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Arsenic Trioxide Induces G2/M Arrest in Hepatocellular Carcinoma Cells by Increasing the Tumor Suppressor PTEN Expression

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ABSTRACT

Arsenic trioxide (As_2O_3), an effective agent against acute promyelocytic leukemia, has been reported to inhibit the viability of solid tumors cell lines recently. The detailed molecular mechanism underlying the As_2O_3-induced inactivation of the cdc2 and possible functional role of PTEN in the observed G2/M arrest has yet to be elucidated. Here, we assessed the role of PTEN in regulation of As_2O_3-mediated G2/M cell cycle arrest in Hepatocellular carcinoma cell lines (HepG2 and SMMC7721). After 24 h following treatment, As_2O_3 induced a concentration-dependent accumulation of cells in the G2/M phase of the cell cycle. The sustained G2/M arrest by As_2O_3 is associated with decreased cdc2 protein and increased phospho-cdc2(Tyr15). As_2O_3 treatment increased Wee1 levels and decreased phospho-Wee1(642). Moreover, As_2O_3 substantially decreased the Ser473 and Thr308 phosphorylation of Akt and upregulated PTEN expression. Downregulation of PTEN by siRNA in As_2O_3-treated cells increased phospho-Wee1(Ser642) while decreased phospho-cdc2(Tyr15), resulting in decreased the G2/M cell cycle arrest. Therefore, induction of G2/M cell cycle arrest by As_2O_3 involved upregulation of PTEN. J. Cell. Biochem. 113: 3528–3535, 2012.

KEY WORDS: ARSENIC TRIOXIDE; G2/M ARREST; AKT; PTEN; cdc2

Hepatocellular carcinoma (HCC) is a common primary malignant tumor of the liver. Despite improved diagnostic and treatment strategies, the prognosis of HCC remains poor. Indeed, most patients were diagnosed at advanced stage or were unable to tolerate surgical resection [El-Serag et al., 2006]. Moreover, HCC is a type of cancer that resistant to conventional chemotherapy and radiotherapy [Bruix et al., 2004]. Therefore, the development of more efficacious therapies for HCC remains urgent.

Arsenic trioxide (As_2O_3), which has been proved to be an effective agent against acute promyelocytic leukemia (APL) [Wang and Chen, 2008], is also effective in the treatment of relapsed and refractory multiple myeloma [Berenson and Yeh, 2006]. Recently, the anticancer activity of As_2O_3 has been extended to a variety of solid tumors and tumor cell lines, including liver, gastric, colorectal, ovarian, cervical, breast, prostate, lung, and bladder cancer [Maeda et al., 2001; Dilda and Hogg, 2007; Han et al., 2008a; Wang et al., 2009; Zheng et al., 2010]. However, clinical response of solid tumors to As_2O_3 has been poor [Chen et al., 2002; Dilda and Hogg, 2007] and much higher As_2O_3 concentrations are required for solid tumors [Liu et al., 2006]. Therefore, better understanding of the mechanisms action of As_2O_3 will facilitate its application.

The anticancer effect of As_2O_3 involved complex mechanisms that are not fully understood. In some cases, induction of G2/M cell cycle arrest by As_2O_3 involves inhibition of cdc2 [Park et al., 2000; Han et al., 2008b], a key regulator of G2/M transition. In eukaryotic cells, entry into mitosis is regulated by activation of cdc2 kinase, a process controlled by cyclin B1 binding. In addition, dephosphorylation of Tyr15 and Thr14 is the critical regulatory step in activating cdc2 [Norbury et al., 1991]. Phosphorylation at Thr14 and Tyr15, which results in inhibition of cdc2 and prevents entry into mitosis,

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can be carried out by Wee1 and Myt1 protein kinases [McGowan and Russell, 1993; Wells et al., 1999].

