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PES1 regulates sensitivity of colorectal cancer cells to anticancer drugs

Wei Xie [1], Like Qu *, Lin Meng, Caiyun Liu, Jian Wu, Chengchao Shou *

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, Peking University Cancer Hospital & Institute, Beijing 100142, China

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ABSTRACT

PES1 (also known as Pescadillo), a nucleolar protein, was involved in biogenesis of ribosomal RNA. Up-regulation of PES1 has been documented in some human cancers, indicating that PES1 may play some crucial roles in tumorigenesis. In our previous study, it was found that silencing of PES1 resulted in decreased proliferation of colorectal cancer cells. We also noticed that depletion of PES1 altered expression profiles of diverse genes. In the present study, we validated the expression changes of a subset of genotoxic stress-related genes in PES1-silenced HCT116 cells by quantitative RT-PCR. The steady and etoposide-induced phosphorylated H2AX (γ-H2AX) were higher in PES1-silenced cells than in control cells. Besides, etoposide-induced γ-H2AX persisted longer in PES1-silenced cells after removing the etoposide. Next, results of comet assay revealed decreased DNA repair after PES1-ablation. PES1-ablated cells were more sensitive to chemotherapeutic agents, which could be reversed by reconstitution with exogenous PES1. Furthermore, deletion of PES1 diminished steady and DNA damage-induced levels of nuclear RAD51. Our results uncover a potential role of PES1 in chemoresistance by regulating DNA damage response in colorectal cancer cells.

1. Introduction

Pescadillo has been shown to play important roles in embryonic development, ribosome biogenesis, and DNA replication [1]. In recent years, abnormal overexpression of human ortholog of Pescadillo (PES1) has been implicated in some cancers, including glioblastomas [2], head and neck squamous cell carcinomas [3], gastric cancer [4] and breast cancers [5]. In addition, deregulated expression of PES1 has been linked to chromosomal instability [6,7]. In our previous work, we have showed that PES1 was overexpressed in colon cancers, and that silencing of PES1 could reduce proliferation and growth of colon cancer cells *in vitro* and *in vivo* [8]. Despite of these findings, the precise role of PES1 in tumorigenesis remains largely unknown.

Chemoresistance is one of the major reasons which cause failure in the chemotherapy for cancer patients. To avoid the toxic effects of the chemotherapy drugs, many cancers developed drug resistance through several molecular mechanisms. The overexpression of energy-dependent transporters, which detect and eject anticancer drugs from cancer cells, is a general mechanism of multidrug resistance [9,10]. These transporters include P-glycoprotein (Pgp), multidrug resistance associated protein (MRP), lung resistance protein (LRP), and breast cancer resistance protein (BCRP). However, some other mechanisms also play critical roles in chemoresistance [11], including suppression of drug-induced apoptosis [12,13], enhancement of glutathione S-transferase (GST) mediated drug-detoxifying system [14] or DNA damage repair. In addition, ribosomal proteins have been demonstrated to be associated with chemoresistance. Ribosomal proteins S13 and L23 promote multidrug resistance by suppressing apoptosis in gastric cancer cells [13]. Besides, ribosomal proteins L4, L5, and S28 were found to be overexpressed in drug resistant cancer cells [15,16].

PES1 contains a conversed BRCA1 C-terminal (BRCT) domain, which is postulated to be a critical structure in the regulation of DNA damage repair, cell cycle checkpoint, and chemoresistance [17]. The feature of this conversed architecture implies that PES1 may play a critical role in DNA damage response and chemoresistance. In this study, we focused on the potential correlation between PES1 and chemoresistance, and provided evidence that deletion of PES1 increased the sensitivity of colon cancer cells to diverse chemotherapy drugs.

2. Materials and methods

2.1. Cell lines, antibodies, and reagents

PES1 protein expression was analyzed in the following cell lines: colorectal cancer cells LoVo, CL187, HT29, RKO, SW480 and

* Corresponding authors. Address: Department of Biochemistry and Molecular Biology, Peking University Cancer Hospital & Institute, 52 Fucheng Road, Beijing 100142, China. Fax: +86 10 88122437.

