Estrogen receptor α induces down-regulation of PTEN through PI3-kinase activation in breast cancer cells

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Abstract. Estrogen receptor α (ER_α) mediates most of the biological effects of estrogen in mammary epithelial cells and stimulates growth signals involving phosphoinositide-3-OH kinase (PI3K)/Akt in breast cancer cells. Phosphatase and tensin homologue (PTEN) is a critical counter-regulator of PI3K signaling and is thus one of the major tumor suppressors in breast cancer. Inhibition of PI3K with an inhibitor, wortmannin, increased the level of PTEN protein in ER_α-positive MCF-7 cells, while levels in ER_α-negative MDA-MB 231 cells were not altered. In addition, the level of PTEN protein in MCF-7 cells was significantly lower than that in MDA-MB 231 cells, which correlated with high levels of phospho-Akt and phosphatidylinositol-3,4,5,-trisphosphate (PIP_3). However, PTEN mRNA expression as measured by real-time PCR showed no differences in either cell line. Notably, the levels of casein kinase 2 (CK2) and phospho-PTEN (Ser380/Thr382/383) in MCF-7 cells were lower than those in MDA-MB 231 cells, indicating that the down-regulation of PTEN protein in MCF-7 cells is caused by low levels of CK2 expression, leading to accelerated PTEN degradation. Collectively, these results suggest that ER_α induces the down-regulation of PTEN through PI3K activation in breast cancer cells.

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Introduction

Phosphatase and tensin homologue (PTEN) on chromosome 10q23 is phosphatidylinositol-3' phosphatase (lipid phosphatase) that dephosphorylates the D3 position of phosphatidylinositol-3,4,5-trisphosphate (PIP_3), a second messenger that is produced by phosphatidylinosititol-3-OH kinase (PI3K) and activates protein kinase B (PKB)/Akt (1-3).

PTEN is known to be one of the major tumor suppressor genes in human cancer and plays a pivotal role in the carcinogenesis of breast cancer cells. PTEN inhibits breast cancer growth through the down-regulation of PI3K signaling, resulting in the blockage of cell cycle progression and the induction of cell death (4). Along the same lines, PTEN has been shown to arrest cells in the G_1 phase of the cell cycle in breast cancer cell lines (5). These observations suggest that PTEN is involved in the modulation of growth and survival in breast cancer cells. Mutations and/or deletions of PTEN are found in a variety of cancer cells, including endometrial and breast neoplasm cells (4,6-12). In addition, reduced expression of PTEN protein has been observed in approximately one third of all breast cancer cases (13). Moreover, the reduced expression of PTEN protein correlates with lymph node metastasis and a worse prognosis in patients with breast cancer (13-15). However, the down-regulating mechanism of PTEN in breast cancer cells remains to be clarified.

Estrogen plays critical roles in the proliferation of breast cancer cells via two estrogen receptors, estrogen receptor $(ER)_\alpha$ and ER_β, which are ligand-dependent transcription factors that belong to the superfamily of nuclear receptors. In breast cancer, the expression of the ERs is correlated with well-differentiated of tumors and indicates a favorable prognosis, with responsiveness to endocrine therapy with anti-estrogen drugs (16,17). The transcription activities of the ERs are regulated by interacting proteins, such as coactivators and kinases as well as ligand-binding proteins (18). ER_α binds to the p85 regulatory subunit of PI3K in a ligand-dependent manner, thereby activating Akt and subsequently its downstream effectors (19). We have also demonstrated that ER_α stimulates growth signals involving PI3K/Akt in breast cancer

cells expressing both ER_α and ER_β (15). These findings indicate that a PI3K-involved signaling system may be related to the biology of ER_α in breast cancer cells.

One of the traits of cancer cells is an imbalance in signal regulation, and ER_α mediates most of the biological effects of estrogen in mammary epithelial cells. To elucidate the role of ER_α and ER_β in the growth of cancer cells, we investigated the regulation of the PI3K signaling system involving PTEN, a counter-regulator of PI3K signaling, in breast cancer cells. Two types of human breast epithelial cell lines were utilized: MCF-7 cells, expressing both ER_α and ER_β, and MDA-MB-231 cells, expressing ER_β only.

Materials and methods

Materials. Antibodies against PTEN and casein kinase 2 (CK2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Akt (p-Akt) and phospho-PTEN (p-PTEN) (Ser380/Thr382/383) were from Cell Signaling Technology (Beverly, MD, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS) and antibiotics were obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA). Hanks balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM) and β-actin antibody were from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MD-231 were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM containing 10% FBS and 1% antibiotics at 37˚C in a humidified atmosphere (5% CO_2, 95% air).

