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Restoring E-cadherin-mediated cell–cell adhesion increases PTEN protein level and stability in human breast carcinoma cells

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Abstract

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a well-characterized tumor suppressor that negatively regulates cell growth and survival. Despite the critical role of PTEN in cell signaling, the mechanisms of its regulation are still under investigation. We reported here that PTEN expression could be controlled by overexpression or knock-down of E-cadherin in several mammary carcinoma cell lines. Furthermore, we showed that the accumulation of PTEN protein in E-cadherin overexpressing cells was due to increased PTEN protein stability rather than the regulation of its transcription. The proteasome-dependent PTEN degradation pathway was impaired after restoring E-cadherin expression. Moreover, maintenance of E-cadherin mediated cell–cell adhesion was necessary for its regulating PTEN. Altogether, our results suggested that E-cadherin mediated cell–cell adhesion was essential for preventing the proteasome degradation of PTEN, which might explain how breast carcinoma cells which lost cell–cell contact proliferate rapidly and are prone to metastasis.

Keywords: E-cadherin; PTEN; Stability; Degradation; Cell–cell adhesion

The tumor suppressor PTEN is a dual-specificity phosphatase, dephosphorylating both lipid and protein substrates [1] which plays critical roles not only in suppressing tumor cells proliferation but also in embryonic development, cell migration, cell signaling, and apoptosis [2]. A loss of heterozygosity or mutation of the PTEN gene is frequently observed in a variety of human malignancies, including cancers of the breast, prostate, brain, melanoma, and glioma [3]. Despite of it, some malignancies including breast cancers exhibit severely reduced PTEN protein level without detectable mutations and altered mRNA expression [4,5]. The modes of regulation of PTEN protein levels at both the synthesis and degradative stages are not completely understood.

The E-cadherin gene, which encodes one of the most important cell adhesion molecules in epithelial cells, is also considered to be a tumor suppressor gene since it can inhibit cells invasion and growth in many types of tumors [6–8]. Recent studies found that the two crucial tumor suppressors could work together. Kotelevets et al. present evidence that PTEN, the p85 submit of PI3K, β-catenin, and E-cadherin form a multi-molecular signalosome, which is critical for stabilization of adherent junctions and suppression of invasiveness [9]. In addition, Vogelmann et al. find that PTEN mediates the TGF-β1 induceddown-regulation of E-cadherin-based cell–cell adhesion by phosphorylation of β-catenin [10]. For all that, previous studies mostly focused on the stabilization of adherent junctions, little work has been done on whether PTEN is regulated by the signalosome complex. In this study, we try to find whether PTEN could be regulated by restoration of E-cadherin in breast carcinoma cells.

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Materials and methods

Cell lines and cultures. Human breast carcinoma cell lines MDA-MB-231, MDA-MB-435, and MCF-7 were obtained from Cancer Hospital, Fudan University (Shanghai, China). These cells were cultured in DMEM complemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2.

Stable transfection. The mammalian expression vector pcDNA3 containing human E-cadherin cDNA was a gift from Dr. Cara J. Gottardi (Memorial Sloan-Kettering Cancer Center, New York, USA). Stable transfections of pcDNA3-E-cadherin plasmid or pcDNA3 empty vector with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) reagent were performed as manufacturer's guidelines. After transfection for 24 h, cells were 1:20 diluted and selected at 800 μg/ml of G418. Stable transfectants were maintained in the following experiments at 200 μg/ml.