E-mail addresses: qulike@bjcancer.org (L. Qu), scc@bjcancer.org (C. Shou).

[1] Current address: Center of Medical Experiment & Technology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China.

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HCT116; immortalized human gastric mucosa epithelial cells GES-1; gastric cancer cells MGC803, SGC7901, BGC823, AGS, N87, and MKN45; esophageal cancer cells E30, E70, E140, E180, E410, E450, E510, and T10; lung cancer cells PG, GLC82, H446, H460, H1299 and A549. All the esophageal cancer cells were kindly provided by Dr. Zhihua Liu of Cancer Institute, Chinese Academy of Medical Sciences. MGC803, BGC823, BEL 7402 and SMMC7901 cells were kept in our lab. Other cell lines were from ATCC. All cells were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA).

Anti-PES1 antibody was described previously [8]. Anti-γ-H2AX (#9718) was obtained from Cell Signaling (Danvers, MA). Anti-GAPDH (60004-1-Ig) was purchased from ProteinTech (Chicago, IL). Anti-RAD51 (H-92, sc-8349) was from Santa Cruz (Santa Cruz, CA). FITC-conjugated anti-rabbit secondary antibody (ZF-0311) was obtained from ZSGB-Bio (Beijing, China). Chemotherapeutical agents, including etoposide, 5-fluorouracil, doxorubicin, and vincristine, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Silencing of PES1, and transfection

For silencing of PES1, pSilencer vector was used to express the following RNAi oligonucleotides to knock down endogenous PES1: PES1-RNAi-1, GGAACACTGTAGAGCGTTTAA; PES1-RNAi-2, GAAGATGCAGAGGCTGGTTCA. Control vector and pSilencer-shRNA-1, -shRNA-2 were transfected into HCT116 cells with lipofectamine 2000 (Invitrogen). Cells were selected with 600 μg/ml G418 (Sigma) for 2 weeks to generate stable cell lines. For the expression of exogenous PES1, cells were transfected with pcDNA3 or pcDNA3-PES1 plasmid using lipofectamine 2000.

2.3. Gene expression microarray and quantitative RT-PCR

Total RNA from HCT116-shRNA cells was isolated with TRIZOL reagent (Invitrogen) for microarray analysis. Gene expression profiles in PES1-silenced HCT116 and control cells were examined using Human Genome CGH Microarray 44 K (Agilent). After normalization, the fold-change of gene expression was calculated. A *P* value less than 0.05 and the fold-change threshold 2 were chosen to identify the statistically significance. Microarray hybridization, data acquisition and analysis were performed by OE Bio-tech (Shanghai, China). Quantitative RT-PCR was performed as previously described [8]. Primers used in quantitative RT-PCR were as follows: *RAD51*-forward, TGACCGGGGTGGAGGTGAAGG, reverse, AGGGCGGTGGCACTGTCTAC; *RAD51AP1*-forward, TTGGTGACTTCGG TGGAC, reverse, CTGCGTATTTCTAATGGTT; *MSH6*-forward, ACTGG AAATGGCTCTCTT, reverse, TCACCACCTCCACTAACG; *AHR*-forward, AGTCTCCCTTCATACCTT, reverse, TTGCATGTGCTTCATCTTCT; *GAPDH*-forward, CATCAAGAAGGTGGTGAAGCAG, reverse, CGTCAAAGGT GGAGGAGTGG. Primers were synthesized by Sangon Biotech (Shanghai, China).

2.4. Cell survival assay

Cells were seeded on 96-well plates at a density of 6×10^3 cells per well. Then the cells were treated with indicated drugs for 72 h, followed by addition of MTT (Methylthiazolyldiphenyl-tetrazolium, 0.5 mg/ml, Sigma) to each well at a final concentration of 0.5 mg/ml for another 4 h at 37 °C. Next, the culture medium was removed and 150 μl dimethyl sulfoxide (DMSO) was added into the wells to solubilize the formazan salt. The absorbance was measured using a microplate reader at a wave-length of 490 nm. All experiments were performed in triplicate. The sensitivity of cells to drugs was expressed as IC50 (the concentration for 50% inhibition of cell growth), which was extrapolated from linear regression analysis of experimental data.

