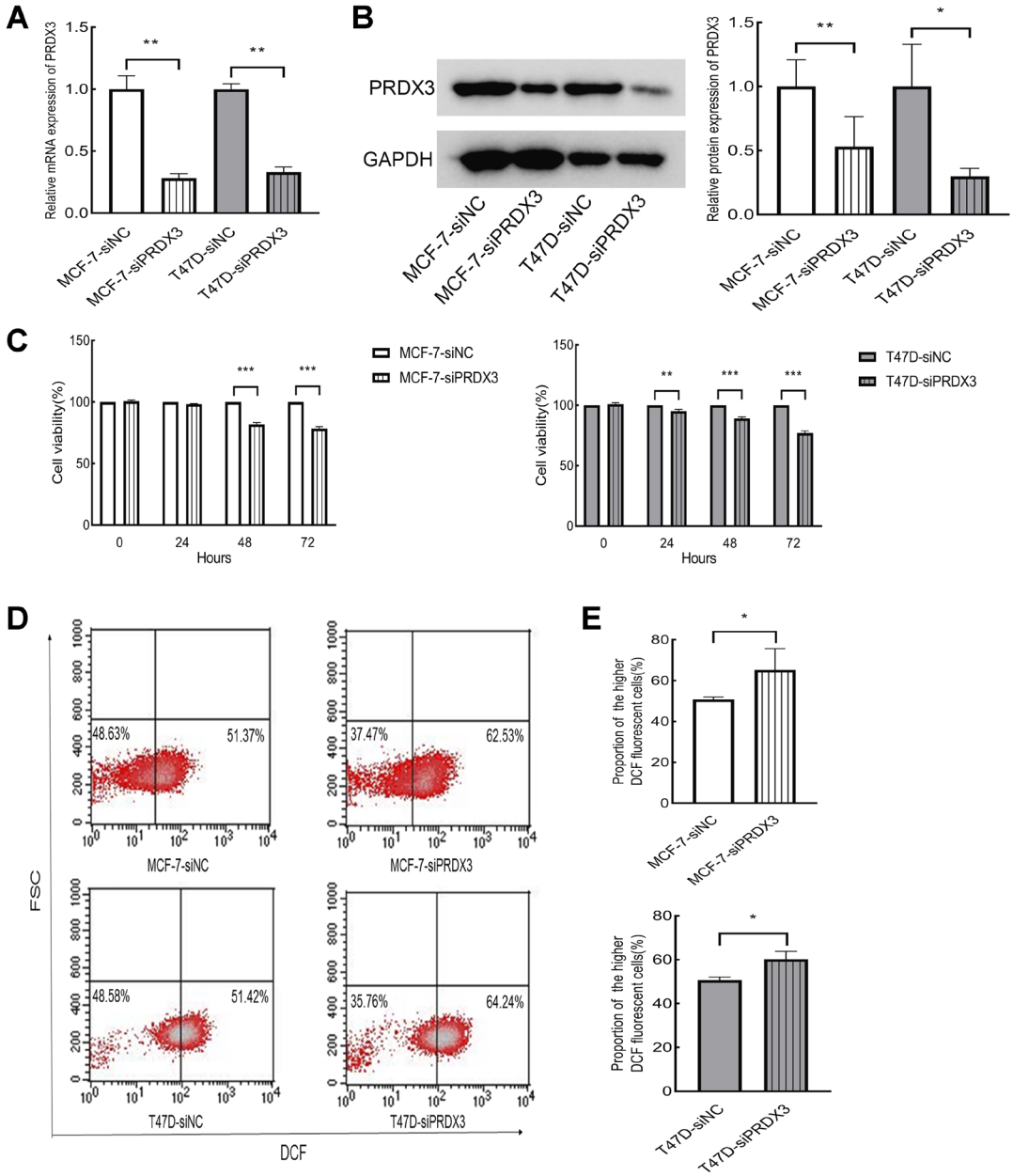
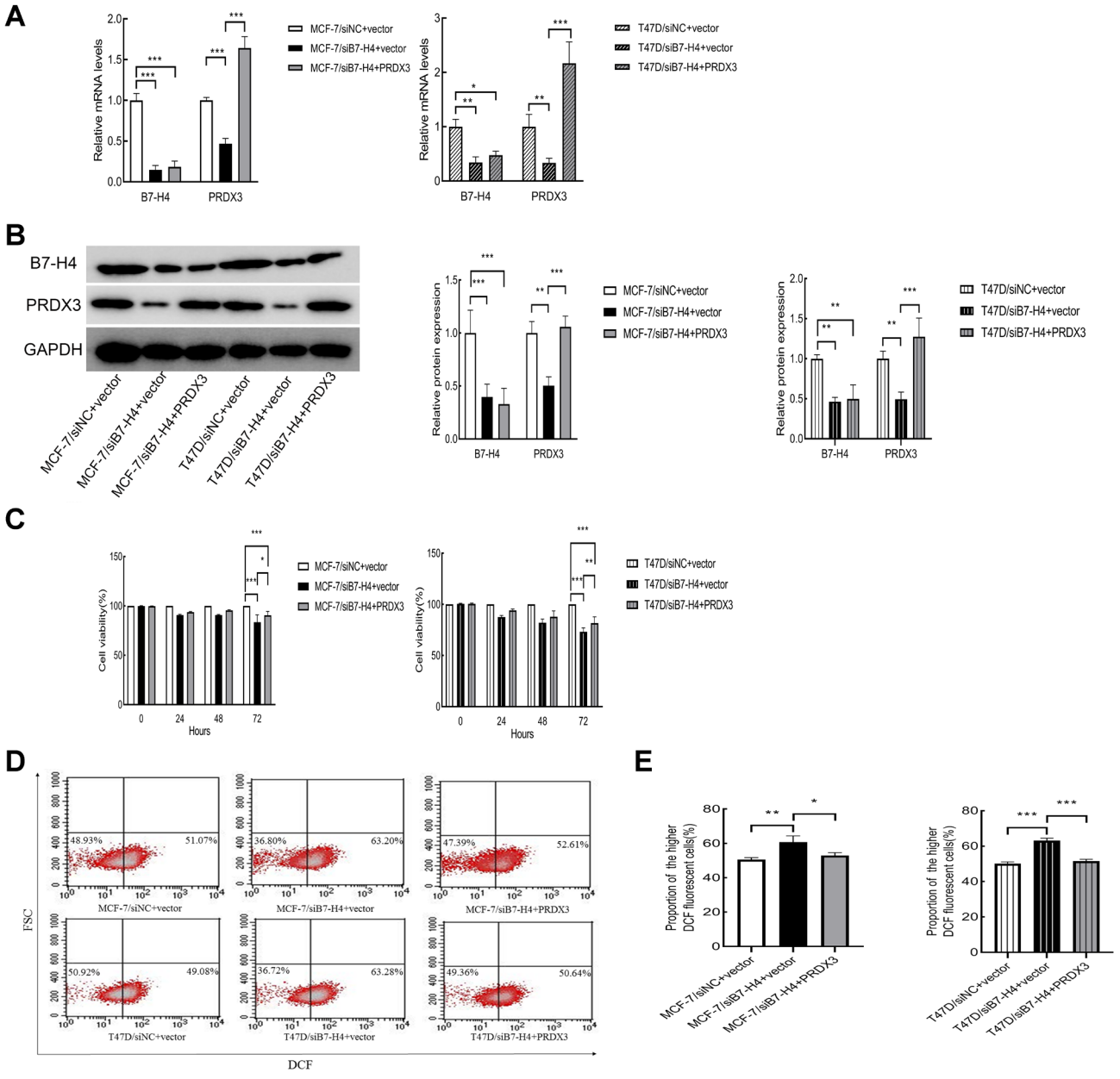


