# PTEN enhances G2/M arrest in etoposide-treated MCF-7 cells through activation of the ATM pathway

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**Abstract.** As an effective tumor suppressor, phosphatase and tensin homolog (PTEN) has attracted the increased attention of scientists. Recent studies have shown that PTEN plays unique roles in the DNA damage response (DDR) and can interact with the Chk1 pathway. However, little is known about how PTEN contributes to DDR through the ATM-Chk2 pathway. It is well-known that etoposide induces G2/M arrest in a variety of cell lines, including MCF-7 cells. The DNA damage-induced G2/M arrest results from the activation of protein kinase ataxia telangiectasia mutated (ATM), followed by the activation of Chk2 that subsequently inactivates CDC25C, resulting in G2/M arrest. In the present study, we assessed the contribution of PTEN to the etoposide-induced G2/M cell cycle arrest. PTEN was knocked down in MCF-7 cells by specific shRNA, and the effects of PTEN on the ATM-Chk2 pathway were investigated through various approaches. The results showed that knockdown of PTEN strongly antagonized ATM activation in response to etoposide treatment, and thereby reduced the phosphorylation level of ATM substrates, including H2AX, P53 and Chk2. Furthermore, depletion of PTEN reduced the etoposide-induced phosphorylation of CDC25C and strikingly compromised etoposide-induced G2/M arrest in the MCF-7 cells. Altogether, we demonstrated that PTEN plays a unique role in etoposide-induced G2/M arrest by facilitating the activation of the ATM pathway, and PTEN was required for the proper activation of checkpoints in response to DNA damage in MCF-7 cells.

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## Introduction

Cellular DNA is constantly challenged by either endogenous [reactive oxygen species (ROS)] or exogenous (UV) factors. To properly transmit the genetic information to the next generation, multiple delicate mechanisms underlying this event have been evolved in eukaryotic cells (1,2). DNA damage response (DDR) is such a crucial mechanism to ensure that genetic information is faithfully transmitted between generations. In response to DNA damages, eukaryotic cells activate a complex signaling cascade to arrest the cell cycle for DNA repair. Dysregulation of DDR may result in severe disorders, such as tumorigenesis (1,3-5). Therefore, it is critical to elucidate the mechanism of DDR in order to treat human diseases, such as cancer. The major regulators of DDR are phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs), which include ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) (3,6,7). ATM and ATR respond to different types of DNA damage; ATM mainly responds to double-strand breaks (DSBs) while ATR mainly responds to replication stress and UV-induced pyrimidine dimers. According to previous studies (1,4,6-9), Ser/Thr kinase checkpoint kinase-1 (Chk1) and -2 (Chk2) are the two most thoroughly investigated substrates of ATR and ATM, respectively. In the situation of DNA damage, these pathways are able to activate *p53*, phosphorylate H2AX and other downstream pathways to activate the checkpoints of DNA damage, which eventually causes cell cycle arrest for DNA repair.

Defects in the ATR/Chk1 and ATM/Chk2 pathways are known to increase cancer risk (1,3). Once DSBs occur, ATM kinases can phosphorylate histone H2AX to form γH2AX foci at the DNA break sites. The formation of γH2AX foci subsequently promotes the gathering of repair factors on damaged

DNA sites and activates the DNA damage arrest. This indicates that γH2AX foci play an important role in cellular DDR. Besides activating the formation of γH2AX nuclear foci, ATM can also induce cell cycle arrest through phosphorylation of its targets, p53 and Chk2. Numerous studies have demonstrated that phosphorylation of Chk2 can induce G2/M arrest by phosphorylating CDC25C, which prevents CDC25C from dephosphorylating CDC2 (or CDK1) (1-3,6,9-11).

The phosphatase and tensin homolog (PTEN) gene encodes a major plasma membrane lipid and protein phosphatase. Scientists have reported that PTEN functions as a highly effective tumor suppressor in various tissues and is frequently mutated in a wide variety of cancers, including endometrial, breast and prostate cancer and glioblastomas. Previous studies revealed that PTEN is a central negative regulator of PI3K/AKT-mediated signaling cascade. The loss of function of PTEN in various human cancers enhances AKT activation, resulting in dysregulated cell proliferation, survival, migration and spreading, which are all important factors in tumor development and progression (12,13). Recent findings have demonstrated various novel functions of PTEN in several cellular processes. For instance, PTEN plays a critical role in DNA damage repair and response. In 2007, Shen *et al* demonstrated that cells deficient in PTEN have defective DNA DSB repair, possibly due to the lack of or downregulation of Rad51 and the lack of PTEN at centromeres (14). In addition, several studies have indicated that PTEN is able to promote the formation of γ-H2AX foci, a marker of DNA damage, which suggests that PTEN functions to reduce DSB levels (15-17). Furthermore, previous studies have shown that PTEN plays a unique role in DDRs through its interaction with the Chk1 and p53 pathways (10,18-21). In the present study, we report that PTEN is required for the proper checkpoint activation in etoposide-treated MCF7 cells. While etoposide treatment induces G2/M arrest in MCF-7 cells by activating the ATM-Chk2-CDC25C pathway, knockdown of PTEN compromises this process, thereby reducing etoposide-induced G2/M arrest in MCF-7 cells.

