Oncogenic function of p34^{SEI-1} via NEDD4-1-mediated PTEN ubiquitination/degradation and activation of the PI3K/AKT pathway

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Abstract. A 34-KD protein encoded by the *SEI-1* gene (p34^{SEI-1}), is a relatively recently discovered oncoprotein that has multiple important biological functions. Our data show that p34^{SEI-1} enhances cancer cell survival and promotes tumorigenesis by downregulating the tumor suppressor PTEN, a negative regulator of the PI3K/AKT signaling pathway, and therefore activating the PI3K/AKT signaling pathway. In this process, p34^{SEI-1} positively affects NEDD4-1 gene expression both at the transcriptional and protein levels. Furthermore, the expression levels of p34^{SEI-1} and NEDD4-1 were found to be coordinated in tumor tissues obtained from patients with breast cancer. We also show that p34^{SEI-1} affects the subcellular localization of PTEN.

Introduction

The PI3K/AKT signaling pathway regulates various cellular functions including tumorigenesis by inhibiting apoptosis and activating proliferation of cancer cells (1-5). Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that phosphorylate phosphatidylinositol-4, 5-biphosphate (PIP_2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) (6). The resulting PIP_3 phosphorylates and activates protein kinase B (AKT, also known as PKB) serine/threonine-specific protein kinase, which plays a key role in multiple cellular processes. Importantly, the activated AKT regulates many events related with tumor malignancies such as metastasis, apoptosis, proliferation, invasion, migration and motility (7-12). Notably, this pathway is often found to be overactive in cancer cells. Many researchers have accordingly tried to develop cancer drugs to inhibit this crucial signaling pathway at some point. The PI3K/AKT pathway is known to be negatively regulated by a well-known tumor suppressor phosphatase and tensin homologue (PTEN), in which PTEN dephosphorylates PIP_3 to PIP_2 preventing activation of AKT (13-15). The PI3K/AKT pathway can be hyper-activated in PTEN defective cells (16-18) and PTEN is frequently found to be mutated or deleted in various human cancers (16-21). Therefore, suppressing the PTEN function is an alternative way for cancer cells to obtain oncogenic activity. Neural precursor cell expressed, developmentally downregulated 4-1 (NEDD4-1) is an effective target in this regard. Wang *et al* suggested that NEDD4-1 degrades PTEN protein by catalyzing PTEN poly-ubiquitination (22). In fact, an inverse relationship between PTEN and NEDD4-1 expression levels is often found in human urinary lung cancer (23). Considering these findings, elimination of NEDD4-1 function seems to be an effective means of inhibiting tumorigenesis in a PTEN-dependent manner.

In an effort to identify the physiological mechanism that triggers the induction of NEDD4-1-mediated PTEN downregulation, we found the involvement of p34^{SEI-1} in this process. p34^{SEI-1} is known to act as a transcriptional regulator, cell cycle regulator, senescence inhibitor and apoptosis inhibitor (22,24-29). Especially, it plays a critical role in tumor pathogenesis acting as an oncoprotein. We previously showed that the expression level of p34^{SEI-1} significantly increased in breast cancer patients relative to healthy subjects (26). Several different research groups have suggested that p34^{SEI-1} exerts oncogenic effects by deregulating several vital pathways. For

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Abbreviations: p34^{SEI-1}, 34-KD protein encoding SEI-1 (selected with Ink4a-1 as bait) gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten; NEDD4-1, neuronal precursor cell-expressed developmentally downregulated 4-1; PI3K, phosphoinositide-3 kinase

Key words: 34-KD protein encoding selected with Ink4a-1 as bait gene, phosphatase and tensin homolog deleted on chromosome ten, neuronal precursor cell-expressed developmentally downregulated 4-1, phosphoinositide-3 kinase/protein kinase B, tumorigenesis

example, $p34^{SEI-1}$ overexpression is associated with upregulation of E2F-mediated transcription, transformation of NIH3T3 fibroblasts, promotion of tumor growth in athymic nude mice, and chromosomal instability (22,24-29). Most importantly, our previous study showed the $p34^{SEI-1}$ protein maximizes oncogenic characteristics by providing an anti-apoptotic function to cancer cells and increasing the survival of tumor cells (26). According to our data, the $p34^{SEI-1}$ confers resistance to various apoptotic stimuli on human breast cancer cells by stabilizing XIAP (X-linked inhibitor of apoptosis protein) (26). In the process of elucidating the mechanism of $p34^{SEI-1}$ mediated tumorigenesis, we suspected that $p34^{SEI-1}$ might obtain its oncogenic potential in part through activation of the PI3K/AKT pathway. Therefore, we tested the involvement of $p34^{SEI-1}$ in the regulation of the PI3K/AKT signaling pathway via NEDD4-1 mediated PTEN degradation.

In this report, we suggest that $p34^{SEI-1}$ oncogenic protein promotes tumor progression by inducing NEDD4-1 mediated PTEN ubiquitination/degradation and activating the PI3K/AKT pathway.

Materials and methods

Cell lines and cell culture. MCF7 breast cancer and HEK293 human epithelial kidney cells were used for this study. Each cell line was cultured in DMEM medium (WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (Gibco-BRL). All of the cells were cultured at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2.