Western blot analysis. After washing with PBS and harvesting, cell pellets were lysed with 40 μl of ice-cold M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations in the lysate were determined using the Bradford method (20). Samples (30 μg) were separated by SDS-PAGE with 10% acrylamide running and 3% acrylamide stacking gels, and then transferred to hybond™-PVDF membranes using a Western blot apparatus. The PVDF membranes were blotted with 1 μg/ml of primary antibodies for PTEN, p-PTEN, p-Akt, CK2 and β-actin. Horseradish peroxidase-conjugated IgG was used as a secondary antibody. Protein expression levels were then determined by analyzing the signals captured on the PVDF membranes using an image analyzer (Las-1000, Fuji-Film, Japan).

Measurement of PIP_3 levels. The intracellular PIP_3 level was determined by immunocytochemistry using a monoclonal antibody against PIP_3 as described by Niswender (21). Briefly, cells mounted on glass slides were equilibrated in PBS at room temperature and then fixed in 4% paraformaldehyde at room temperature for 5-10 min. After blocking in a blocking buffer containing 5% normal goat serum and 2% bovine serum albumin, samples were incubated with a PIP_3 monoclonal antibody (Echelon at a 1:100 dilution) overnight at 4˚C. The negative control for the antibody was an equivalent concentration of non-immune mouse IgM. The immunoreactivity of PIP_3 was detected with goat anti-mouse IgM-TRITC at a 1:200 dilution by incubation for 1 h at 4˚C. Samples were mounted in aqueous mounting medium with anti-fading agents. Images were acquired with a confocal laser scanning biological microscope (FV 1000; Olympus, Japan).

Quantitative real-time PCR assay. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined at 260/280 nm. DNA strands were synthesized from 1 μg total RNA using oligo-dT primers and AMV reverse transcriptase (Takara, Japan). The expression of PTEN mRNA was determined by real-time reverse transcription-PCR using the ABI-PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq (Takara). The primers for real-time reverse transcription-PCR measurement were PTEN primer (NM 002424.2) and 18SrRNA primer (X03205.1) (SuperArray Bioscience Co., Frederick, MD, USA). To control variations in mRNA concentrations, the results were normalized to a housekeeping gene, 18sRNA. Relative quantitation was performed using a comparative ΔΔCt method according to the manufacturer's instructions.

Statistical analysis. Statistical data analysis was performed using ANOVA and Duncan's test. Differences with a p-value <0.05 were considered statistically significant.

Results

PI3K modulates PTEN protein levels in human breast cancer cells. To examine the effect of PI3K on PTEN expression, ER_α-positive MCF-7 and ER_α-negative MDA-MB 231 cells were incubated with 1 and 5 μM of wortmannin, a PI3K inhibitor, for 24 h, and the levels of PTEN protein were determined by Western blotting. As shown in Fig. 1, treatment of MCF-7 cells with the inhibitors resulted in a dose-dependent increase in the levels of PTEN protein; PTEN levels were increased by approximately 130% compared to the control cells. Supporting these results, the levels of p-Akt were decreased (Fig. 1A). However, PTEN levels in MDA-MB 231 cells were not affected by treatment with wortmannin (Fig. 1B).

ER_α down-regulates PTEN protein levels in human breast cancer cells. We and others have shown that ER_α activates PI3K in breast cancer cells (15,19). The above results indicate that PI3K modulates PTEN expression. Therefore, PTEN expression profiles and p-Akt levels in ER_α-positive MCF-7 cells and ER_α-negative MDA-MB 231 cells were determined by Western blotting. PTEN protein levels in ER_α-positive MCF-7 cells were significantly lower compared to those in ER_α-negative MDA-MB 231 cells, whereas p-Akt levels in MCF-7 cells were higher than those in MDA-MB 231 cells (Fig. 2). To verify these observations, PIP_3 levels were also measured using immunocytochemistry. As shown in Fig. 3, PIP_3 levels in ER_α-positive MCF-7 cells were approximately 20-fold higher than those in ER_α-negative MDA-MB 231 cells.