## Materials and methods

*Materials and reagents*. PTEN shRNA and related products were purchased from Invitrogen. ECL Western Blotting kit, anti-p53, anti-β-actin, anti-γH2AX, anti-H2AX and anti-CDC25C antibodies were purchased from Abcam. Anti-phospho-ATM, anti-Chk2, anti-phosph-Chk2 (T68), anti-phospho-p53 (S15) and anti-phospho-CDC25C (S216) antibodies were purchased from Cell Signaling. Anti-phospho-ATM (S1981) and anti-ATM antibody, FITC-donkey anti-rabbit IgG and Rhodamine-donkey anti-mouse IgG were purchased from Sigma. Immobilon-P membranes were purchased from Millipore.

*Cell lysis and western blotting*. The cells were lysed with a buffer containing 20 mM Tris (pH, 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. Total cell lysate (50 μg) was separated on SDS-PAGE gel and transferred onto Immobilon-P membranes. The membranes were first blocked with 5% skim milk, and then incubated with primary antibodies at room temperature for 2 h. The ECL Western Blotting kit was utilized to detect the signals (22). The concentrations of antibodies were as follows: anti-p53 (FL-353 at 1 μg/ml), anti-phospho-p53 (S15) (1:1,000), anti-ERK (1:500), anti-H2AX (1:1,000), anti-γH2AX (1:1,000), anti-phospho-ATM (S1981) (1:500), anti-ATM (1:500), anti-phospho-CHK2 (T68) (1:500), anti-CHK2 (1:1,000), anti-phospho-CDC25C (S216) (1:500), anti-CDC25C (1:1,000) and anti-β-actin (1:1,000).

*Immunofluorescence staining*. Cells were treated as described in the figure legends. The cells were fixed with pre-chilled (-20˚C) acetone-methanol for 15 min. The primary antibodies anti-γH2AX (0.5 μg/ml; Upstate) and anti-phospho-ATM (S1981) (1:100; Cell Signaling) were then added onto the slides and incubated at 4˚C overnight. The slides were gently washed with 1X phosphate-buffered saline (PBS) twice, and the FITC-conjugated secondary antibodies (donkey anti-rabbit IgG and Rhodamine-donkey anti-mouse IgG; 1:200; Jackson ImmunoResearch Laboratories) were added onto the slides and incubated at room temperature for 1 h. Afterwards the slides were covered with Vectashield mounting medium containing DAPI (CA94010; Vector Laboratories, Inc., Burlingame, CA, USA). Images were captured with a fluorescence microscope (Axiovert 200; Carl Zeiss) (22).

*Cell cycle assessment*. Cells in the logarithmic growth phase were cultured in 6-well plates for 24 h and different concentrations of etopside were added into the plates. After 18 h of incubation, cells were trypsinized and transferred into a 15-ml tube, followed by centrifugation at 1,000 rpm in a 5840R cooled centrifuge (Eppendorf, Germany) for 5 min. The cells were then washed with 1X PBS and the centrifugation was repeated twice. The supernatant was discarded and 1 ml propidium iodide (PI) solution (0.05 mg/ml) was added into each tube. The samples were kept in the dark at room temperature for 30 min, and then the phases of the cell cycle were analyzed using a flow cytometer (22).

*Statistical analysis*. Data were analyzed using one-way ANOVA followed by a Tukey's test. Statistical significance was achieved if the p-value was ≤0.05. Data values are expressed as the mean ± standard error (SE).

## Results

*PTEN promotes etoposide-induced p53 phosphorylation*. *PTEN* was initially identified as a tumor suppressor, which has been found to be mutated in a large number of cancers at a high frequency (23). Mutations of *PTEN* are a critical step in the development of many cancers and this gene may play an important role in DDRs (12,14,20,21). In order to investigate how *PTEN* acts on the etoposide-induced DDR, we knocked down this gene in MCF-7 cells (Fig. 1A). As observed, depletion of *PTEN* did not significantly affect proliferation of the MCF-7 cells (data not shown). It has been widely recognized that *p53* phosphorylation is a crucial event of DDR by affecting MDM2, 14-3-3σ, DADD45 and $p21^{Cip1}$ (24-27). Therefore, we first investigated the impact of *PTEN* deple-