2.5. Western blot analysis

Cells were lysed with 1X SDS sample buffer and sonicated for 30 s. Protein samples were resolved by SDS–PAGE and electroblotted onto nitrocellulose membranes, which were blocked in 5% skim milk in PBST and probed with the indicated antibodies. Protein bands were visualized with enhanced chemoluminescence system (Thermo Scientific, Rockford, IL).

2.6. Comet assay

The single cell gel electrophoresis assay was performed using OxiSelect™ Comet Assay Kit (Cell Biolabs, San Diego, CA). The cell slides were viewed by Leica TCS SP5 fluorescent microscope with a FITC filter. The circular nuclei in microscopic images indicated undamaged DNA, while the damaged DNA fragments had migrated out from the nucleus to form a comet-like shape. More than 150 cells were counted for the percentage of cells with damaged DNA.

2.7. Immunofluorescence

Cells were grown on coverslips to 60% confluence, and fixed with cold methanol/acetic acid (1:1). Next, cells were permeabilized with 0.5% Triton X-100 in PBS, washed with PBS, blocked with 5% goat serum (Sigma) at room temperature for 1 h, and incubated with anti-RAD51 antibody (1: 500 dilution) at 4 °C for 16 h. After washing with 0.1% Tween 20 in PBS for 5 times, cells were incubated with FITC-conjugated secondary antibody (1: 500 dilution) at room temperature for 45 min, followed by washing with 0.1% Tween 20 in PBS, and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using a Leica TCS SP5 laser confocal microscope with identical setting at room temperature.

3. Results and discussion

3.1. Overexpression of PES1 in diverse cancer cells

With a previously generated anti-PES1 monoclonal antibody [8], we examined PES1 expression in a panel of cancer cell lines originated from diverse human tissues, including stomach, colorectal tract, lung, and esophagus. Results of Western blot analysis revealed high levels of PES1 in majority of cancer cell lines (Fig. 1A). These results were accordant with previous studies [4,5] and confirmed that PES1 protein was overexpressed in different human cancer cells.

3.2. PES1 regulates expression of a subset of DNA damage and repair-related genes

To investigate the potential roles of PES1 in the cellular response to chemotherapeutical drugs, we first knocked down endogenous PES1 by two specific shRNAs in HCT116 colon cancer cells, which was confirmed by Western blot analysis (Fig. 1B). Next, cDNA microarray analysis was performed with RNA extracted from PES1-silenced cells and control cells [8]. Genes with same changing pattern in both shRNA-1 and shRNA-2 cells were picked up. Among these genes, a subset of DNA damage repair-related genes were found to be upregulated in PES1-silenced cells, including *RAD51*, *MSH6*, and *RAD51AP1*. RAD51 protein could bind to single- and double-stranded DNA and exhibits DNA dependent ATPase activity, which is critical for homologous recombination repair of DNA breaks [18]. RAD51AP1 could interact with RAD51 and enhanced the ATPase activity of RAD51 [19]. MSH6 has been shown to play a role in DNA mismatch repair [20]. Additionally, we found that *AHR* (aryl hydrocarbon receptor) was down-regulated upon