ER_α destabilizes PTEN protein by dephosphorylation in human breast cancer cells. Phosphorylation of PTEN by CK2

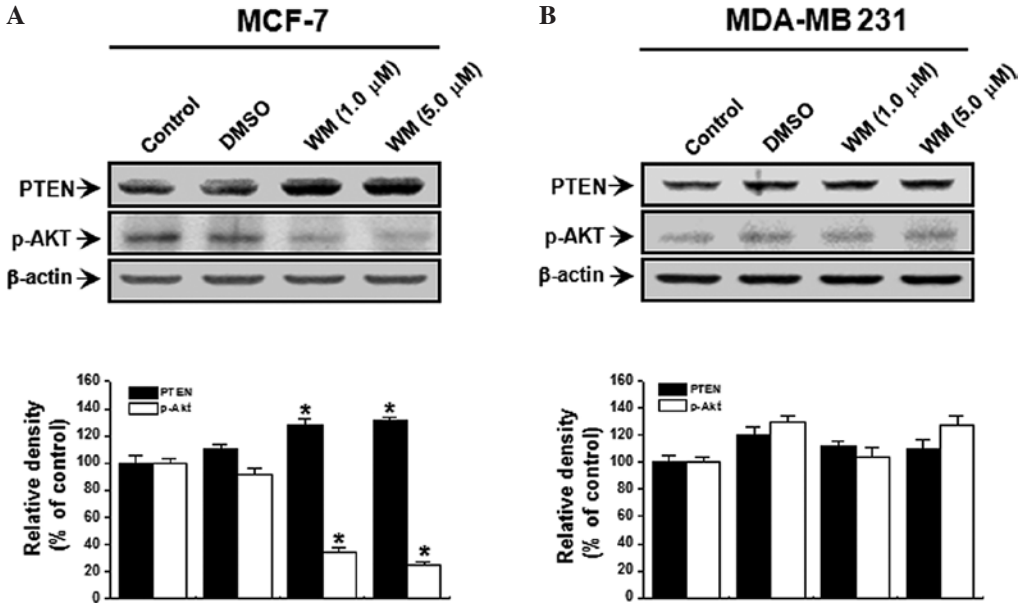

Figure 1. Effect of PI3-Kinase on PTEN expression in breast cancer cells. (A) MCF-7 and (B) MDA-MB 231 human breast cancer cells ($2x10^5$) were treated with 1 and 5 μM wortmannin for 24 h. Upper panel, cell lysates analyzed by Western blotting for PTEN and p-Akt. β-actin was used as a loading control. Lower panel, values expressed as a percentage of the control. Error bars, SE; n=4 in each group. *P<0.05 vs. the control.

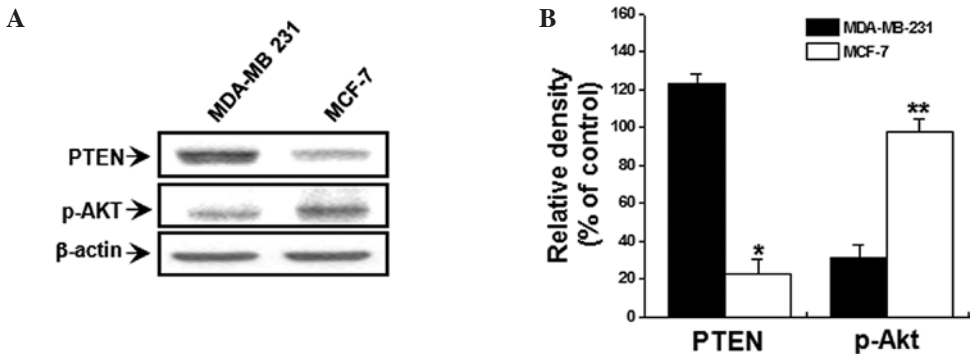

Figure 2. Effect of ER_α on PTEN and p-Akt expression in breast cancer cells. MCF-7 and MDA-MB 231 human breast cancer cells ($2x10^5$) were lysed for Western blotting. (A) Western blot analysis for PTEN and p-Akt. β-actin was used as a loading control. (B) Values expressed as a percentage of β-actin. Error bars, SE; n=4 in each group. *P<0.05, **P<0.01 vs. MDA-MB 231 cells.

has been shown to modulate the stability of PTEN protein (22). To elucidate the mechanism of PTEN down-regulation in ER_α-positive MCF-7 cells, the levels of PTEN mRNA, p-PTEN and CK2 were determined. There were no differences in the expression of PTEN mRNA between ER_α-positive MCF-7 cells and ER_α-negative MDA-MB 231 cells (Fig. 4A). However, the levels of p-PTEN (Ser380/Thr382/383) in MCF-7 cells were significantly lower than those in MDA-MB 231 cells (Fig. 4B). In addition, the levels of CK2 in MCF-7 cells were significantly lower than those in MDA-MB 231 cells (Fig. 4C). These results suggest that the down-regulation of PTEN protein in ER_α-positive breast cancer cells is caused by low levels of CK2 expression, leading to a decrease in the phosphorylation of PTEN.