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), that is mutated or deleted with high frequency in various cancers, plays critical roles in cell growth, migration, and death [Sawai et al., 2008; Peyrou et al., 2010; Baig et al., 2011]. As a dual specificity phosphatase, PTEN catalyses the dephosphoryltation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), resulting in inhibition of the Akt signaling pathway, which plays an important role in regulating cell survival, proliferation, and invasiveness [Carnero et al., 2008]. Thereby, PTEN functions as a tumor suppressor by negatively regulating PI3K/Akt pathway [Chalhoub and Baker, 2009]. Akt is reported to promote cell cycle progression at the G2/M transition through inactivation of Wee1 or inhibition of Myt 1 [Kandasamy and Srivastava, 2002; Okumura et al., 2002]. Several previous studies suggested that upregulation or restoration of PTEN results in G2/M arrest through downregulation of the Akt pathway [Hang et al., 2005; Zhang et al., 2010; Arafa el et al., 2011]. Loss of PTEN facilitated the transition from G2/M to G1 and override G2/M arrest induced by gamma irradiation [Kandel et al., 2002]. These findings suggest that upregulation of PTEN induced G2/M arrest through inhibition of Akt pathway [Selvendiran et al., 2007; Fei et al., 2009].

Earlier studies have reported that As_2O_3 induces the G2/M arrest in cancer cells in vitro [Han et al., 2008b; Li et al., 2009], and PTEN induction was found in the apoptosis of B-chronic lymphocytic leukemia cell induced by As_2O_3 [Redondo-Munoz et al., 2010]. The detailed molecular mechanism underlying the As_2O_3-induced inactivation of the cdc2 and possible functional role of PTEN in the observed G2/M arrest has yet to be elucidated, and we have addressed this in the present report.

MATERIALS AND METHODS

CELL CULTURE

The human HCC cell lines HepG2 and SMMC-7721 (Heilongjiang cancer institute, China) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under an atmosphere of 95% air and 5% CO_2. Cells were routinely sub-cultured every 2–3 days and cell samples used were all in the logarithmic growth phase.

REAGENTS AND ANTIBODIES

A stock solution of arsenic trioxide (As_2O_3; Sigma, St. Louis, MO) was prepared at the concentration of 1 mmol/L by dilution with phosphate-buffered saline (PBS) after dissolving the reagent in 0.1 mol/L NaOH, and was further diluted to the working concentration before use. Methyl thiazolyl tetrazolium (MTT), trypsin, propidium (PI) were purchased from Sigma Chemical Co.

Antibodies against total Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), cdc2, phospho-cdc2(Tyr15), Wee1, Phospho-Wee1(Ser642), and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PTEN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

CELL VIABILITY ASSAY

Effect of As_2O_3 on the viability of hepatocellular carcinoma cells was performed using MTT conversion assay. Briefly, cells were seeded at 4×10^3 cells/well in 96-well plates and incubated for 20 h, and then different concentrations of As_2O_3 were added and further incubated for 24 h. Thereafter, 20 μl of MTT solution (5 mg/ml) was added to each well and the cells were incubated for another 4 h at 37°C. After removal of the culture medium, the cells were lysed in 100 μl of dimethylsulfoxide and then the optical density (OD) was measured at 490 nm using a microplate reader (Bio-rad, Hercules, CA).The experiment was repeated three times. The following formula was used: relative percentage of cell viability = (OD of the experimental sample/OD of the control group) × 100%.

CELL CYCLE PHASE ANALYSIS

Phase distributions of the cell cycle were determined by flow cytometry (BD Biosciences, San Jose, CA). Cells were treated with varying concentrations of As_2O_3 for 24 h, then 1×10^6 cells collected and fixed with ice-cold 70% ethanol overnight at 4°C. The cell pellets were incubated with 50 μg/ml propidium iodide (PI) and 10 μg/ml RNase A for 30 min in the dark. Finally, samples were evaluated by flow cytometry. The experiment was repeated three times.