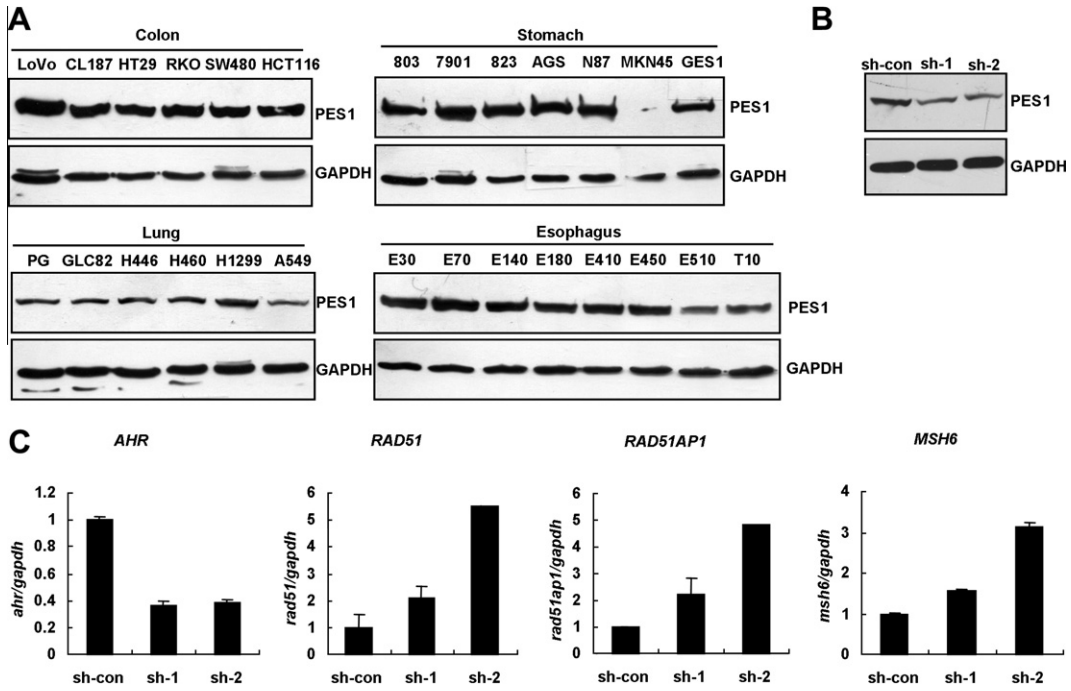

Fig. 1. PES1 expression in cancer cell lines and silencing of PES1 in HCT116 cells. (A) Western blot analysis of PES1 expression levels in diverse cancer cell lines. GAPDH was shown as a loading control. (B) Western blot of PES1 in HCT116 cells transduced with shRNA-control and PES1-shRNA1/2, respectively. (C) Validation of some upregulated and downregulated genes' expression by quantitative RT-PCR in HCT116 cells. *Gapdh* was used as a housekeeping gene.