E-cadherin SiRNA plasmid construct. A reported DNA sequence of the type AA(N19) (GCAGAATTGCTCACATTTC) was selected to design SiRNA targeting E-cadherin. Synthetic sense and antisense oligonucleotides (5′-GATCCGCAGAATTGCTCACATTTCTTCAAGAGAGAAATGTGAGCAATTCTGCTTTTTTGGAAA-3′ and 5′- AGCTTTTCCAAAAAAGCAGAATTGCTCACATTTCTCTCTTGAAGAAATGTGAGCAATTCTGCG-3′ Sangon, Shanghai, China) constitute the template for generating RNA. Each oligonucleotide was annealed and then ligated into pSilencer 2.0 vector. The constructed plasmid was named E-cadherin SiRNA. The negative control vector SiRNA (control) was obtained from Ambion, which is a plasmid with a similar structure but encoding a nonsense minigene with no homology to any known sequences in the human genome.

Western blot analysis. Cells (5×10^6) were lysed in 1× SDS lysis buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, and 10% glycerol) with proteasome inhibitors 1 mM PMSF, 1 mM Na_3VO_4, and 10 mM NaF. Sample treatment and Western blot analysis were described previously [11]. The primary antibodies used in the present study were as followed: monoclonal anti-PTEN antibody, PTEN A2B1, polyclonal goat anti-Akt1/2 antibody, and monoclonal anti-β-catenin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phosphorylation Ser473 Akt antibody and antibody against E-cadherin were purchased from Cell Signaling Technologies (Beverly, MA, USA) and BD Transduction Laboratories (San Jose, CA, USA), respectively. Primary antibody against GAPDH and HRP conjugated of anti-mouse, anti-rabbit, and anti-goat secondary antibodies were acquired from Kang-Cheng Biotech (Shanghai, China).

RT-PCR assay of PTEN mRNA expression. mRNAs were extracted from 10^7 cells by using the mRNA Purification Kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, China) according to the manufacturer's guidelines. Then the concentration of mRNA was measured and reverse transcription was performed on 2 μg of mRNA by using reverse transcriptase MMLV (Promega, USA) for first-strand cDNA synthesis with Oligo (dT) primer. One tenth of the cDNA products were used for PCR amplification with PTEN primers designed as reported [12] to amplify a region from 117 to 741 bp (5′-CAGAAAGACTTGAAGGCGTAT and 5′-AACGGCTGAGGGAACTC). PCR was carried out as follows: an initial denaturation of 3 min at 94 °C was followed by 22–28 cycles of 45 s at 94 °C, 45 s at 54 °C, and 1 min at 72 °C, followed by 10 min of final elongation at 72 °C. Control PCR amplifications were performed with β-actin-specific primers which were purchased from Waston Biotech (Shanghai, China).

Cell aggregation assay. Cells were incubated in HCMF buffer (160 mM NaCl, 0.6 mM Na_2HPO_4, 0.1% w/v Glucose, and 10 mM Hepes, pH 7.4) containing 0.01% trypsin and 2 mM $CaCl_2$, for 30 min at 37 °C. They were then washed and resuspended in HCMF with 2 mM $CaCl_2$ and the concentration was adjusted to 10^5 cells/ml. After incubation for 30 min at 37 °C with constant rotation at 70 rpm, the cells were fixed by adding an equal volume of 5% formaldehyde in PBS. The total numbers of particles (cells or cell aggregates) were counted with a hemacytometer, and the aggregation index was calculated by $(N_0 - N_t)/N_0$, where N_0 is the number of particles before the experiment started, and N_t is the number at the final time. The assay was repeated three times, and the means and SDs were determined [13].

Cadherin blocking experiments. Cadherin blocking experiment was performed as described previously [14]. In brief, neutralizing antibody against E-cadherin (DECMA-1 Antibody from Sigma) was mixed at a concentration of 2 or 10 μg/ml with confluent cells in DMEM media containing 10% fetal bovine serum. After 24 h, cells were lysed and PTEN protein levels were assessed by Western blot.