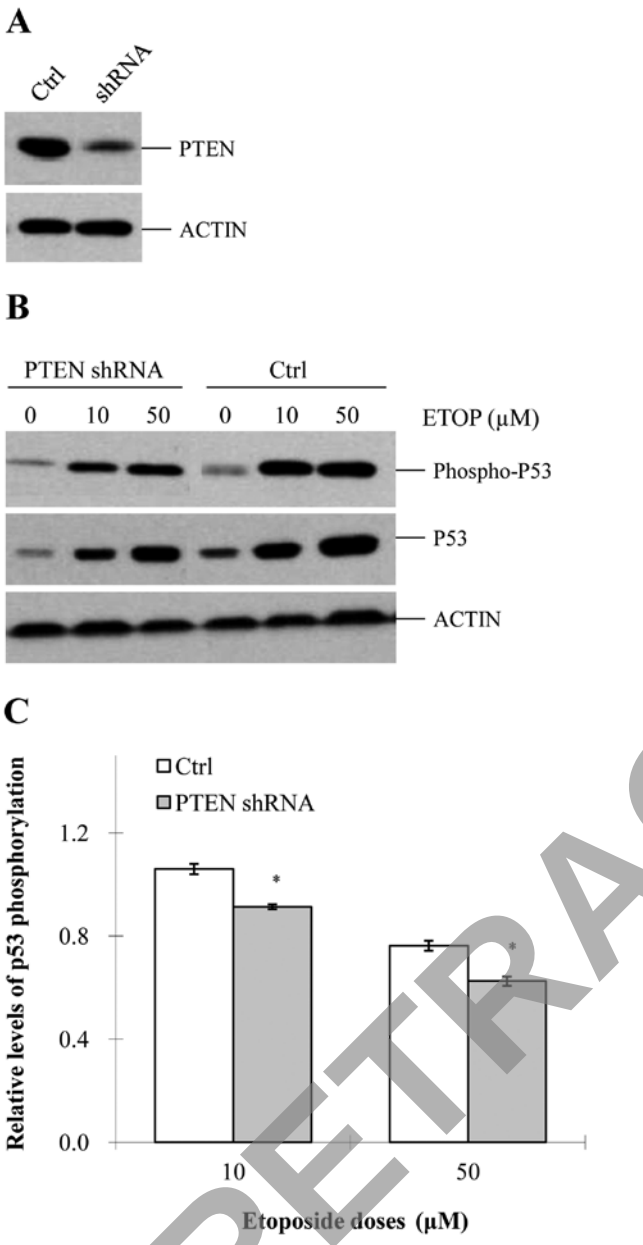


Figure 1. Depletion of *PTEN* reduces etopside-induced p53 phosphorylation. (A) MCF-7 cells were stably infected with an empty vector (control), or PTEN shRNA, followed by investigation of the expression level of PTEN and actin by western blotting using specific antibodies. (B) The expression levels of p53 phosphorylation (phospho-p53), total p53 and actin were detected by western blotting (n=3) in the PTEN-knockdown and control cells treated with different doses of etoposide (ETOP) for 8 h. (C) The relative level of phospho-p53 against β-actin was quantified in both the control and PTEN-knockdown MCF-7 cells as determined in B. Data are shown as means ± SE (n=3). *p<0.05 in comparison to the respective expression level of phospho-p53 in the control.

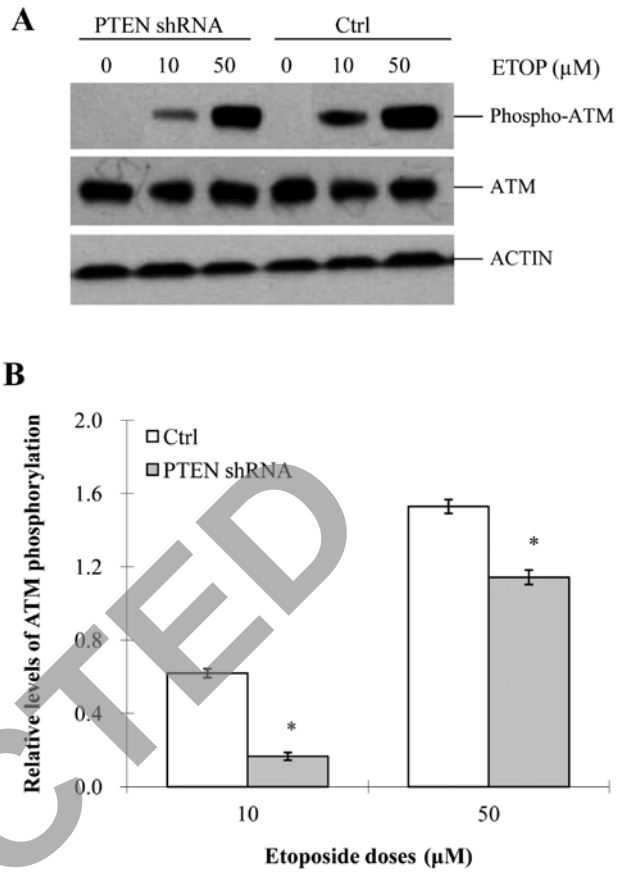


Figure 2. PTEN promotes etoposide-induced ATM activation. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h followed by western blotting for phosphorylated ATM and total ATM. Representative images of western blotting from the control and knockdown group are presented (n=3). (B) The relative expression level of phospho-ATM was quantified against total ATM and shown as means ± SE (n=3). *p<0.05 in comparison to the respective level of phosphorylated ATM in the control group.



tion on etoposide-induced *p53* phosphorylation. In both the control and *PTEN*-knockdown cells, etoposide induced *p53* phosphorylation in a dose-dependent manner (Fig. 1B). However, at each specific concentration of etoposide, the level of *p53* phosphorylation was significantly reduced in the *PTEN*-knockdown cells, compared with that in the control cells (Fig. 1B and C). These data suggest that PTEN is able to promote etoposide-induced *p53* phosphorylation.