WESTERN BLOT ANALYSIS

Western Blotting was performed using standard techniques. Cells in logarithmic growth phase were seeded at 1×10^6 cells/bottle in 25 ml culture flasks and then different concentrations of As_2O_3 were added and further incubated for 24 h. Cells were washed twice with PBS buffer and lysed in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris–Cl pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 2 μg/ml aprotinin) on ice. Protein concentration was determined by Bradford method. Total proteins 50 μg were subjected to sodium dodecyl sulfate–polyacrylamide gel ectrophoresis (SDS–PAGE) and transferred to PVDF membranes (Roche Diagnostics, Mannheim, Germany). Membranes were blocked with 5% skim milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween20) at room temperature for 1 h and incubated with the corresponding primary antibodies at room temperature for 2 h. After washing with TBST, the membrane was reacted with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After extensive washing with TBST, proteins were visualized by chemiluminescent reagents (EZ–ECL, Beit–Haemek, Israel).

PTEN SMALL INTERFERING RNA ASSAY

Cells were seeded onto 6-well plates at a density of 1×10^5. The next day, the cells were transfected with PTEN siRNA or control siRNA using FuGENE HD Transfection Reagent (Roche) at a final concentration of 100 nmol/L, according to the manufacturer's protocol. At 48 h post-transfection, the cells were treated with or without 8 μmol/L As_2O_3 for another 24 h.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. The oneway analysis of variance (ANOVA) was used for statistical analyses. $P < 0.05$ was considered as statistically significant.

RESULTS

As_2O_3 DECREASED CELL VIABILITY AND ARRESTED CELLS AT G2/M PHASE

To investigate the effect of As_2O_3 on the viability of liver cancer cells, HepG-2 and SMMC-7721 cells were treated with increasing concentrations of As_2O_3 (1–8 μmol/L) and their viability was determined by MTT assay. Dose-dependent inhibition of cell viability was found in both cell lines (Fig. 1A). Then, flow cytometry analysis showed that As_2O_3 induced a significant G2/M phase arrest in both cell lines (Fig. 1B).

As_2O_3 DECREASED THE EXPRESSION OF cdc2 ACCOMPANIED BY THE ALTERNATION OF Wee1

To explore the molecular mechanisms of the As_2O_3-induced G2/M phase arrest, the expression levels of cdc 2 and its inactive form (the Tyr15-phosphorylated form) in HepG2 and SMMC-7721 cells were examined by Western blot. As shown in Figure 2, 2–8 μmol/L As_2O_3, which arrested the two cell lines in G2/M, decreased the cdc2 expression and increased the expression of Phospho-cdc2(Thr15). Next, we observed the levels of protein kinases Wee1, that regulates the phosphorylation at Tyr15 residue of cdc2 kinase, were significantly increased in both cell lines (Fig. 2). Furthermore, Western blot analysis showed that As_2O_3 treatment decreased the phospho-Wee1(Ser642) levels (Fig. 2), which promotes its cytoplasmic localization and abolished the Wee1-induced G2/M arrest [Katayama et al., 2005]. Thus, these results suggested that inhibition of cdc2 kinase due to the induction of Wee1 of As_2O_3, induced a G2/M phase arrest.

As_2O_3 INHIBITED AKT ACTIVATION BY UPREGULATING PTEN

To analyze the effect of As_2O_3 on Akt signaling pathway, which promotes G2/M-phase transition by phosphorylating Ser642 of human Wee1 [Katayama et al., 2005], we focused on the alteration of PI3K/AKT related proteins in both cell lines. As shown in Figure 3, phospho-Akt (Ser473) and phospho-Akt (Thr308) were decreased after As_2O_3 treatment for 24 h in both cell lines, whereas the levels of Akt were unchanged. Moreover, As_2O_3 treatment dose-dependently increased PTEN protein expression levels.