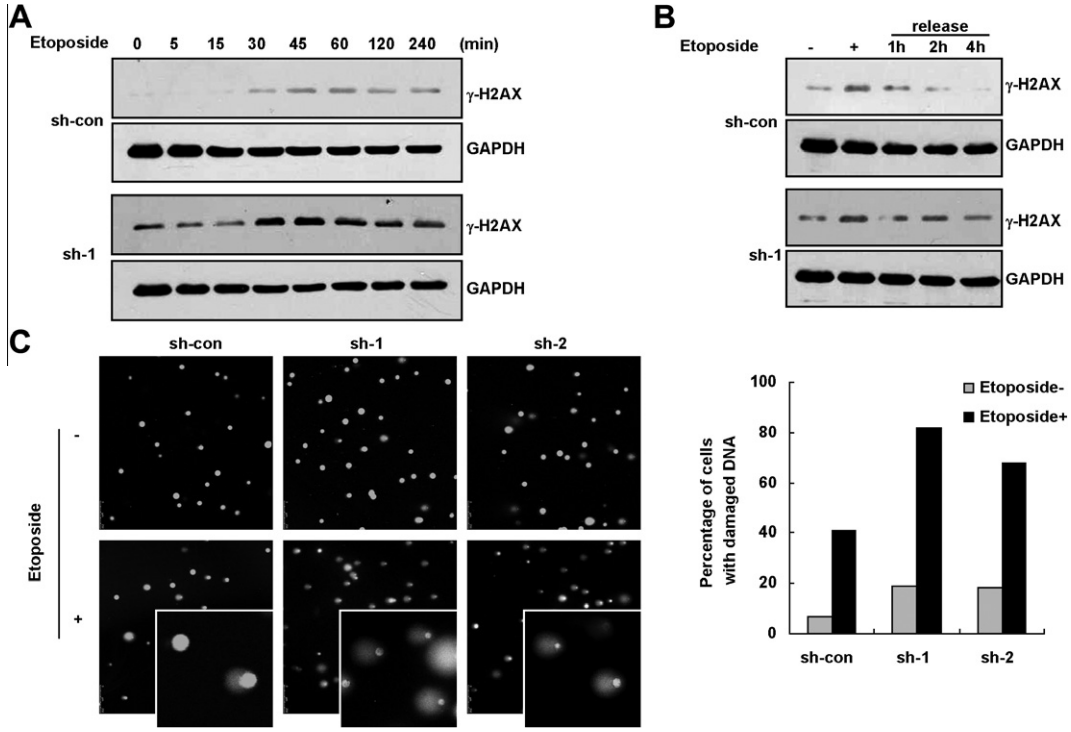

Fig. 2. PES1 deficiency resulted in increased DNA damage response and decreased DNA repair. (A) Detection of γ-H2AX in HCT116-sh-1 and control cells after treatment with etoposide (20 μM) for different times. GAPDH was used as loading control. (B) The cells were exposed to etoposide for 1 h, then cells were released with fresh medium and cultured for indicated times. Levels of γ-H2AX were detected by Western blot. (C) Comet assay to assess the DNA damage in PES1-depleted cells. The left panel showed the representative photos of cell electrophoresis. The percentage of cells with damaged DNA was calculated by counting more than 150 cells and shown in the right panel.

PES1 ablation. AHR is essential for controlling DNA damage response by functioning as a transcription factor responsible for the induction of drug-metabolizing enzymes [21]. By quantitative RT-PCR, we validated the results of microarray analysis (Fig. 1C). These observations suggested that PES1 may play a role in DNA damage response.

3.3. Deletion of PES1 increased DNA damages and disturbed DNA repair

Since previous studies demonstrated that DNA damage response could booster *RAD51*, *MSH6*, and *RAD51AP1* expression at transcriptional level [22–24], we are interested to explore whether there was any changes in DNA damage response in PES1-ablated HCT116 cells. Compared with control cells, PES1-shRNA-1 cells exhibited higher levels of phosphorylated form of H2AX (γ-H2AX), a hallmark of DNA damage response [25]. Furthermore, we treated the cells with chemotherapeutic agent etoposide to induce genotoxic stress. Robust γ-H2AX was induced in the PES1-shRNA-1 cells (Fig. 2A). These data suggested that inhibition of PES1 increased the DNA damage status and rendered cells to be more sensitive to genotoxic agent. In the next experiment, we tested whether PES1 deficiency might affect DNA repair. Cells were treated with etoposide for 1 h and were released into drug-free medium. We found that the level of γ-H2AX was reduced after the removing of etoposide (Fig. 2B), indicating the DNA breaks were repaired. However, the decrease of γ-H2AX was relatively slower in PES1-shRNA-1 cells than in control cells, suggesting that deletion of PES1 reduced the rate of DNA repair.

We next utilized single cell gel electrophoresis assay (comet assay) to directly assess the levels of DNA damage in PES1-depleted HCT116 cells. This assay is a sensitive technique for the detection of DNA damages in individual cells [26]. PES1 ablation resulted in higher percentage of cells with damaged DNA than in control cells (19.05% and 18.44% versus 6.44%) (Fig. 2C). After treatment with etoposide, PES1 ablation further resulted in a significantly higher percentage of cells with damaged DNA (81.59% and 67.49% versus 41.01%). Thus, PES1 silencing compromises cells' capacity to repair DNA damage.

3.4. PES1 contributes to chemotherapeutic drugs sensitivity

The sensitivities of HCT116-shRNA-control and HCT116-PES1-shRNA cells to a panel of chemotherapeutic agent (etoposide, doxorubicin, 5-fluorouracil, and vincristine) were evaluated by MTT assay and IC50 calculation. Compared to control cells, PES1-ablated cells were more sensitive to drugs-induced growth inhibition, as indicated by decreased IC50 values (Fig. 3A). To further confirm essential role of PES1 in the chemoresistance, we re-introduced the exogenous PES1 into the PES1-silenced cells (Fig. 3B). As shown in Fig. 3C, expression of exogenous PES1 increased the IC50 values in both control and PES1-abalted cells, confirming that PES1 was indeed associated with cells' sensitivity to chemotherapeutic agents.