Western blot analysis. Cells were washed in an ice-cold PBS buffer and lased in RIPA lysis buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.025% NaN_3 and 1.0 mM protease inhibitor). The protein amount was quantified using a protein assay kit (Bio-Rad, Seoul, Korea). Each protein sample was subjected to SDS-PAGE and transferred to an Immobilon Transfer Membranes (Millipore, cat. no. IPVH00010, Billerica, MA, USA). The filter was blocked in 5% non-fat dry milk/0.1% Tween/TBS followed by incubation with each corresponding antibody. Immune-detection was done by using the Power Opti-ECL Western blotting Detection reagent (Bionote, Hwaseong, Korea). Antibodies used in this study were purchased as follows: $p34^{SEI-1}$ (Enzo Life Sciences, ALX-804-645, Farmingdale, NY, USA), pAKT (Ser473) (Cell Signaling, cat. no. 9271, Danvers, MA, USA), PTEN (Santa Cruz Biotechnology, sc-7974, Santa Cruz, CA, USA), NEDD4-1 (Santa Cruz Biotechnology, sc-25508), and γ-tubulin (Santa Cruz Biotechnology, sc-7396).

Overexpression and knockdown of $p34^{SEI-1}$ and NEDD4-1. For ectopic overexpression of $p34^{SEI-1}$, MCF7 or HEK293 cells were transfected for 12, 24 or 48 h with 6-8 μg of either C-terminal EGFP-tagged $p34^{SEI-1}$ expression vector ($p34^{SEI-1}$-EGFP) or a control vector (pEGFP) by using Lipofectamine 2000 (Invitrogen, Seoul, Korea). To knockdown endogenous $p34^{SEI-1}$ and NEDD4-1, MCF7 or HEK293 cells were transiently transfected with $p34^{SEI-1}$ or NEDD4-1 specific siRNA (20 pmol siRNA final concentration) with a control of scrambled siRNA. The following target sequences were used to generate $p34^{SEI-1}$ or NEDD4-1 siRNA; $p34^{SEI-1}$ siRNA (5-CCGAAUUGGACUAC CUCAUdTdT-3) and NEDD4-1 siRNA (5-UUCCAUGAAUC UAGAAGAACATT-3 (30). $p34^{SEI-1}$ siRNA was obtained from Santa Cruz Biotechnology (sc-62988) and NEDD4-1 oligonucleotides were chemically synthesized by ST Pharm Co. Ltd (Seoul, Korea).

Immunoprecipitation. To analyze the interaction between $p34^{SEI-1}$ and NEDD4-1, HEK293 cells were co-transfected with EGFP-tagged $p34^{SEI-1}$ ($p34^{SEI-1}$-EGFP) and HA-tagged NEDD4-1 (pHA-NEDD4-1) plasmids and cell lysates were immunoprecipitated with either anti-EGFP (Abcam, ab290, Cambridge, MA, USA) or anti-HA (Sigma, H9658, St. Louis, MO, USA) antibodies. IP was performed by lysing cells in IP buffer (50 mM of Tris-HCl pH 7.4, 150 mM of NaCl, 10 mM of NaF, 10 mM of Na_3VO_4, 1 mM of PMSF, 1% of NP-40) with protease inhibitors, followed by pre-clearing with protein A/G Sepharose (Santa Cruz Biotechnology, sc-2003). Pre-cleared lysates were incubated with each antibody for 16 h at 4˚C with continuous agitation, and then protein A/G Sepharose was added. After 4 h, the lysate-antibody-agarose A/G bead complex was collected by centrifugation at 10,000 x g for 5 min, the complex was then washed three times with IP buffer, and proteins were eluted from the beads by boiling them in SDS sample buffer and analyzed by using a western blot with the indicated a ntibodies. Proteins were probed with the corresponding antibodies.

In vivo PTEN-ubiquitination assay. HEK293 cells were transfected with $p34^{SEI-1}$-EGFP plasmid and/or NEDD4-1 siRNA in the presence of HA-Ub. Forty-eight hours after transfection, cells were treated with 10 μM proteasome inhibitor MG132 (A.G. Scientific Inc, M-1157, San Diego, CA, USA) for 16 h. The cells were lysed with RIPA buffer with protease inhibitors. The lysates were centrifuged to obtain cytosolic proteins. Ubiquitinated PTEN was immunoprecipitated by anti-PTEN antibody (Santa Cruz Biotechnology, sc-7974), followed by immunoblotting with anti-Ub antibody (Santa Cruz Biotechnology, sc-8017).