Results

E-cadherin up-regulated PTEN protein level in mammary carcinoma cells

To determine whether the PTEN was regulated by E-cadherin, firstly, MDA-MB-435 and MDA-MB-231 cells (E-cadherin null) were transiently transfected with pcDNA3-E-cadherin expression plasmid or pcDNA3 empty vector for 48 h, and then the endogenous PTEN protein level was examined. As shown in Fig. 1A, positive expression of E-cadherin significantly increased PTEN protein level in both MDA-MB-435 and MDA-MB-231 cells. Next, we examined the PTEN expression in the G418-resistance MDA-MB-435 transfectants with different E-cadherin expression. As shown in Fig. 1B, higher expression of E-cadherin correlated to more elevated PTEN protein level in a dose-dependent manner. This result indicated that PTEN was up-regulated by overexpression of E-cadherin in MDA-MB-435 cells. For next investigation, one of E-cadherin expressing stable clones named Ecad6-435 and an empty vector pcDNA3 stable clone named Neo1-435 were selected. Then, the Akt phosphorylation level on Ser473 was examined to confirm PTEN activity. Consistent with previous result, we found that the relative Akt phosphorylation level on Ser473 was reduced in Ecad6-435 cells compared to Neo1-435 cells (Fig. 1C).

On the other hand, we tested this hypothesis by down-regulation of E-cadherin using siRNA. Another mammary carcinoma cell line MCF-7, which highly expresses E-cadherin, was used. Transfection of E-cadherin specific siRNA resulted in a marked down-regulation of total E-cadherin expression when compared with transfection with non-targeting control siRNA. And we observed that the down-regulation of E-cadherin resulted in a decrease of PTEN protein level and an increase of Akt phosphorylation level on Ser473 (Fig. 1D).

Maintenance of cell–cell adhesion mediated by E-cadherin is necessary for PTEN regulation

To confirm the role of cell–cell adhesion, we next investigated whether the functional-blocking cell–cell adhesion but not E-cadherin itself would affect PTEN. The calcium chelator EGTA and a neutralizing antibody against E-cadherin (DECMA-1) were utilized to specifically block E-cadherin mediated adherens junction formation in Ecad6-435 and MCF-7 cells [16]. IgG antibody was used as a control. In Ecad6-435 cells, we observed that either 2 mM EGTA,