*PTEN promotes etoposide-induced ATM activation*. In cells, one of the key responses to DNA damage is ATM activation, which occurs via auto-phosphorylation of ATM (S1981). ATM activation can facilitate the activation of *p53* given that S15 of *p53* is the substrate of ATM (28-30). As previously reported (31,32), etoposide activates ATM and thereby promotes a series of downstream responses leading to G2/M arrest. This finding was further supported by our results which showed that etoposide was able to enhance the phosphorylation level of ATM (Fig. 2A and B). In addition, the phosphorylated level of ATM was increased in an etoposide dose-dependent manner. However, the activation of ATM was largely compromised in the *PTEN*-knockdown cells at any specific concentration of etoposide (Fig. 2A and B), which indicated that *PTEN* is needed for the etoposide-induced activation of ATM/ATR.

*PTEN enhances the etoposide-induced production of γH2AX*. Histone H2A is one of the main histone proteins involved in the structure of chromatin in eukaryotic cells and is responsible for maintaining the shape and structure of the nucleosome (33). H2AX is a variant of H2A and its C-terminus is involved in DNA repair. A known response of H2AX to DNA damage

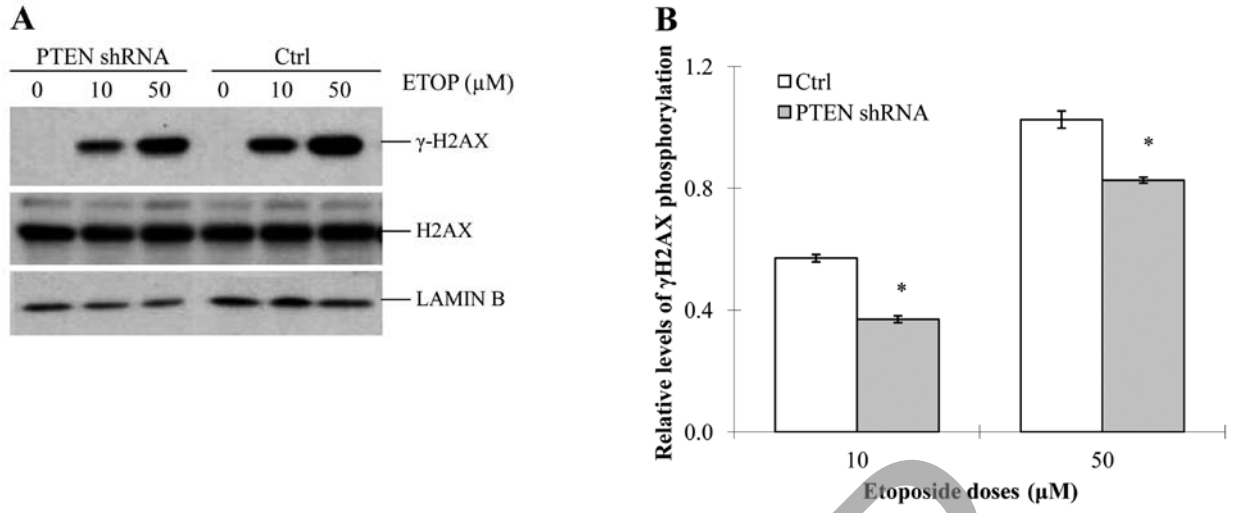


Figure 3. PTEN enhances etoposide-induced production of γH2AX. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) as indicated for 8 h. The expression levels of γH2AX, H2AX and lamin B were analyzed by western blotting (n=3). Representative images of western blotting are presented. (B) The relative expression level of γH2AX was quantified against the total level of H2AX that was first normalized by lamin B. Data are shown as means ± SE (n=3). *p<0.05 in comparison to the respective phosphorylated H2AX (γH2AX) in the control group.