Immunohistochemistry analysis. Four-micrometer-thick sections were sliced onto Silane Coated Micro Slides (Muto Pure Chemicals Corp., Tokyo, Japan) and incubated at 60˚C for 2 h. The slides were then deparaffinised by application of xylene and incubation (5 min x 3) at room temperature. Sections were hydrated by applying graded alcohol and endogenous peroxidase activity was quenched by incubating the sections in methanol with 0.3% H_2O_2 for 30 min at room temperature. After washing the slides in PBS (5 min x 2), antigen retrieval was performed by heating the slides in citrate buffer (0.01 M, pH 6.0) using a microwave in a pressure cooker for 15 min. After heating, the samples were allowed to cool for 2 h at room temperature followed by washing with PBS (5 min x 2). An immunohistochemical analysis was performed using $p34^{SEI-1}$ (Biorbyt, Cambridge, UK) and NEDD4-1 (Proteintech, cat. no. 13690-1-AP, Chicago, IL, USA) rabbit polyclonal antibodies with 1:50 dilution each by PBS. The tissues were incubated with primary antibodies for 2 h at room temperature and washed three times with PBS followed by incubation using an Ultra Vision Quanto Detection System HRP DAB (Lab Vision Corp., Fremont, CA, USA) according to the

Table I. Oligonucleotide sequences and conditions for RT-PCR analysis.

Primer name	Primer sequence (5'→3')[a]	Amplicon	Conditions size (bp)[b]	Source
pRT-SEI-1	F: AGGACCTCAGCCACATTGAG R: GGTGCCCAAAGTTCATTGTC	142 bp	60˚C 27 cycles	This study
pRT-NEDD4-1	F: GGAGTTGCCAGAGAATGGTT R: TTGCCATGATAAACTGCCAT	151 bp	60˚C 27 cycles	This study
pRT-NF-κB	F: CCGCACCTCCACTCCATCC R: ACATCAGCACCCAAGGACACC	121 bp	62˚C 26 cycles	Sarma *et al* (37) Du and Galan (38)
pRT-ACTB	F: AGGTCGGAGTCAACGGATTTG R: GTGATGGCATGGACTGTGGT	377 bp	58˚C 21 cycles	This study

F, forward primer; R, reverse primer. [a]All sequences are shown in the 5'→3' direction. [b]Conditions are shown in the order of annealing temperature (˚C) and number of cycles.

manufacturer's instructions. The immunostained slides were examined by two independent observers and a consensus score was determined for each specimen. A positive reaction for both antibodies was scored into 4 grades, according to the intensity of the staining: 0, 1+, 2+ and 3+. The percentages of positive cells were also scored into 4 categories: 0, 0%; 1, 1-30%; 2, 31-60%; and 3, 61-100%. The final score, calculated as the product of the intensity score multiplied by the percentage score, was classified as follows: 0, negative; 1-3, weak; 4-6, moderate; and 7-9, strong. Samples with a final score less than 3 were grouped together as expression negative while those with a score greater than 4 were grouped together as expression positive.

Reverse transcription (RT)-PCR. The total RNA was extracted from HEK293 cells after transfection with either pEGFP or $p34^{SEI-1}$-EGFP using an RNeasy mini kit (Qiagen, Hilden, Germany). For reverse transcription, 1 μg RNA of each sample was subjected to cDNA synthesis using an oligo (dT) primer and the ImProm-II™ Reverse Transcription System (Promega, A3800, Madison, WI, USA) following the manufacturer's instructions. Each gene product was amplified using 10 ng cDNA, the corresponding pair of primers, and an AccuPower PCR PreMix system (Bioneer, Daejeon, Korea), in which the β-actin gene product was used as an internal control (Table I).

Reporter assay. Cells were co-transfected with $p34^{SEI-1}$-EGFP and pGL4-NF-κB plasmids using Lipofectamine 2000 (Invitrogen) transfection reagent following the manufacturer's protocol, in which the NF-κB genes were subcloned into the pGL4 basic luciferase reporter vector (pGL4.1; Promega). The following day, 14-18 h later, cells were lysed and luciferase assays were performed using Luciferase Assay System (Promega, cat. no. E1501) and the luciferase activity levels were measured after standardization against pGL4.1.

Results

Effect of $p34^{SEI-1}$ on the alteration of pAKT, PTEN, and NEDD4-1 protein levels. As part of an effort to identify how $p34^{SEI-1}$ promotes tumor progression in various cancer cells, we initially tested the effect of $p34^{SEI-1}$ on the PI3K/AKT signaling pathway because of its vital roles in tumorigenesis. After MCF7 and HEK293 cells were transfected with the EGFP control vector (pEGFP) and C-terminal EGFP-tagged $p34^{SEI-1}$ ($p34^{SEI-1}$-EGFP) plasmids, AKT phosphorylation on serine 473 residue was examined because phosphorylation on this residue is known to promote signal transduction related with tumor malignancies. Our data showed that $p34^{SEI-1}$ overexpression significantly increased AKT phosphorylation on this residue (Fig. 1A). In contrast, the AKT phosphorylation level was decreased when MCF7 cells were treated with $p34^{SEI-1}$-siRNA compared to scrambled siRNA-treated control cells (Fig. 1B). This strongly suggests that $p34^{SEI-1}$ has a positive effect on AKT phosphorylation and therefore activation of the PI3K/AKT signaling pathway. Next, we tested the alteration of PTEN and NEDD4-1 expression levels in response to ectopic expressed $p34^{SEI-1}$ because they are the main components affecting the PI3K/AKT signaling pathway. Interestingly, the western blot analysis revealed that $p34^{SEI-1}$ overexpression slightly decreased PTEN but increased NEDD4-1 at the protein levels (Fig. 1A). We then tested PTEN and NEDD4-1 protein levels in MCF7 cells transfected with $p34^{SEI-1}$-siRNA. As expected, NEDD4-1 expression significantly decreased relative to the scrambled siRNA-treated cells. However, no change in the PTEN protein level was detected, likely due to a weak or even no effect of NEDD4-1 on downregulation of PTEN (Fig. 1B). Taken together, our results implicate that $p34^{SEI-1}$ activates the PI3K/AKT signaling pathway at least in part by regulating NEDD4-1 and PTEN.