We next investigated the alterations of cdc2 related proteins expression in cells pretreated with PTEN siRNA for 48 h followed by As_2O_3 in order to show a direct connection between PTEN induction by As_2O_3 and G2/M arrest. PTEN siRNA pretreatment abrogated the induction of PTEN by As_2O_3 in these cell lines, accompanied with increase of Akt phosphorylation (Ser473 and Thr308). Furthermore, the suppression of PTEN by PTEN siRNA in As_2O_3-treated cells resulted in increase of phospho-Wee1(-Ser642), decrease of phospho-cdc2(Tyr15) and decreased the G2/M cell cycle arrest as compared with As_2O_3-treated groups (Fig. 4). These results suggest that inactivation of Akt due to the induction of PTEN by As_2O_3 treatment, arrested cell cycle at G2/M phase.

DISCUSSION

As_2O_3, which is currently successfully employed for the treatment of APL, has potential anticancer activity against some solid tumors including HCC [Liu et al., 2011]. Understanding the mechanism by which As_2O_3 inhibits cell viability may help to identify potential targets for combination therapies of cancer. In this study, we found that As_2O_3 treatment decreased the level of cdc2 protein and increased phospho-cdc2(Thr15). Furthermore, it increased the levels of Wee1 kinase and decreased phospho-Wee1(Ser642). From these results, we conclude that As_2O_3 arrested HCC (HepG2 and SMMC-7721 cells) at G2/M phase by inhibiting cdc2 dephosphorylation/activation due to the induction of Wee1. As_2O_3 also inhibited Akt activation by upregulating PTEN, and these effects were inhibited by PTEN siRNA. Taken together, these results suggest that the effect of As_2O_3 on the levels of phospho-cdc2(Thr15) and phospho-Wee1(Ser642) are mediated, at least in part, by Akt inactivation via an upregulation of PTEN.

PTEN, as an inhibitor of the PI3K/Akt pathway, plays critical roles in cell proliferation, cell cycle and apoptosis [Chalhoub and Baker, 2009]. The induction of PTEN was reported to arrest cell cycle at the G1/G0 or G2/M phase through both Akt-dependent and -independent mechanisms [van Duijn et al., 2010; Zhang et al., 2010; Arafa el et al., 2011; Xiong et al., 2011]. Recently it was shown that Akt inactivation is involved in As_2O_3-induced G2/M phase arrest in gastric cancer cells [Li et al., 2009]. In this study, we observed that As_2O_3-induced upregulation of PTEN resulted in down-regulated the pAkt (Ser473 and Thr308) and G2/M cell cycle arrest in HCC cells. These results are consistent with those reports that pointed upregulation of PTEN induced G2/M arrest through the inhibition of the Akt pathway [Cao et al., 2006; Arafa el et al., 2011]. The siRNA experiments against PTEN showed significantly increased pAkt (Ser473 and Thr308) and decreased G2/M arrest in As_2O_3-treated HCC cells, further supporting the idea that upregulation of PTEN might be involved in the G2/M arrest through the inhibition of the Akt pathway.

Several studies have shown that some anticancer agents induced G2/M arrest, in part, by blocking the activation of Akt kinase [Weir et al., 2007; Huang et al., 2011; Weng et al., 2012]. It is reported that Akt-mediated Wee1 phosphorylation on Ser642 residue induced Wee1 binding to 14-3-3θ and cytoplasmic localization upon growth factor stimulation, promoting G2/M cell cycle progression [Katayama et al., 2005]. In mammalian cells, the cdc2 kinase, a key regulator of G2/M transition, is in part regulated by inhibitory phosphorylation of Tyr15, catalyzed by Wee1 [McGowan and Russell, 1993; Nigg, 2001]. Another phosphorylation of cdc2, on the Thr-161 residue located in the T-loop of the protein, is believed to be essential for cdc2 kinase activity [Desai et al., 1995; De Smedt et al., 2002]. At the G2/M transition, cdc25C phosphatase is responsible for removal of phosphates at Thr14 and Tyr15 and subsequent activation of cdc2 [Gautier et al., 1991]. Although Thr161 on cdc2 and cdc25C have a putative Akt consensus sequence, Akt could not phosphorylate either of these site [Katayama et al., 2005]. In this study, we found that As_2O_3 decreased the level of cdc2 protein, which is consistent with those reports that suggested that induction of G2/M cell cycle arrest