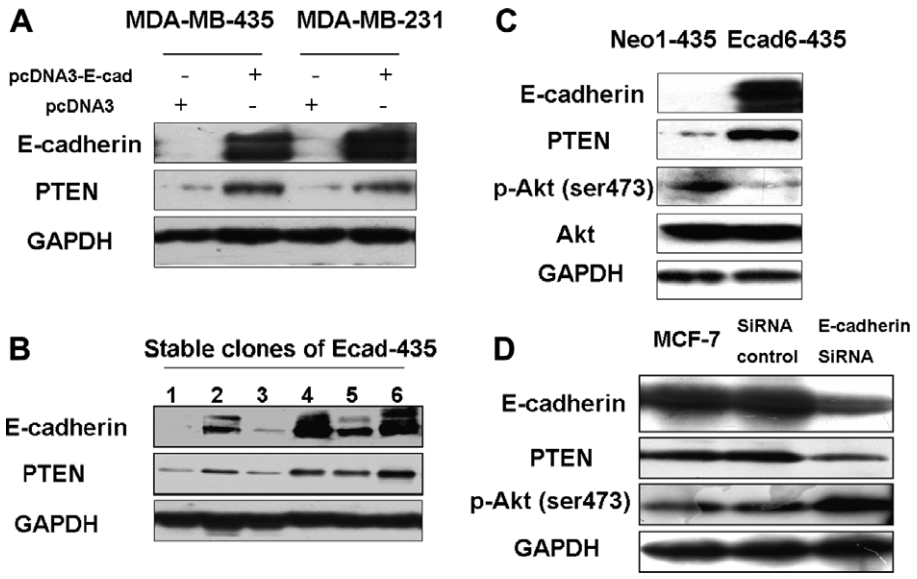

Fig. 1. E-cadherin up-regulated PTEN protein level in mammary carcinoma cells. (A) MDA-MB-435 and MDA-MB-231 cells were transfected with pcDNA3-E-cadherin expression plasmid or pcDNA3 vector only for 48 h, then all cells were lysed, and measured for E-cadherin and PTEN expression by Western blot. (B) MDA-MB-435 cells were stable transfected with E-cadherin as described in Materials and methods. After G418 selection, all the transfectants were tested for the expression of PTEN and E-cadherin by Western blot. (C) The endogenous PTEN and Ser-473 phosphorylated Akt were examined by Western blot in Ecad6-435 and Neo1-435 cells. (D) SiRNA interference of E-cadherin in MCF-7 resulted in decreased protein level of PTEN. MCF-7 cells were transfected with non-targeting control siRNA or siRNA specific for E-cadherin. After 48 h, total cell lysates were prepared and E-cadherin, PTEN, Ser-473 phosphorylated Akt, and GAPDH were detected by Western blot as indicated.

2 μg/ml DECMA-1, or 10 μg/ml DECMA-1 disrupted E-cadherin mediated cell–cell adhesion and decreased cell aggregation (Fig. 2A). Meanwhile, one could find that the disruption of cell–cell adhesion led to a decrease of PTEN protein level, which was consistent with the decrease of cell aggregation (Fig. 2B). The decrease of PTEN by DECMA-1 was in a dose-dependent manner. Moreover, we found the same effect in MCF-7 cells as shown in Fig. 2C. This observation suggested that the maintenance of cell–cell adhesion was necessary for the regulation of PTEN.

Exogenous E-cadherin expression increased PTEN protein stability

The increase of PTEN protein level might be caused mainly by either increased PTEN mRNA level or decreased protein degradation. Firstly, we investigated the effect of E-cadherin on PTEN's mRNA levels using semi-quantitative RT-PCR. As shown in Fig. 3A, we observed that expression levels of PTEN mRNA remained unchanged in all 22–28 cycle PCR products, implying that E-cadherin mediated cell–cell adhesion is not likely to be responsible for regulating PTEN at transcriptional level.

Generally, elevated protein expression with no changes in mRNA levels suggests a decreased degradation process. We next investigated whether the half-life of PTEN was altered in this model. Since protein synthesis can be inhibited by cycloheximide treatment, it is possible to survey the degradation, and therefore the half-life, of any protein of interest. In Neo1-435 cells, PTEN was unstable and had an estimated half-life of about 2 h (Fig. 3B). However, in Ecad6-435 cells, the basal PTEN amount was increased and more stable than in the Neo1-435 cells (Fig. 3C). In fact, the E-cadherin transfected cells prolonged PTEN half-life to about 6 h (Fig. 3D). This result indicated that E-cadherin mediated cell–cell adhesion regulated PTEN protein stability.

The proteasome-dependent degradation of PTEN was delayed by E-cadherin mediated cell–cell adhesion

It has been reported that PTEN is polyubiquitinated in 293T cells [15], and hence we examined if the observed PTEN stabilization by E-cadherin occured through a delay in the 26S proteasome mediated protein degradation. In order to confirm this possibility, we used MG132 to inhibit the 26S proteasome. In Neo1-435 cells, treatment with MG132 led to a time-dependent increase in PTEN protein abundance and to the stabilization of the protein throughout the experiment. PTEN protein levels in Neo1-435 cells with 12 h MG132 treatment were almost equal to Ecad6-435 cells (Fig. 4A, compare lanes 5 and 6). In contrast, MG132 did not accumulate PTEN protein in Ecad6-435 cells. (Fig. 4B) This result indicated that PTEN might undergo a rapid proteasome-dependent degradation in Neo1-435 cells; whereas in Ecad6-435 cells, this degradation process was delayed due to the existence of E-cadherin mediated cell–cell adhesion.