3.5. Repression of PES1 disturbed redistribution of RAD51 in response to DNA damage

In the microarray analysis, *RAD51* was found to be upregulated in PES1-ablated cells (Fig. 1C). RAD51 has been identified as a key DNA recombinase mediating DNA homologous recombinational

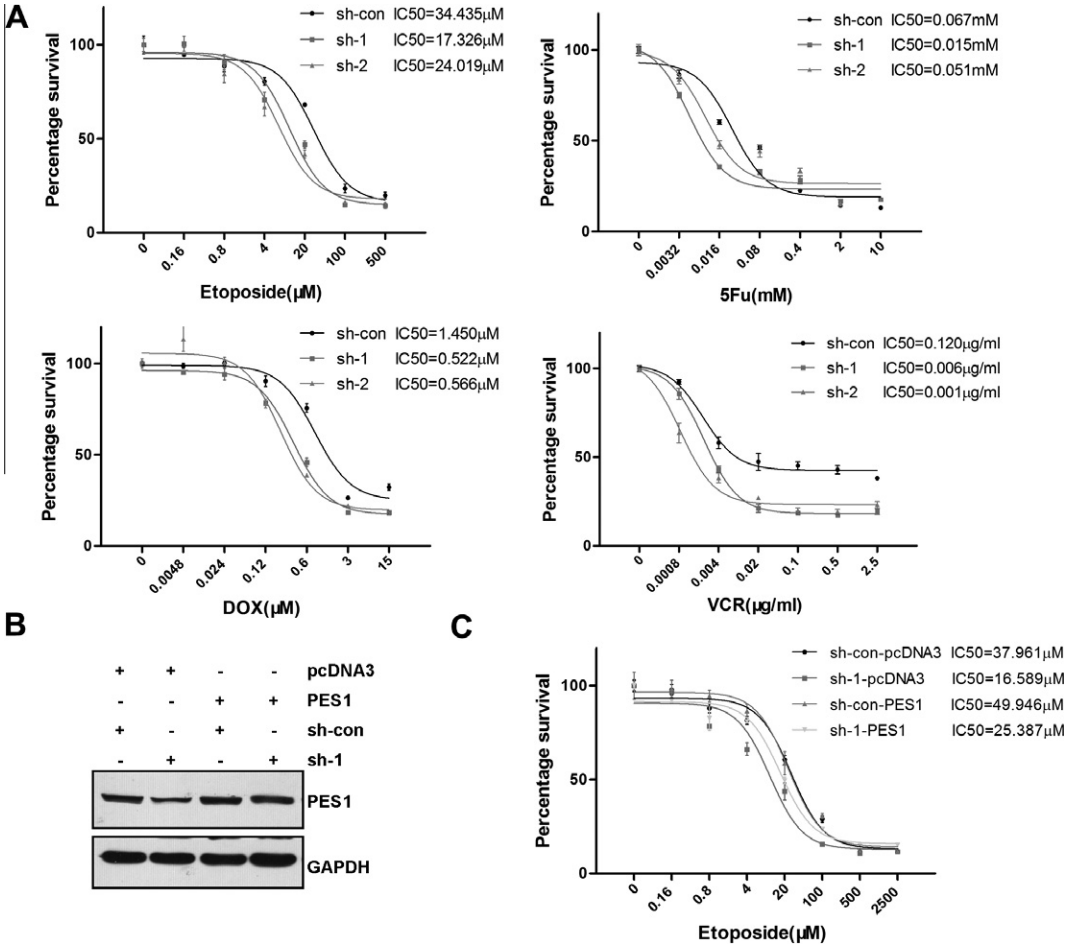

Fig. 3. PES1 regulates the sensitivity of colon cancer cells to anticancer drugs. (A) MTT assay to evaluate the sensitivities of HCT116 cells to chemotherapeutic agents. The cells were treated with indicated concentrations of agents for 72 h and cell survival was examined by MTT assay. The percentage survival of HCT116-sh-control cells with no drugs was set to 100%. The IC50 values of HCT116-shRNA cells were shown as indicated. (B) Expression of exogenous PES1 in PES1-ablated HCT116 cells. (C) Expression of exogenous PES1 in HCT116-shRNA-1 cells could decrease the sensitivity to etoposide and increase the IC50 values.