$p34^{SEI-1}$ induced ubiquitination and degradation of PTEN in a NEDD4-1 dependent way. Our results imply that $p34^{SEI-1}$ may downregulate PTEN in a NEDD4-1-dependent manner. However, it is possible that $p34^{SEI-1}$ may affect other proteins or pathways and indirectly cause PTEN downregulation. For example, $p34^{SEI-1}$ might activate different types of E3 ligases rather than NEDD4-1 and downregulate PTEN. In fact, two different research groups suggested apparently discrepant results. Wang *et al* suggested that NEDD4-1 directly binds to PTEN and induces its ubiquitination and degradation (31). However, Fouladkou *et al* showed a contradictory result that NEDD4-1 is dispensable for the regulation of PTEN stability

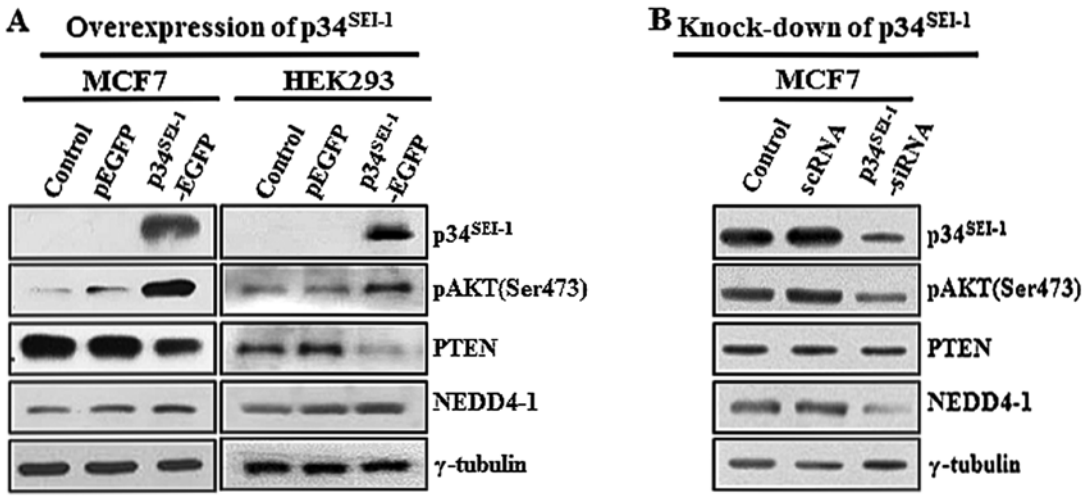

Figure 1. Effect of p34^{SEI-1} on the alteration of endogenous pAKT, PTEN and NEDD4-1 protein levels. (A) MCF7 and HEK293 cells were transfected with either an empty control vector (pEGFP) or C-terminal EGFP-tagged p34^{SEI-1} overexpressing vector (p34^{SEI-1}-EGFP) as indicated in Materials and methods. (B) MCF7 cells were transfected with either scrambled RNA (scRNA) or p34^{SEI-1} siRNA for 48 h. Endogenous pAKT, PTEN and NEDD4-1 levels was detected by western blot analysis using each corresponding antibody.