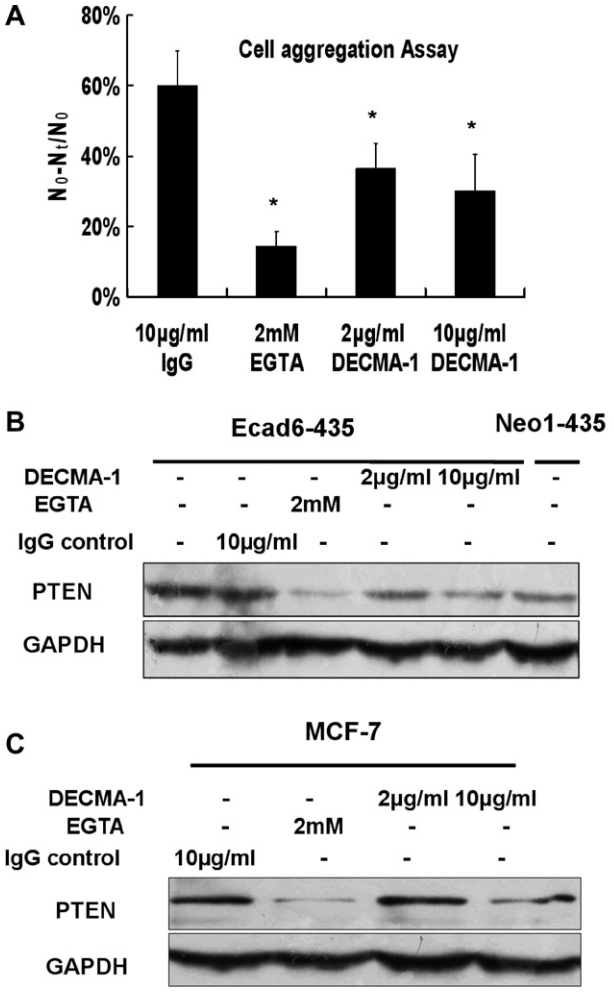

Fig. 2. Disruption of cell–cell adhesion decreased PTEN protein level in Ecad6-435 and MCF-7 cells. (A) Ecad6-435 cells were seeded into 6-well plate, treated with 2 mM EGTA, 2 μg/ml DECMA-1, and 10 μg/ml DECMA-1, and 10 μg/ml IgG control, then cell aggregation assay were performed as described in Material and methods. Here shown are representative data from three independent experiments. Bars indicate means of three results ± SD. Asterisks show statistically significant difference ($p < 0.01$) from IgG control treatment. (B) Western blot analysis of PTEN in the Ecad6-435 cells with the same treatment as above (lanes 2–5). Lane 1: Ecad6-435 without treatment; lane 6: Neo1-435 without treatment. (C) MCF-7 cells were seeded into 6-well plate, treated with 2 mM EGTA, 2 μg/ml DECMA-1, and 10 μg/ml DECMA-1, and 10 μg/ml IgG control, then endogenous PTEN expression was examined in these cells by Western blot.