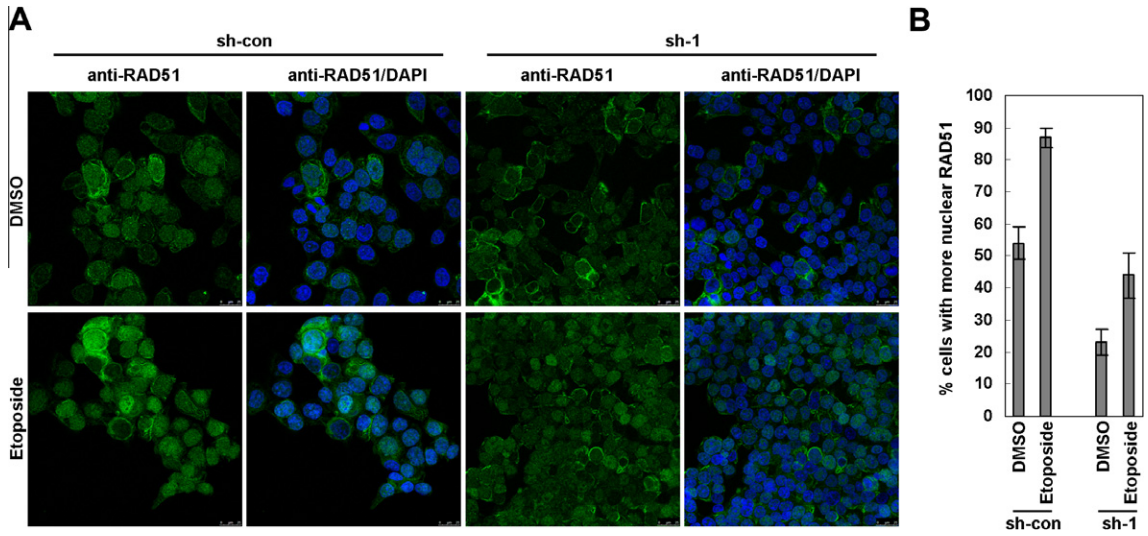

Fig. 4. Silencing of PES1 disturbed redistribution of RAD51 in response to DNA damage. (A) Immunofluorescence analysis of RAD51 distribution in HCT116 cells treated with DMSO or 20 μM etoposide for 8 h. The nuclei were counterstained with DAPI. (B) The percentage of cells with more nuclear RAD51 was calculated by counting more than 200 cells. Data show mean values ± SD from two independent experiments.

repair [18,27]. However, PES1 ablation had no obvious effect on the protein level of RAD51 (data not shown), indicating the changes in the mRNA level of PES1 is likely to be an adaptive response to elevated DNA damage, as reported previously [23]. Since DNA damage-induced cytoplasmic to nuclear redistribution of RAD51 and recruitment to damaged DNA are crucial for RAD51 to perform its DNA recombinase function [28,29], we examined the cellular distribution of RAD51 via immunofluorescence assay. It was found that deletion of PES1 decreased the steady and DNA damage-induced levels of nuclear RAD51 (Fig. 4A and B). These results suggested that PES1 plays a role in enhancing the nuclear entry of RAD51.

Chemoresistance is a major challenge to the cancer therapy. By showing that ablation of PES1 in colorectal cancer cells resulted in elevated DNA damage response and enhanced sensitivity to chemotherapeutic agents, our present study demonstrates that overexpression of PES1 in diverse types of cancer may contribute to chemoresistance. Although the precise mechanisms underlying PES1-regulated chemoresistance need further investigation, we demonstrated that reduced nuclear entry of RAD51 may be associated with decreased DNA repair and increased sensitivity to chemotherapeutic drugs in PES1-ablated cells. Our study further suggests that PES1 could be a potential target to improve the sensitivity of colorectal cancer cells to chemotherapeutic drugs.

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