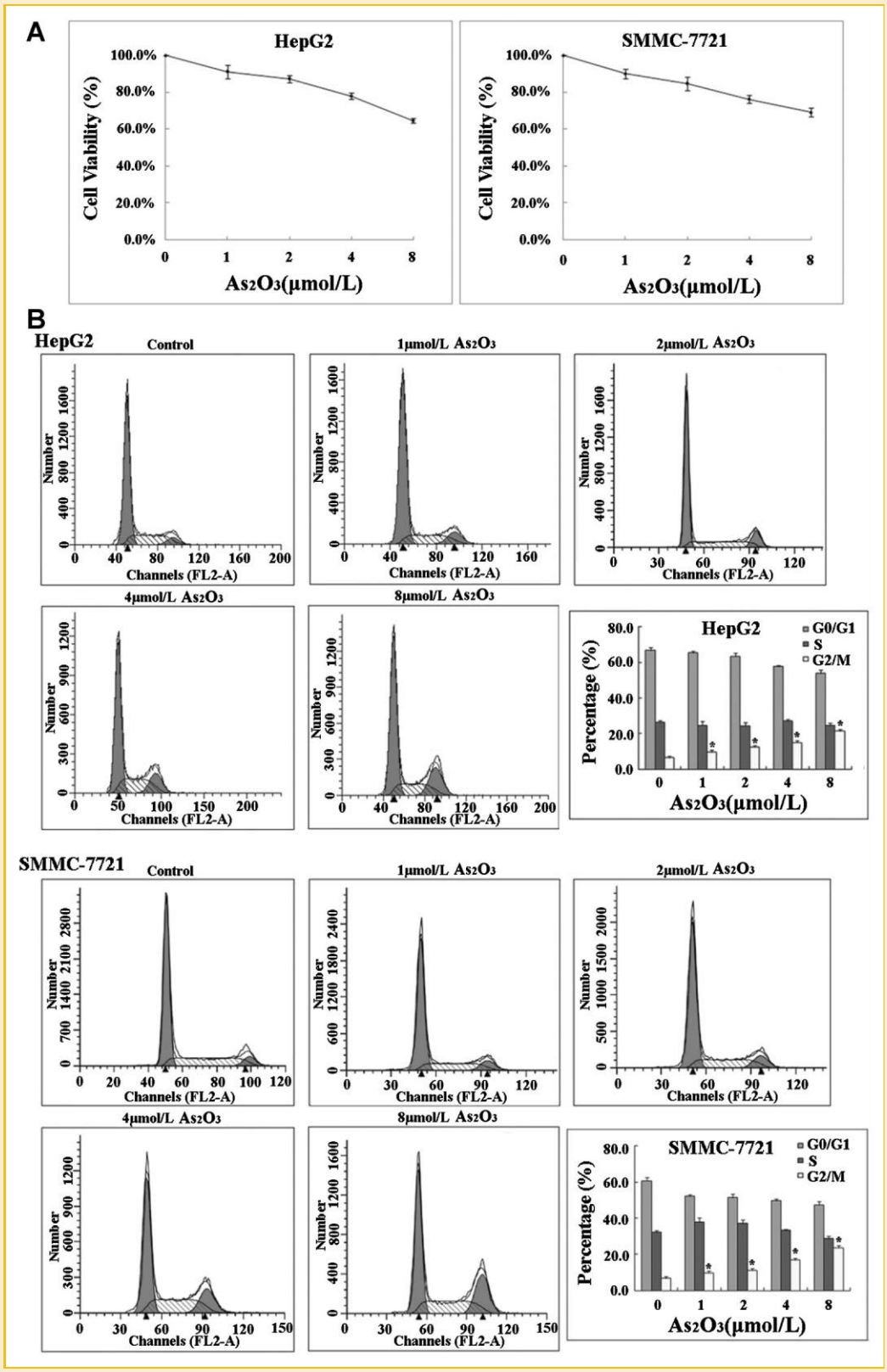

Fig. 1. As_2O_3 decrease cell viability in HCC cancer cells and arrests cells at G2/M phase. A: Effect of As_2O_3 on the viability of HepG2 cells and SMMC-7721 cells. Both HCC cell lines were treated with the indicated concentrations of As_2O_3 for 24 h and the cell viability was determined by MTT assay. Absorbances were measured at 490 nm and the results were expressed as the percentage viability with respect to control cell. Data are shown mean ± SD for three separate experiments in which each treatment was repeated in five wells. B: As_2O_3 arrested HCC cells at G2/M Phase. Cells were treated with