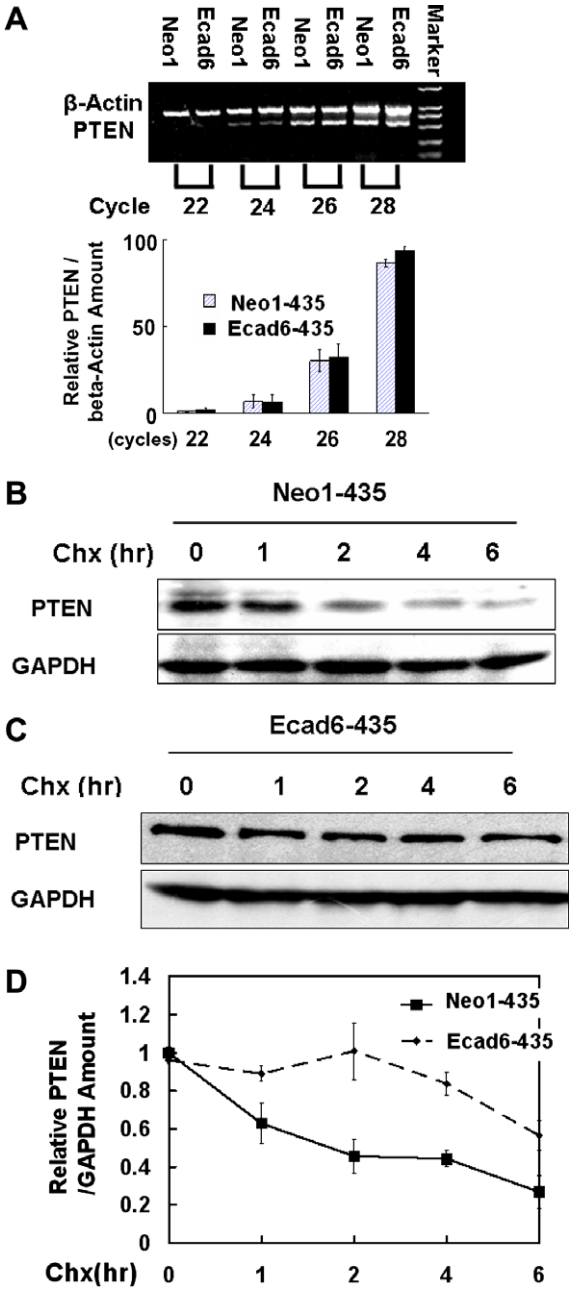

Fig. 3. Exogenous E-cadherin expression increased PTEN protein stability (A) RT-PCR analysis of PTEN mRNA level in Neo1-435 and Ecad6-435 cells. (B) Neo1-435 cells were treated with Chx (50 μg/ml) for 0, 1, 2, 4, or 6 h. Total protein of treated cells were prepared and analyzed for PTEN expression. (C) Ecad6-435 cells were treated with Chx (50 μg/ml) for 0, 1, 2, 4, or 6 h. Total protein of treated cells were prepared and analyzed for PTEN expression. (D) Relative densitometry of PTEN protein were calculated and showed in the diagram. The half-life of PTEN was about 2 h in Neo1-435 cells and more than 6 h in Ecad6-435 cells.

Discussion

Both E-cadherin and PTEN are recognized individually as important tumor suppressors involved in breast carcinomas [16]. Although PTEN has been demonstrated to play roles in stabilization of E-cadherin mediated adherent junctions [9,17], the effects of cell–cell adhesion on PTEN protein expression is under investigation. In this study, we demonstrated E-cadherin mediated cell–cell adhesion could control PTEN expression by stabilizing it. This finding is consistent with previous work by Subauste et al. [14], in which PTEN protein levels for the first time were proven to be controlled by vinculin, via maintaining the interaction of MAGI-2 with β-catenin. But this work focused more on the effect of cell adhesion molecule E-cadherin but not vinculin. By artificially restoring of E-caherin mediated cell–cell adhesion in breast carcinoma cells, E-cadherin was demonstrated to act as an outside-in signaling receptor, which transduced signals from cell–cell adhesion