Figure 3. Silencing of PRDX3 induced intracellular oxidative stress and cell viability inhibition in BC cells. A) Decreased RNA expression of PRDX3 after transfected with siRNA in both MCF-7 cells and T47D cells; B) Decreased protein expression of PRDX3 after transfected with siRNA in both MCF-7 cells and T47D cells; C) Silencing of PRDX3 led to cell viability inhibition in MCF-7 and T47D cells at 48 h and 72 h; D, E) Silencing of PRDX3 resulted in ROS levels elevating in MCF-7 and T47D cells. Error bars represent the means ± SEM, *p<0.05, **p<0.01, ***p<0.001

lated with patient prognosis and infiltration of T cells in the tumor microenvironment. Many studies have also shown that B7-H4 is involved in the regulation of the immune microenvironment in a variety of tumors. B7-H4 promotes renal cell carcinoma progression by recruiting tumor-associated neutrophils [19]. In metastatic colorectal cancer, the overexpression of B7-H4 is associated with immune cells infiltration and poor prognosis [20]. Denarda *et al.* found that using a human B7-H4 recombinant antibody to neutralize B7-H4 could effectively overcome tumor cell immune escape and enhance the antitumor effect of T cells in the tumor microenvironment [21]. Therefore, B7-H4 may serve as a promising target in cancer immunotherapy to reactivate anti-tumor T cell function. Across numerous BC types, B7-H4 is more highly expressed in cancer samples than in normal breast tissues [22]. Some scholars have carried out studies on the