the indicated concentrations of As_2O_3 for the 24 h and then the percentage of cells in each cell cycle phase (G1/G0, S, and G2/M) was determined by flow cytometry ($n = 3$). $^*P < 0.05$ versus control group.

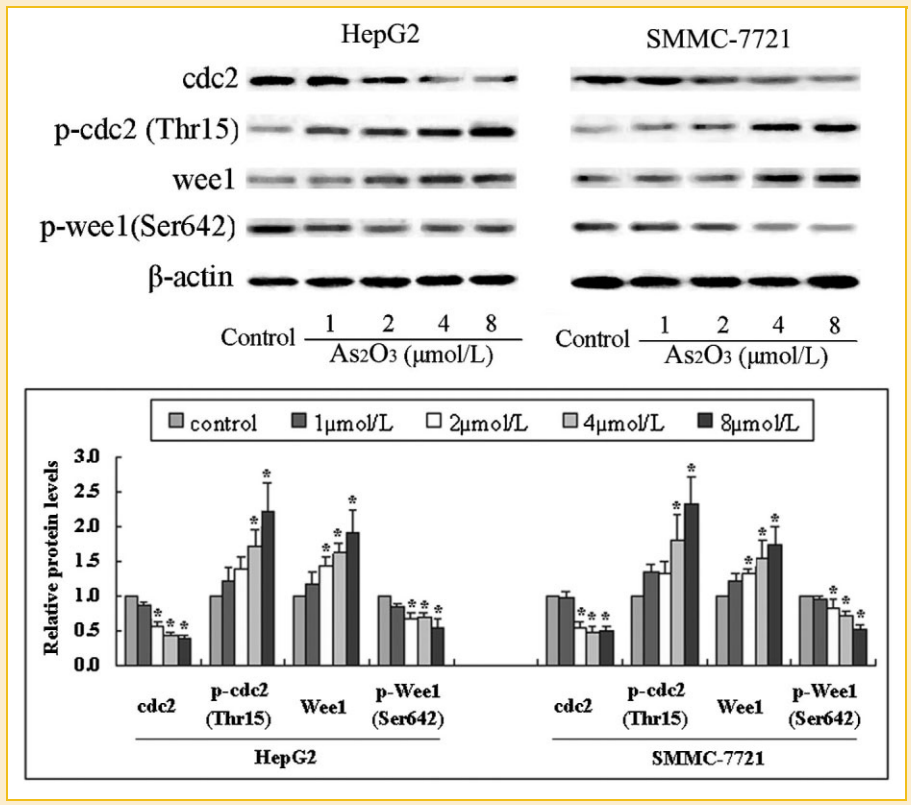

Fig. 2. Effects of As_2O_3 on the expression of cdc2 and related proteins. Exponentially growing cells were treated with the indicated concentrations of As_2O_3 for 24 h and the levels of cdc2, p-cdc2(Thr15), Wee1, and p-Wee1(Ser642) proteins were evaluated using Western blot. The data are shown mean relative protein levels ±SD for three separate blots (lower). $^*P < 0.05$ versus control group.