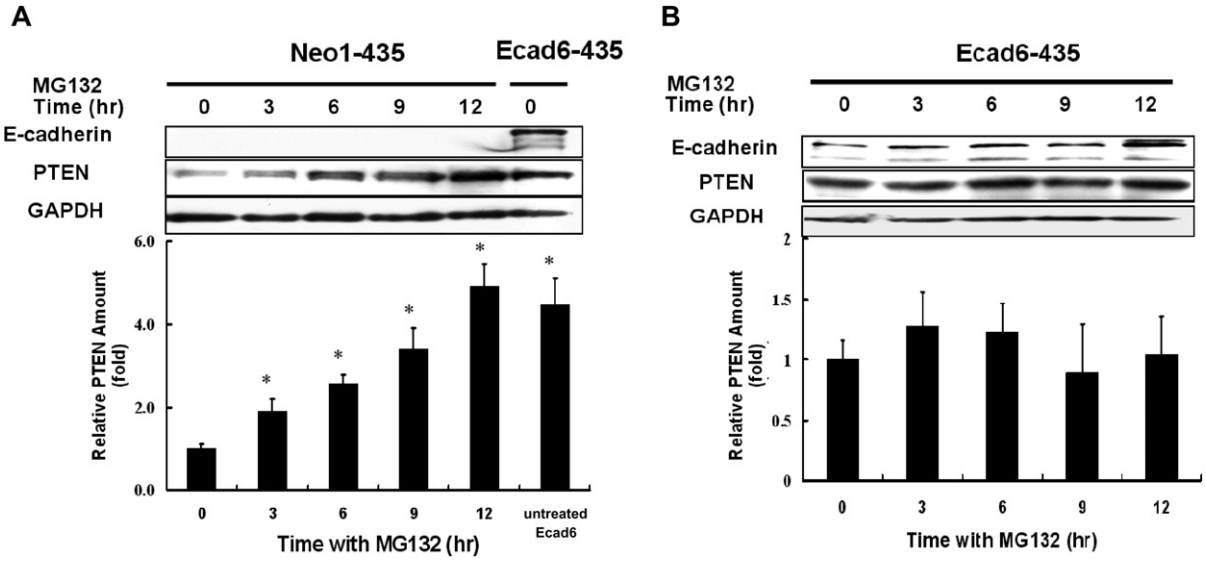

Fig. 4. The proteasome-dependent degradation of PTEN is delayed by E-cadherin Neo1-435 (A) or Ecad6-435 (B) cells were exposed to 10 mM MG132 for 0, 3, 6, 9, or 12 h, harvested, and then subjected to Western blot analysis for E-cadherin and PTEN. GAPDH was tested as the loading control. There is a time-dependent increase of PTEN protein level with MG132 treatment in Neo1-435 cells (A), but not in Ecad6-435 cells (B). A representative picture from one of three individual experiments is shown there. Densitometric analysis of each band was performed with image software and shown in the lower diagram. Bars indicate means of three results ± SD. Asterisks show statistically significant difference compared with no treatment ($p < 0.01$).

to PTEN. Moreover, the stability of PTEN was also investigated in this study. Not only did we show that the half-life of PTEN was prolonged, but also we found that the proteasome-dependent degradation pathway was delayed by E-cadherin. Previous studies have found some factors which can affect PTEN degradation, such as BMP2 treatment, the exposure to Zn^{2+} ions and protein–protein interaction to PTEN [18–21]. Here we extended the research of PTEN post-translational regulation and reported that PTEN stability was also increased due to E-cadherin mediated cell–cell adhesion.

In addition, this report also provides novel insights into the function of cell–cell adhesion process. As reported, disruption of normal cell–cell adhesion in transformed cells may contribute to tumor cells enhanced migration and proliferation, leading to invasion, and metastasis [22,23]. The regulation of PTEN by cell–cell adhesion suggests the possibility of the cell–cell adhesion executing these functions via regulating PTEN which is a multi-functional tumor suppressor. In fact, the phosphorylation of Ser473 Akt was demonstrated to be decreased in E-cadherin expressing cells (Fig. 1C). Therefore we propose that alteration in PTEN protein levels may be a more important cause of changes in biological behavior induced by cell–cell adhesion than previously thought. Hence, in following experiments, it would be interesting to study the biological significance of the up-regulation of PTEN stability.

In summary, here we suggest a critical role of E-cadherin mediated cell–cell adhesion in up-regulating PTEN protein and stability. These findings may be relevant in the understanding of tumor suppression function of E-cadherin mediated cell–cell adhesion in breast tumors, and also provide a novel insight on the study of PTEN regulation.

Acknowledgments

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