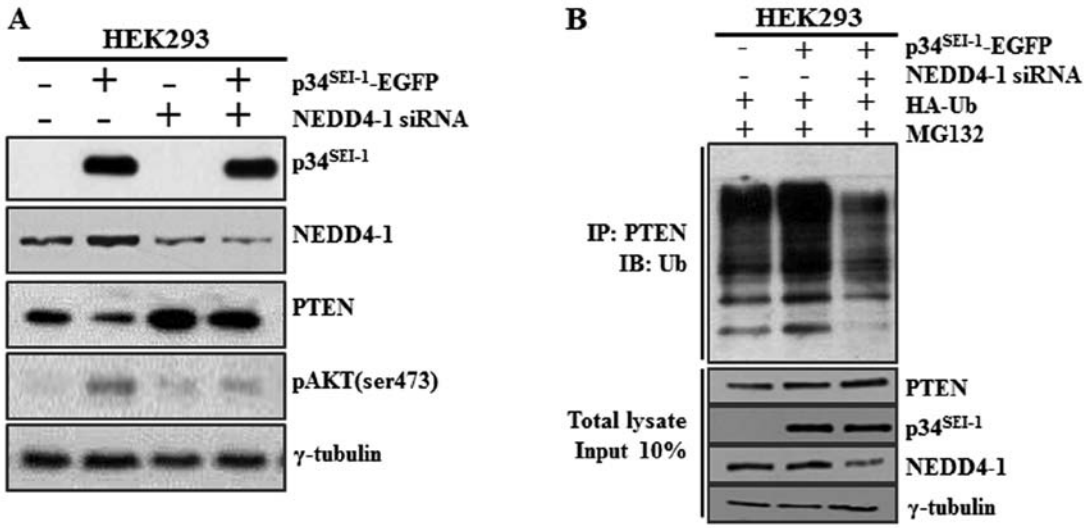

Figure 2. Positive effect of p34^{SEI-1} on the NEDD4-1 stability. (A) HEK293 cells were co-transfected with p34^{SEI-1}-EGFP and/or NEDD4-1 siRNA vector with controls. Expression levels of p34^{SEI-1}, NEDD4-1, PTEN and pAKT were examined by using a western blot analysis. (B) p34^{SEI-1} induced PTEN poly-ubiquitination. HEK293 cells were transfected with p34^{SEI-1}-EGFP and/or NEDD4-1 siRNA in the presence of HA-Ub and MG132. Cell lysates were immunoprecipitated (IP) with anti-PTEN antibody and immunoblotted (IB) with anti-ubiquitin. The total lysates before immunoprecipitation (Input) and the immunoprecipitate supernatants (Sup) were then subjected to immunoblot analysis with corresponding antibodies.

(23,32). To test whether PTEN downregulation by p34^{SEI-1} is NEDD4-1 dependent, the PTEN protein level was checked after HEK293 cells were transfected with p34^{SEI-1}-EGFP vector and/or NEDD4-1 siRNA (Fig. 2A). Our result showed that siRNA-induced NEDD4-1 silencing induced an increase of endogenous PTEN protein level and a concurrent decrease of AKT phosphorylation. Importantly, p34^{SEI-1} overexpression alone reduced the PTEN protein level but co-transfection of p34^{SEI-1}-EGFP and NEDD4-1 siRNA had no effect on the PTEN protein level (Fig. 2A). This finding suggests that p34^{SEI-1} decreases the PTEN protein level in a NEDD4-1 E3 ligase-dependent manner. The data strongly imply that p34^{SEI-1} is responsible for the PTEN ubiquitination and its subsequent degradation. Therefore, a ubiquitination assay was performed to determine whether downregulation of PTEN stimulated by p34^{SEI-1} is derived from ubiquitination-mediated protein degradation. HEK293 cells were transfected with p34^{SEI-1}-EGFP vector and/or NEDD4-1 siRNA in the presence of HA-Ub and MG132. The resulting cells were subjected to immunoprecipitation (IP) and immunoblot (IB) analyses as mentioned in Materials and methods. As shown in Fig. 2B, endogenous PTEN was significantly ubiquitinated in p34^{SEI-1} overexpressing cells compared to control cells. However, this did not occur in cells co-transfected with p34^{SEI-1}-EGFP and