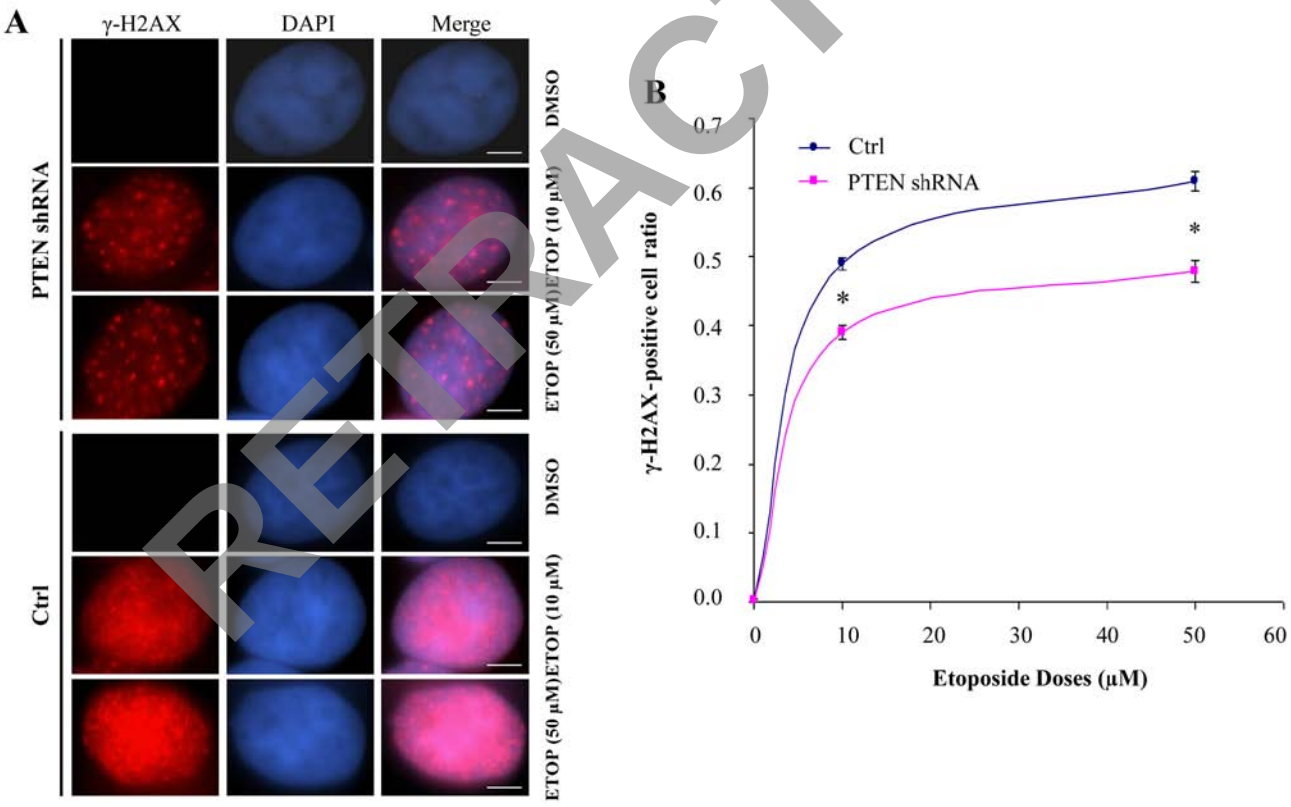


Figure 4. Knockdown of *PTEN* inhibits the formation of γ-H2AX nuclear foci in etoposide-treated MCF-7 cells. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide or DMSO for 8 h followed by immunostaining for γ-H2AX (red). Nuclei were stained with DAPI (blue). Scale bar, 5 μm. (B) Quantification of γ-H2AX-positive cells in both *PTEN*-knockdown and control cells. More than 200 cells in four randomly selected fields were counted to assess the ratio of γ-H2AX-positive cells. Data are presented as means ± SE (n=3). *p<0.05 in comparison to the control cells.

is the formation of γH2AX (34). According to previous studies (35-37), ATM activation promotes the phosphorylation of H2AX, and this phosphorylated form, named γH2AX, may bind to SDBs and form nuclear γH2AX foci, which are critical for DNA repair or G2/M arrest (38). Given the role of γH2AX in DDRs, in the present study, we also investigated whether *PTEN* contributes to etoposide-induced production of γH2AX. As shown in the control cells, the higher the dose of etoposide, the more γH2AX was produced in the MCF-7 cells. However, γH2AX was significantly reduced in the *PTEN*-knockdown MCF-7 cells (Fig. 3A and B). In addition, the formation of γH2AX was dose-dependent on etoposide in both the control