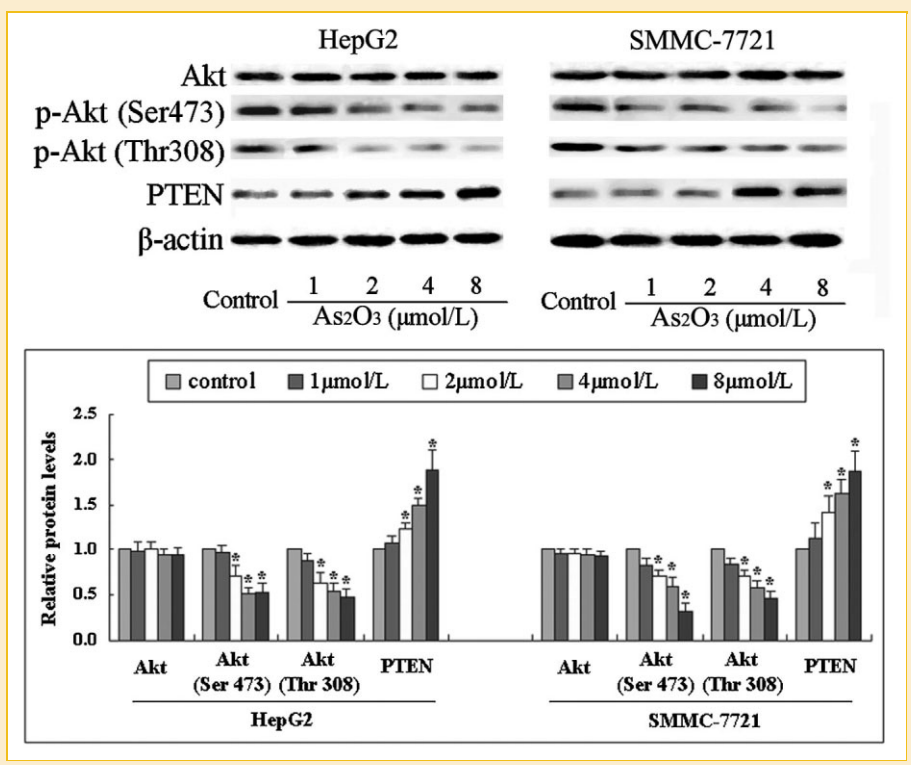

Fig. 3. The effect of As_2O_3 on Akt signaling pathway. Exponentially growing cells were treated with the indicated concentrations of As_2O_3 for 24 h and the Akt, phospho-Akts and PTEN were evaluated using western blot. The data are shown mean relative protein levels ±SD for three separate blots (lower). $^*P < 0.05$ versus control group.