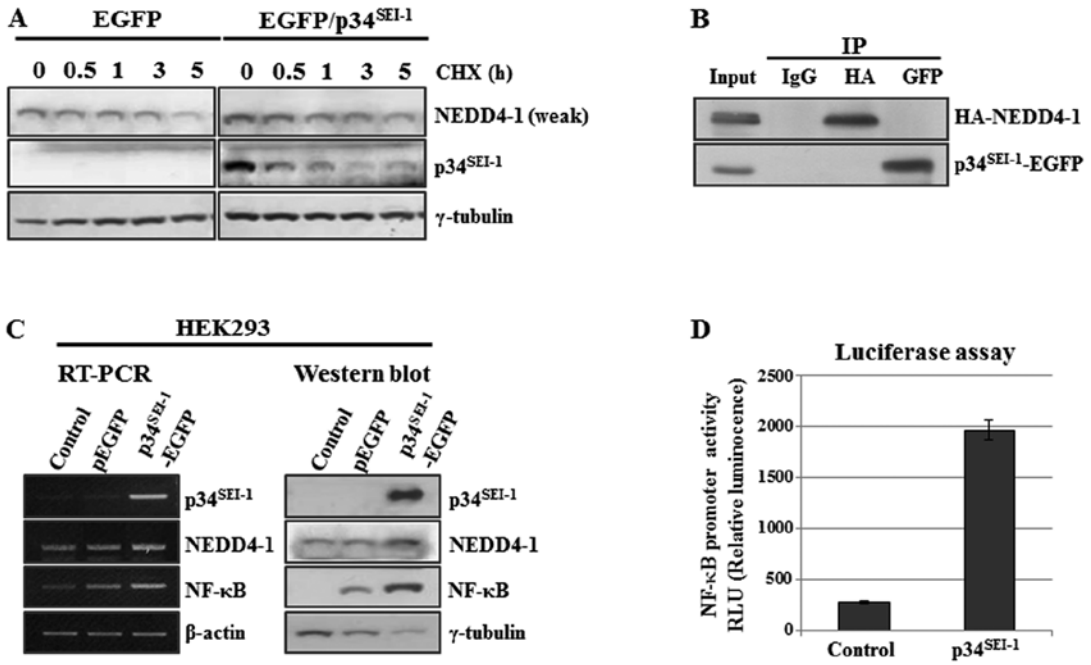

Figure 3. Positive effect of p34^{SEI-1} on NEDD4-1 expression. (A) Cycloheximide chase experiment. HEK293 cells were transfected with pEGFP and p34^{SEI-1}-EGFP plasmids for 24 h and treated with cycloheximide (20 μg/ml) for the indicated hours. The resultant samples were then subjected to a western blot analysis with corresponding antibodies. (B) Immunoprecipitation analysis to examine direct interaction between p34^{SEI-1} and NEDD4-1 proteins. HEK293 cells were transfected with p34^{SEI-1}-EGFP and NEDD4-1-HA plasmids and then the lysates were subjected to co-immunoprecipitation as mentioned in Materials and methods. (C) RT-PCR and western blot analyses of NF-κB and NEDD4-1 expression in response to overexpressed p34^{SEI-1}. HEK293 kidney cells were transfected with either pEGFP or p34^{SEI-1}-EGFP vectors. The RT-PCR and western blot analyses were performed as described in Materials and methods. β-actin and γ-tubulin were used as internal controls. (D) Luciferase assay to examine effect of overexpressed p34^{SEI-1} on transcriptional activation of NF-κB. HEK293 cells were co-transfected with pGL4-NF-κB and p34^{SEI-1}-EGFP. NF-κB promoter activity was measured as indicated in Materials and methods.

NEDD4-1 siRNA indicating that NEDD4-1 is required for p34^{SEI-1}-mediated PTEN ubiquitination. Taken together, these results strongly suggest that p34^{SEI-1} induces PTEN ubiquitination in a NEDD4-1 dependent manner and thereby targets PTEN for proteasomal degradation.

Positive effect of p34^{SEI-1} on NEDD4-1 expression both at the transcriptional and protein levels. Our data indicate that p34^{SEI-1} might exert positive effect on NEDD4-1 expression probably by controlling NEDD4-1 turnover. This hypothesis was examined by employing cycloheximide chase experiment. Briefly, HEK293 cells were transfected with pEGFP and p34^{SEI-1}-EGFP plasmids in the presence of cycloheximide (CHX, 20 μg/ml), an inhibitor of protein biosynthesis and NEDD4-1 protein levels were checked at the indicated times of treatment as shown in Fig. 3A. NEDD4-1 expression slowly decreased in p34^{SEI-1}-EGFP transfected cells compared to the control cells (Fig. 3A). This strongly suggests that p34^{SEI-1} stabilizes NEDD4-1 by preventing proteasome-dependent protein degradation. The next question was how p34^{SEI-1} can stabilize NEDD4-1 protein. One possibility is that p34^{SEI-1} may stabilize NEDD4-1 via direct interaction with it. To test this hypothesis, HEK293 cells were co-transfected with p34^{SEI-1} and NEDD4-1 overexpressing vectors (p34^{SEI-1}-EGFP and HA-NEDD4-1) and cell lysates were used for co-immunoprecipitation using anti-EGFP (i.e., p34^{SEI-1}-EGFP) or anti-HA (i.e., HA-NEDD4-1) antibodies. However, no direct interaction was detected between p34^{SEI-1} and NEDD4-1 protein (Fig. 3B). Moreover, we also found the same result from an examination of molecular interactions between endogenous p34^{SEI-1} and NEDD4-1 (data not shown).

In addition, the positive effect of p34^{SEI-1} on NEDD4-1 expression was also checked at the transcriptional level because p34^{SEI-1} is a well-known transcriptional co-activator. NEDD4-1 transcription was measured by using RT-PCR after HEK293 cells were transfected with either pEGFP or p34^{SEI-1}-EGFP expression vector. Our data showed that NEDD4-1 transcription was slightly increased by p34^{SEI-1} overexpression (Fig. 3C). However, our luciferase assay showed no direct effect of p34^{SEI-1} on NEDD4-1 transcription (data not shown). Considering that p34^{SEI-1} is a transcriptional co-activator, we could not exclude the other possibility that p34^{SEI-1} might indirectly activate NEDD4-1 transcription by working with other transcriptional activator. To test this hypothesis, we first searched transcription factor binding sites in the NEDD4-1 gene promoter by using the TRANSFAC database (http://www.genome.ad.jp). Many putative transcription factor binding sites were found in the NEDD4-1 gene promoter, including NF-κB, FOXO3b, FOXO3a, FOXD1, FOXO1a, FOXO4 and Nkx2-2. Among them, we initially focused on NF-κB because the PI3K/AKT signaling pathway is known to be strongly related with NF-κB activity (16,33,34). More importantly, Judge *et al* suggested that NEDD4 is one of the targets of NF-κB transcription factor (35). As shown in Fig. 3C, NF-κB expression was slightly or significantly increased by p34^{SEI-1} overexpression both at the