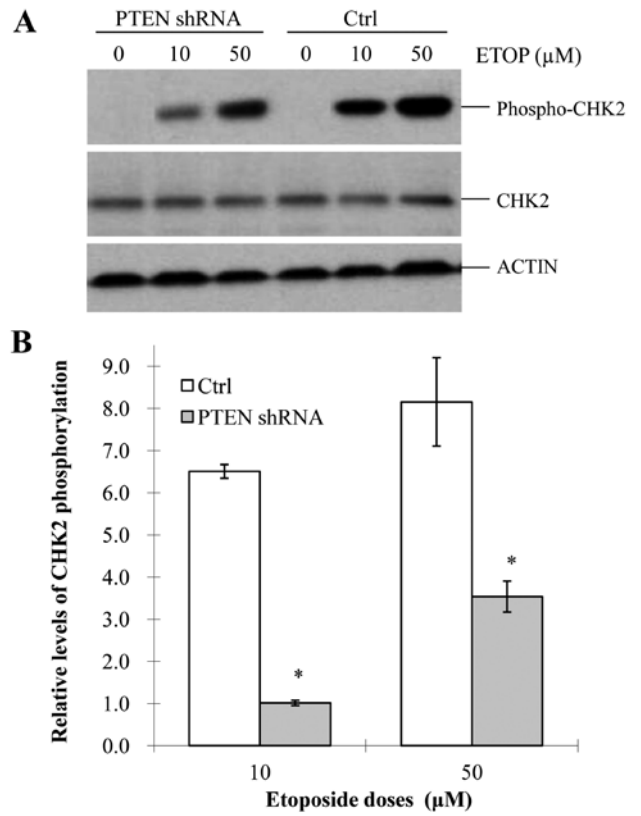


Figure 5. PTEN facilitates etoposide-induced Chk2 activation. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h followed by evaluation of Chk2 activation using western blotting. Representative images of western blotting are presented (B). The relative expression level of phospho-Chk2 was quantified against the total Chk2 that was first normalized by β-actin. Data are presented as means ± SE. *p<0.05 in comparison to the respective phospho-Chk2 in the control group.

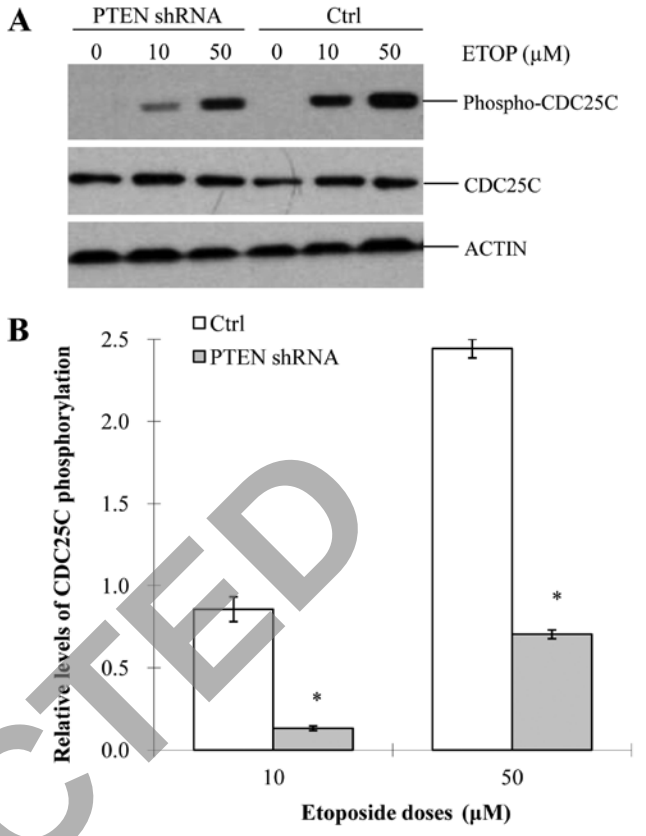


Figure 6. PTEN promotes etoposide-induced inactivation of CDC25C. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h and phospho-CDC25C (inactive form) was detected by western blotting. Representative images of western blotting are presented for both control and PTEN-knockdown groups. (B) The relative expression level of phosphorylated CDC25C was quantified against total CDC25C that was first normalized by β-actin. Data are presented as means ± SE. *p<0.05 in comparison to the respective phospho-CDC25C (S216) in the control group.



and *PTEN*-depleted cells (Fig. 3A and B). In order to confirm these results, we performed immunostaining to examine the formation of γH2AX nuclear foci in the etoposide-treated *PTEN*-knockdown MCF-7 cells. The γH2AX nuclear foci were strikingly reduced in the *PTEN*-knockdown etoposide-treated cells, compared with that in the control cells (Fig. 4A). In addition, the production of γH2AX nuclear foci was dose-dependent on etoposide in both groups. Furthermore, the number of γH2AX-positive cells was significantly reduced in the *PTEN*-knockdown MCF-7 cells, compared with that in the control cells. This quantitative difference between the control and *PTEN*-knockdown cells was also dose-dependent on etoposide (Fig. 4B). These data demonstrated that *PTEN* facilitated etoposide-induced γH2AX formation. Given that γH2AX foci not only functions in DNA damage repair but also activates DNA damage checkpoints, together with the data we presented above, we concluded that *PTEN* plays an important role in etoposide-induced G2/M arrest.

*PTEN facilitates etoposide-induced G2M/arrest in MCF-7 cells*. During DDR, ATM/ATR can be activated and thereby influences their substrates such as *p53* and *Chk2* (29). Similar with *p53*, phosphorylated Chk2 modulates downstream CDC25C and CDC2 (39-41). As a typical substrate of ATM, Chk2 kinase can be phosphorylated and activated by ATM (42). To further confirm our results and examine the role of *PTEN* in the DNA damage pathway, we investigated whether PTEN regulates Chk2 in the etoposide-treated MCF-7 cells. Our data showed that phospho-Chk2 (T68) was strikingly reduced in the *PTEN*-knockdown cells, compared with that in the control cells (Fig. 5A and B). In addition, the activation of Chk2 was dose-dependent on etoposide in both groups (Fig. 5A and B). These results demonstrated that *PTEN* promoted etoposide-induced Chk2 phosphorylation, which is consistent with our above finding that *PTEN* facilitated etoposide-induced ATM activation. It is known that the activation of CDC2 is needed for cells to pass G2/M during a cell cycle. Normally, CDC2 is dephosphorylated and activated by CDC25C, which can be phosphorylated and activated by Chk2. Once CDC25C is phosphorylated, cells cannot proceed through G2/M and thus G2/M arrest occurs. Therefore, in addition to assessing etoposide-induced Chk2 activation, we examined whether PTEN could activate CDC25C in the MCF-7 cells. Similar to our previous results, phosphorylated CDC25C was significantly reduced in the *PTEN*-knockdown MCF-7 cells, compared with that in the control cells (Fig. 6A and B). We also observed that etoposide induced the phosphorylation of CDC25C in a dose-dependent manner. To further confirm the role of *PTEN* in