Discussion

ER_α mediates most of the biological effects of estrogen in mammary epithelial cells and stimulates growth signals involving PI3K/Akt in breast cancer cells (15). To further elucidate the roles of the ERs in breast cancer traits, the regulation of the PI3K signaling system involving PTEN, was evaluated in MCF-7 cells expressing ER_α and ER_β and in MDA-MB-231 cells expressing ER_β only. The results revealed that the levels of PTEN protein in ER_α-positive MCF-7 cells were significantly lower than those in ER_α-negative MDA-MB 231 cells. Moreover, p-Akt levels in MCF-7 cells were higher than those in MDA-MB 231 cells. In addition, treatment with a PI3K inhibitor, wortmannin, increased the levels of PTEN protein in ER_α-positive MCF-7 cells, but not in ER_α-negative MDA-MB 231 cells. These results suggest that PI3K signaling reduces the levels of PTEN protein in breast cancer cells expressing ER_α, and that the expression of ER_α may differentiate the traits of breast cancer cells.

Deletion or reduced expression of PTEN is associated with poor prognosis, resistance to conventional therapeutic agents and relapse following initial treatment of breast cancer (23-25). PTEN dephosphorylates the D3 position of PIP_3, the

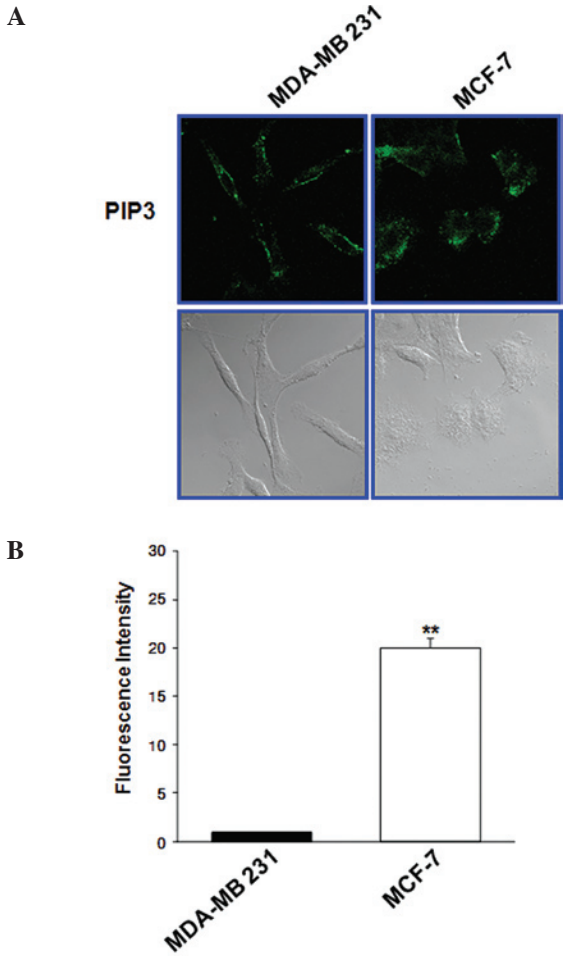

Figure 3. Effect of ER_α on PIP_3 in breast cancer cells by immunocytochemistry. To determine PIP_3 levels, human breast cancer cells ($2x10^5$) were fixed with 4% paraformaldehyde, incubated with anti-PIP_3 antibody, stained with TRITC-labeled anti-mouse IgM antibody and visualized with confocal fluorescence microscopy. Images were obtained by overlaying fluorescent images and differential interference contrast images. (B) Values are expressed as the mean ± SE fold increases of cellular PIP_3 (n=4). $^{**}P<0.01$ vs. MDA-MB 231 cells.