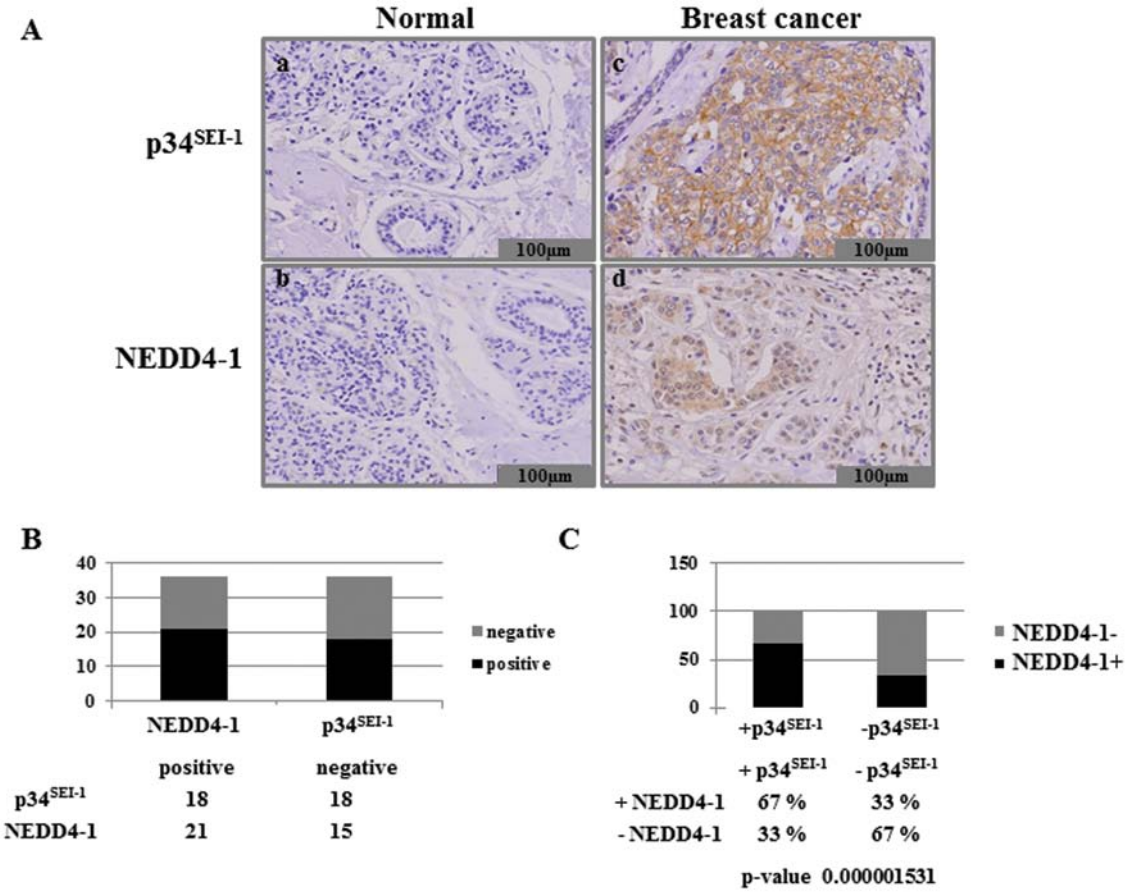

Figure 4. Expression of p34^{SEI-1} and NEDD4-1 in breast tissues containing adjacent histologically normal and invasive ductal carcinoma tissues. (A) Representative images from normal and cancerous tissues. Weak or strong immune-reactivity against p34^{SEI-1} and NEDD4-1 are detected in normal breast epithelial cells (panels a and b) or in breast cancer cells (panels c and d), respectively. (B) Approximately half (i.e., 18 of 36) of the tumor samples from patients with breast cancer reacted with the anti-p34^{SEI-1} antibody, whereas 21 of the tumor samples reacted with the anti-NEDD4-1 antibody. (C) The correlation in p34^{SEI-1} and NEDD4-1 expression levels of tissue samples. The statistical correlation between the expression of p34^{SEI-1} and NEDD4-1 in human breast cancer was determined using an chi-square test (i.e., P=0.000001531).

transcriptional and protein levels, respectively. In a further study, a luciferase assay was employed to examine the direct effect of p34^{SEI-1} on the NF-κB transcriptional activation. The luciferase assay was performed by transfecting pGL4-NF-κB and p34^{SEI-1}-EGFP into HEK293. As shown in Fig. 3D, over-expressed p34^{SEI-1} significantly activated NF-κB transcription. Our data suggest that p34^{SEI-1} seems to indirectly activate NEDD4-1 transcription through NF-κB transcription factor. We showed that p34^{SEI-1} overexpression significantly increased NF-κB transcription. Our further search of transcription factor binding sites in the NF-κB gene promoter revealed the putative binding site of p300, a known p34^{SEI-1} interacting protein. Therefore, it is possible that p34^{SEI-1} may enhance transcriptional activation of NF-κB via the interaction with p300. However, this needs to be tested further. Considering all these data, we conclude that p34^{SEI-1} exerts a positive effect on NEDD4-1 stability but not through direct interaction with NEDD4-1, whereby p34^{SEI-1} positively affects NEDD4-1 both at the transcriptional and protein levels.