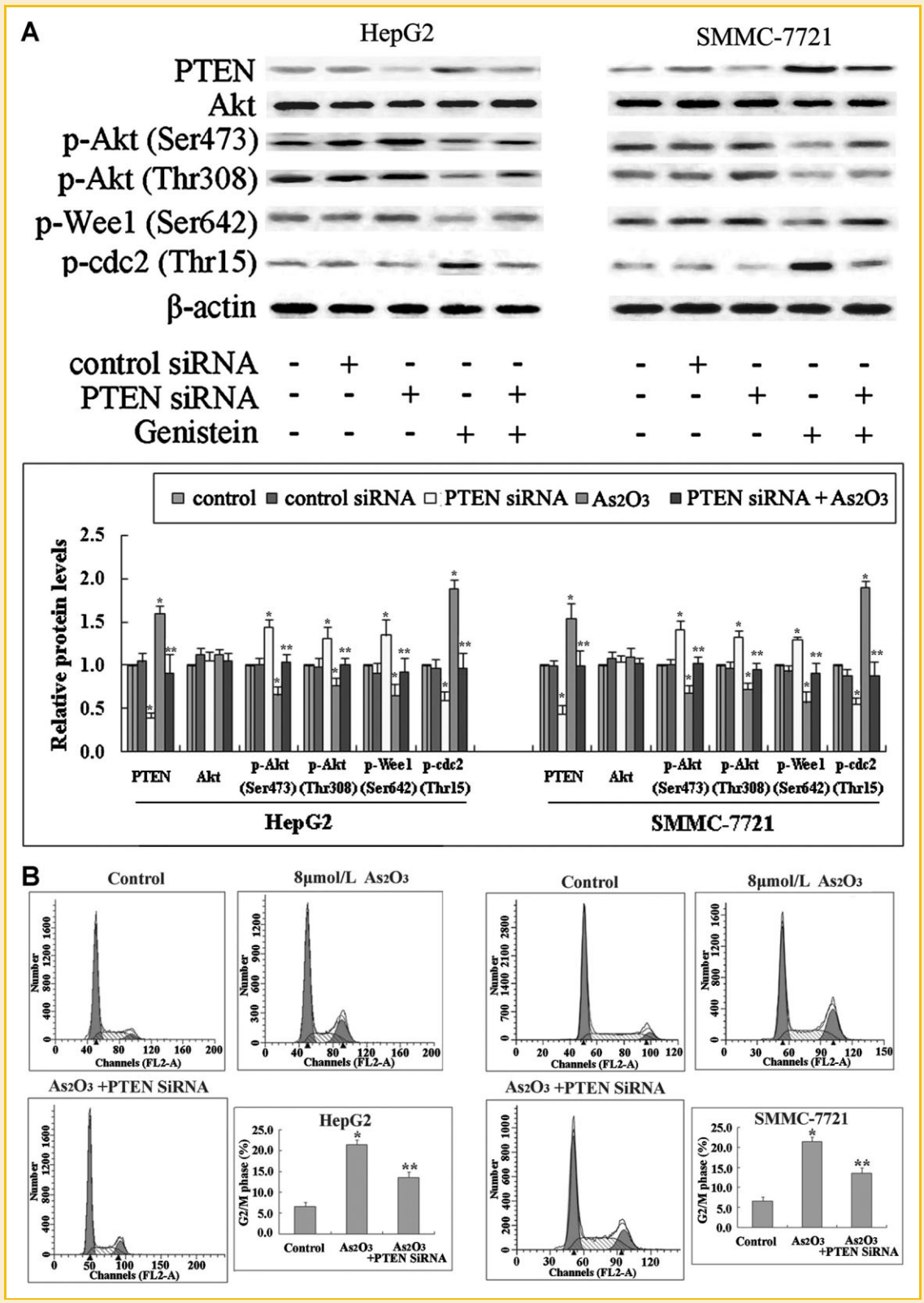

Fig. 4. PTEN siRNA partially attenuated As_2O_3 -induced G2/M phase arrest. HepG2 cells and SMMC-7721 cells were transfected with PTEN siRNA or control siRNA according to the manufacturer's protocol. At 48 h post-transfection, the cells were treated with or without 8 μmol/L As_2O_3 for another 24 h. A: The PTEN, Akt, phospho-AKTs p-Wee1(Ser642) and p-cdc2(Thr15) were evaluated using Western blot. B: Phase distribution of cell cycle was determined by flow cytometry. $^{*}P < 0.05$ versus control group, $^{**}P < 0.05$ versus non-transfected cells treated with genistein.

by As_2O_3 involves inhibition of cdc2 [Park et al., 2000; Han et al., 2008b]. In addition, we used Western blots to show that As_2O_3 increased phospho-cdc2(Thr15) and decreased phospho-Wee1 (Ser642). These effects of As_2O_3 were attenuated by PTEN siRNA. Thus, upregulation of PTEN might be involved in inhibition of cdc2 through decreasing phospho-Wee1(Ser642) and increasing phospho-cdc2(Thr15).

In summary, we have shown that As_2O_3 increased PTEN expression in HCC cells. The upregulation of PTEN resulted in a decrease in Akt and phospho-Wee1(Ser642) and an increase in phospho-cdc2(Thr15), thereby inducing G2/M arrest. Our study provided a basic mechanism for the anticancer effect of As_2O_3 in the HCC cells. Future in vivo studies using animal models and other cancer cell lines could ascertain whether this inducing G2/M arrest effect of As_2O_3 might contribute to its overall effect in the fight against cancer and possibly show novel therapeutic applications. This work may provide mechanistic insights for understanding the molecular basis of the anticancer effect of As_2O_3.

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