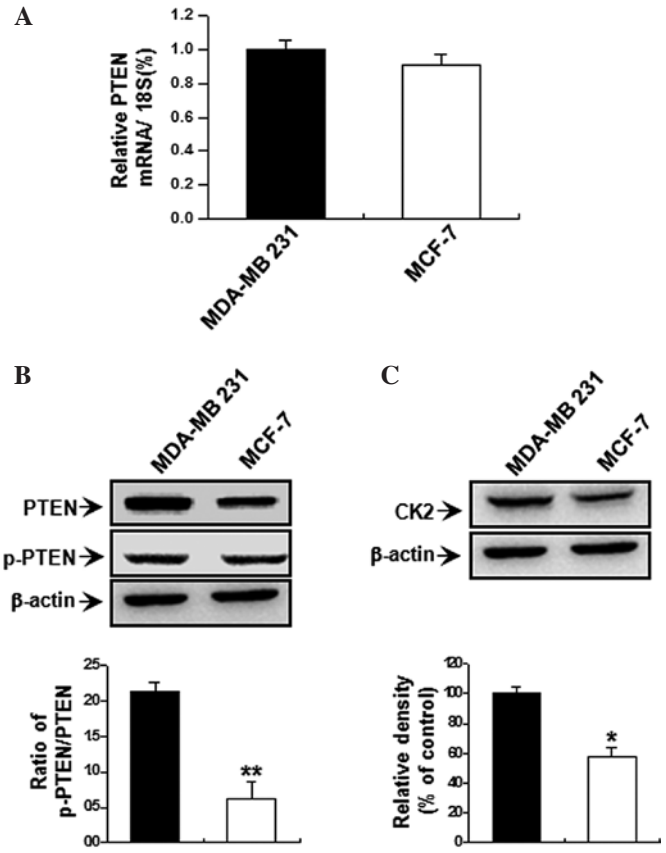

Figure 4. Effect of ER_α on PTEN mRNA, CK2 and p-PTEN expression in breast cancer cells. Human breast cancer cells ($2x10^5$) were cultured for realtime PCR (PTEN mRNA) and Western blotting (p-PTEN and CK2). (A) PTEN mRNA expression analyzed by real-time PCR. The cell lysates were analyzed by Western blotting for p-PTEN (B) and CK2 (C). Real-time PCR for PTEN was performed as described in Materials and methods. Error bars, SE; n=4 in each group. $^{*}P<0.05$, $^{**}P<0.01$ vs. MDA-MB 231 cells.

second messenger produced by the activation of PI3K (3). Thus, PIP_3 levels are controlled directly by the balance of activity between PI3K and PTEN. The results of this study showed that the levels of PTEN protein in ER_α-positive MCF-7 cells were lower than those in MDA-MB-231 cells expressing ER_β only. Supporting these observations, the levels of p-Akt and PIP_3 in ER_α-positive MCF-7 cells were higher than those in MDA-MB-231 cells.

In this study, we found that a decrease in PTEN levels in ER_α-positive MCF-7 cells was due to the degradation of the protein, but not its synthesis. Thus, the levels of PTEN mRNA were similar to each other in both ER_α-positive MCF-7 cells and ER_α-negative MDA-MB-231 cells. However, the levels of p-PTEN (Ser380/Thr382/383) in ER_α-positive MCF-7 cells were significantly lower than those in ER_α-negative MDA-MB 231 cells. Structurally, PTEN is composed of an N-terminal dual specificity phosphatase-like enzyme domain and a C-terminal regulatory domain, which binds to the phospholipid membrane (26). The C-terminal PTEN domain is rich in putative phosphorylation sites, and phosphorylation of the PTEN C-terminus has been reported to affect PTEN stability and function (27).

Protein kinase CK2 is a messenger-independent serine/threonine-kinase that phosphorylates a wide variety of substrates involved in essential cell processes, including cell cycle progression and growth (28,29). CK2 is the major kinase involved in the phosphorylation of PTEN, and CK2 phosphorylation sites in PTEN are located within a C-terminal cluster of Ser/Thr residues (30). In addition, inhibition of PTEN phosphorylation with a CK2 inhibitor reduces PTEN protein content, suggesting that proper phosphorylation of PTEN by CK2 is important for the stability of PTEN protein in proteasome-mediated degradation (22). In the present study, we found that the levels of CK2 in MCF-7 cells were significantly lower than those in MDA-MB 231 cells. Taken together, these observations suggest that the down-regulation of PTEN protein in ER_α-positive breast cancer cells is caused by low levels of CK2 expression, leading to accelerated PTEN degradation.

In conclusion, our results indicate that PTEN is down-regulated in ER_α-positive, but not in ER_α-negative, breast cancer cells, and that the down-regulation of PTEN is caused by the reduced phosphorylation of PTEN. The results also suggest that PTEN expression is regulated through PI3K signaling involving ER_α in breast cancer cells. Since deletion or reduced expression of PTEN is known to be associated with poor outcome in breast cancer, our findings may aid in the design

of novel therapeutic strategies as well as in the diagnosis of ER_{α}-positive breast cancer.

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