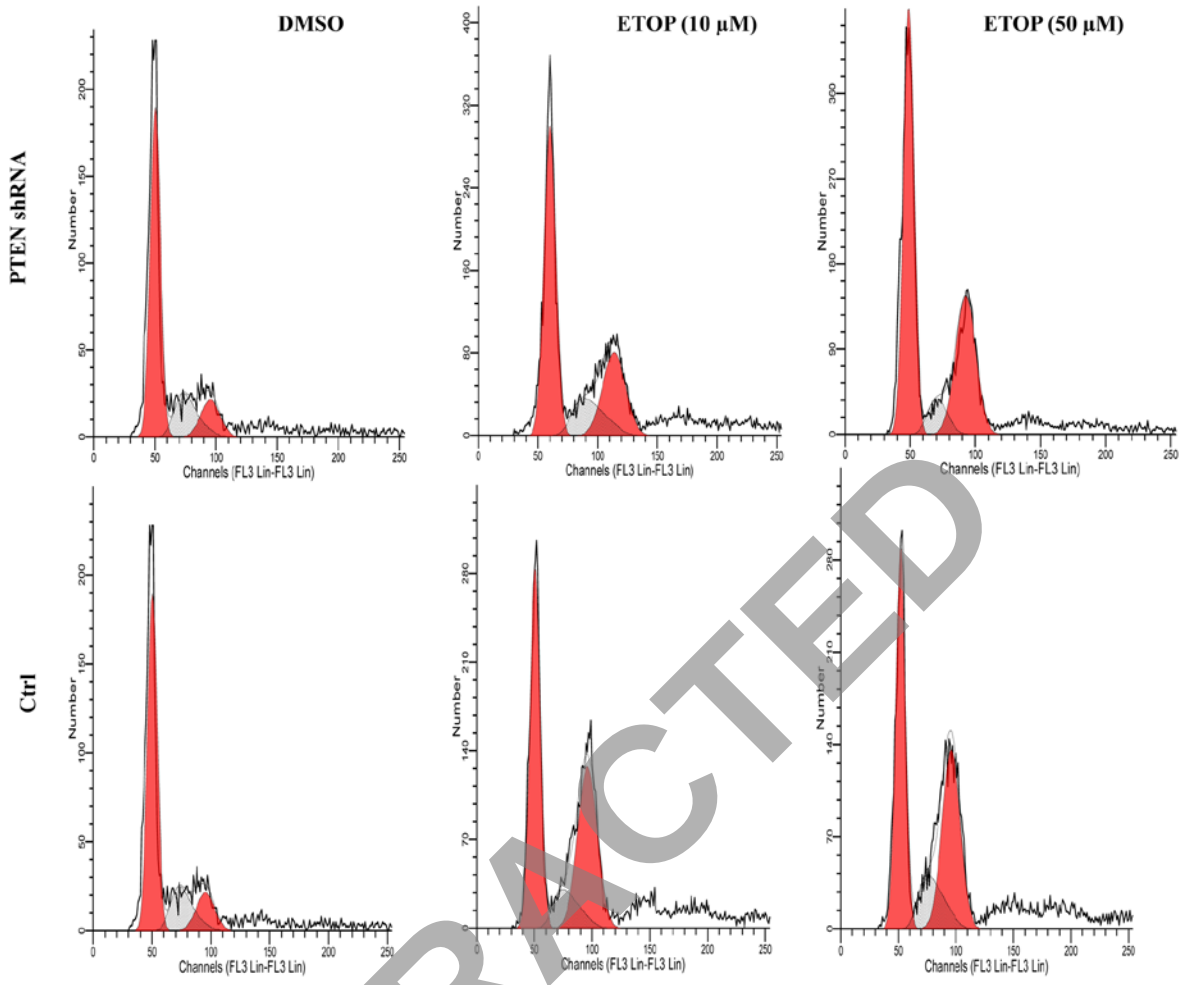


Figure 7. Knockdown of *PTEN* reduces the etoposide-induced G2/M arrest in MCF7 cells. Control and *PTEN*-knockdown MCF-7 cells were treated with DMSO or different doses of etoposide for 24 h, followed by the determination of cell cycle progression using flow cytometry.