Figures 4. Overexpression of PRDX3 partially attenuated B7-H4 silencing-induced oxidative stress and cell viability inhibition in BC cells. A) Changes in RNA expression after overexpression of PRDX3 in B7-H4-knocked down cells; B) Western blot analysis showed successful overexpression of PRDX3 in B7-H4-knocked down cells; C) Overexpression of PRDX3 partially attenuated B7-H4 silencing-induced cell viability inhibition of MCF-7 and T47D cells; D, E) Overexpression of PRDX3 reversed ROS levels in B7-H4-knocked down cells. Error bars represent the means ± SEM, *p<0.05, **p<0.01, ***p<0.001

role of B7-H4 in TNBC. For example, it was found that pharmacological inhibition of B7-H4 glycosylation contributes to the treatment of TNBC [17]. Anti-B7-H4 checkpoint synergistic trastuzumab therapy helps eradicate BC [23]. Most of the earlier studies focused on the role of B7-H4 in the regulation of tumor immunity, whereas nowadays, more and more studies are beginning to concentrate on the effects of B7-H4 on tumor cells themselves and the underlying mechanisms. However, the underlying mechanism and downstream molecules of B7-H4 in BC are not well defined.

In our study, we firstly found that silencing of B7-H4 could downregulate PRDX3, a key enzyme regulating intracellular hydrogen peroxide levels, in BC cells. As a hydrogen peroxide reductase, PRDX3 can affect the biological phenomenon of cells by regulating the level of hydrogen peroxide in cells. Studies have shown excessive hydrogen peroxide levels

can inhibit cell growth [24-26]. Silencing of PRDX3 inhibited cell growth and causes cell cycle arrest in MDA-MB-231 cells [27]. Meanwhile, PRDX3 can protect MCF-7 cells from doxorubicin-induced toxicity by alleviating oxidative stress [28]. Therefore, we further detected the intracellular ROS level as well as cell viability changes after the silencing of B7-H4 in BC cells. Consistent with what we speculated; intracellular ROS levels were elevated accompanied by inhibition of cell viability after silencing of B7-H4 in BC cells. Then, we verified the PRDX3 role as one of the B7-H4 downstream effectors by directly using siRNA to silence PRDX3 expression in BC cells, then we observed the changes of intracellular ROS level and cell viability, which were similar to those results from silencing of B7-H4.

From the above results, we hypothesized that B7-H4 is causing intracellular oxidative stress and suppressing cell viability changes that might be mediated by downstream PRDX3. To further confirm this role played by PRDX3, we did a revertant experiment by forced overexpressing PRDX3 in B7-H4-knocked down BC cells, and then detected the changes in cell viability and intracellular ROS levels. Consistent with the assumption, not only the cell viability inhibition but also the upregulation of intracellular ROS levels were alleviated with the elevated expression of PRDX3 in B7-H4-knocked down BC cells. For all of these reasons above, a conclusion was reached that the silencing of B7-H4 induced intracellular oxidative stress and decreased cell viability of BC cells via downregulating PRDX3. To our knowledge, this is the first study to show that B7-H4 may be involved in ROS metabolism by regulating PRDX3 in BC cells.

At the same time, we found that the silencing of B7-H4 caused the G1-S phase arrest of the cell cycle in both MCF-7 and T47D cells. However, when directly silencing PRDX3, only the MCF-7 cell line produced the same result. The overexpression of PRDX3 did not significantly affect the cell cycle changes caused by B7-H4 knocking down in MCF-7 cells (results are not shown). In other words, PRDX3 may not play a significant role in mediating cell cycle arrest resulting from the silencing of B7-H4 in BC cells. Combined with the changes in ROS levels described above, PRDX3 may affect cell viability mainly by regulating the intracellular ROS levels. However, the elevated ROS levels could be reduced too close to the control groups by overexpressing PRDX3 in B7-H4-knocked down BC cells, but cell viability/proliferation did not restore to the same extents as in the control groups. These results indicated that B7-H4 might also regulate the cell cycle through other downstream molecules and pathways, thereby affecting cell viability/proliferation, which requires further experiments to explore.

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