Table I. Knockdown of *PTEN* reduces the etoposide-induced G2/M arrest in MCF-7 cells.

| | DMSO | | ETOP (10 $\mu$M) | | ETOP (50 $\mu$M) | |
|---|---|---|---|---|---|---|
| Cell cycle phase | Control | PTEN shRNA | Control | PTEN shRNA | Control | PTEN shRNA |
| G1 | 63.7±5.3 | 61.5±5.2 | 36.7±3.2 | 47.4±3.9[a] | 24.8±4.3 | 42.8±1.3[a] |
| S | 14.3±5.7 | 21.5±4.4 | 9.0±3.0 | 17.2±2.4[a] | 6.2±1.9 | 11.2±1.6[a] |
| G2/M | 18.7±9.3 | 17.0±1.1 | 54.4±1.4 | 35.4±3.4[a] | 69.0±6.2 | 48.1±2.1[a] |

Cell cycle distributions are presented as mean ± SE (n=3), [*]p<0.05 in comparison to the respective cell cycle distributions of the control group. PTEN, phosphatase and tensin homolog; DMSO, dimethyl sulfoxide; ETOP, etoposide.

G2M arrest, we performed flow cytometry to analyze the cell cycle profile in the *PTEN*-knockdown cells. As shown in Fig. 7 and Table I, etoposide induced G2/M arrest in a dose-dependent manner in both the control and *PTEN*-knockdown cells. However, when *PTEN* was knocked down in the etoposide-treated cells, the rate of G2/M arrest was significantly reduced. These data, together with other data shown above, suggest that PTEN may enhance etoposide-induced G2/M arrest through activation of the ATM-*p53*/Chk2-CDC2C pathway.

## Discussion

*PTEN* is one of the most commonly mutated tumor suppressors in human cancers (12,14,19). During tumor progression, mutations of *PTEN* can result in increased cell proliferation and decreased cell death. PTEN is involved in the regulation of cell proliferation, at a normal rate. PTEN has been one of the targets for drug candidates such as the oncomiR, miR-21 (12,43-45). It is widely recognized that PTEN dephos-

phorylates phosphatidylinositol (3,4,5)-trisphosphate [PtdIns (3,4,5)$P_3$ or $PIP_3$] (46), which leads to the inhibition of the AKT signaling pathway. It was reported that PTEN interacts with *p53* and PTK2 and modulates the progression of various cancers (23,47).

Over the last decades, it has been commonly accepted that DNA damage response (DDR) is one of the most important factors to maintain genomic integrity. In fact, DDR is not only essential to DNA repair, but is also critical to distinct cellular events, such as cell proliferation, apoptosis and gene transcription. The AKT signaling pathway has been demonstrated to function in these cellular processes while PTEN was also reported to be partially involved in progression of DDR (48-50). The contributions of molecular factors or pathways to DDR are variable and cell type-specific. Although some mechanisms underlying DDR are well understood, more factors associated with DDR, such as PTEN, remain to be further explored and elucidated. PTEN has certain impacts on some DNA repair pathways, for example the AKT pathway (50-53). To date, the effects of PTEN on the ATM pathway, particularly on etoposide-induced ATM-Chk2-CDC2 activation, have not yet been systematically investigated. Indeed, PTEN can be an important player in DDR given that it can functionally interact with a number of key important factors such as *p53*.

Etoposide is a medicine commonly used as a chemotherapy agent to treat cancers, such as Kaposi's sarcoma, Ewing's sarcoma, lung and testicular cancer, lymphoma, non-lymphocytic leukemia and glioblastoma multiforme. It is also used in a conditioning regimen prior to bone marrow or blood stem cell transplant (54). The cancer cell killing activity of etoposide is indeed dependent on the activation of the ATM pathway followed by G2/M arrest (31,55,56). Considering that the ATM/*p53* pathway is critical in DDR, scientists have made efforts to investigate this pathway. Recently, BMI1, ERK1 and ERK2, have been reported to affect etoposide-induced ATM activation, indicating that they are also required for etoposide-induced G2/M arrest (22,57).

In the present study, we provide evidence that PTEN facilitates etoposide-induced ATM activation, and also contributes to etoposide-induced changes of other factors in the ATM pathway, including activation of Chk2 and inactivation of CDC25C, eventually leading to G2/M arrest. Our data demonstrated that PTEN is required for proper activation of the ATM pathway in response to etoposide treatment. It is not clear yet how PTEN functions in activation of the ATM pathway and ATM pathway-mediated G2/M arrest. It is possible that PTEN facilitates the etoposide-induced activation of the ATM pathway through interacting with ATM directly or other proteins such as AKT, *p53* and BRCA1 indirectly. Further studies are required to clarify these questions.

Taken together, our finding that PTEN regulates DNA damage through activation of the ATM-Chk2 pathway provides a new perspective with which to understand the relationship between PTEN and initiation/progression of cancer cells; it also provides a new potential therapeutic target for cancer treatment. In addition, the fact that PTEN affects etoposide-induced G2/M arrest in cancer cells provides us with important information when patients are treated with etoposide, which is critical for precision medicine.



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