Correlation between p34^{SEI-1} and NEDD4-1 expression levels in breast tissues containing adjacent histologically normal and invasive ductal carcinoma tissues. Considering the role of p34^{SEI-1} as a positive regulator of NEDD4-1, we hypothesized that high level of p34^{SEI-1} in cancer cells might contribute to similar high levels of NEDD4-1 protein in cancer cells. We therefore conducted an immunohistochemical analysis of both p34^{SEI-1} and NEDD4-1 expression in 36 tissue samples from patients with breast cancer. We assessed p34^{SEI-1} expression in formalin-fixed and paraffin-embedded samples obtained from surgical resections of 36 patients with breast cancer. Normal and cancer breast tissues showed immune-negative and strong immune-positive staining, respectively, for both p34^{SEI-1} and NEDD4-1 (Fig. 4A). This suggests that expression levels of p34^{SEI-1} and NEDD4-1 are very low in normal breast tissue but strong in breast cancer cells. The immunohistochemical analysis also revealed that significant overexpression of p34^{SEI-1} and NEDD4-1 was detected in 18 (50%) of the 36 and in 21 (58%) of the 36 breast cancer tissue samples, respectively (Fig. 4B). Interestingly, NEDD4-1 levels were found to be consistently higher in the areas of high p34^{SEI-1} protein level. Our examination of the co-expression of p34^{SEI-1} and NEDD4-1 in breast cancer cells showed that NEDD4-1 was expressed in 67% of the p34^{SEI-1} positive samples (Fig. 4C).

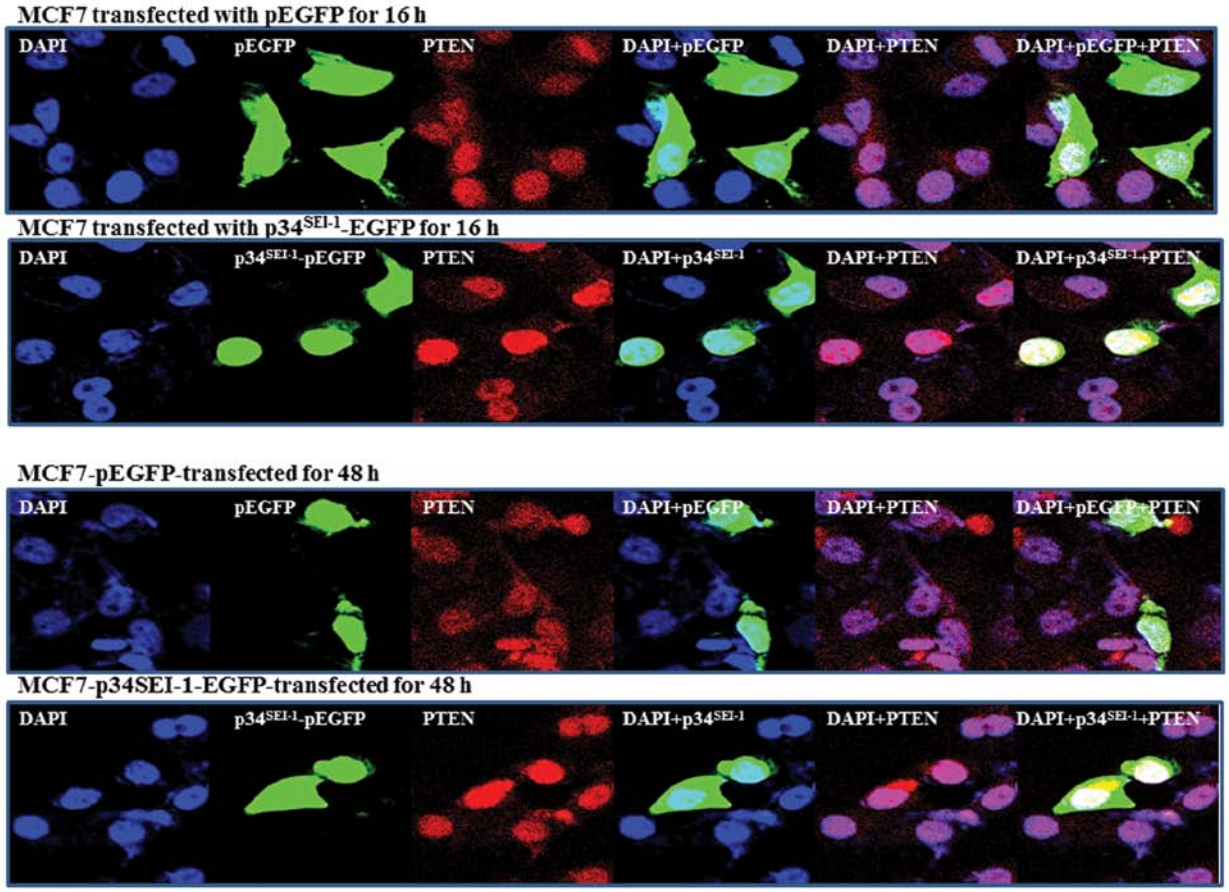

Figure 5. Change of subcellular localization of PTEN in response to overexpressed p34^{SEI-1}. MCF7 cells were transfected with pEGFP and p34^{SEI-1}-EGFP vectors for indicated times and PTEN sub-localization was detected with immunofluorescence staining as indicated in Materials and methods.

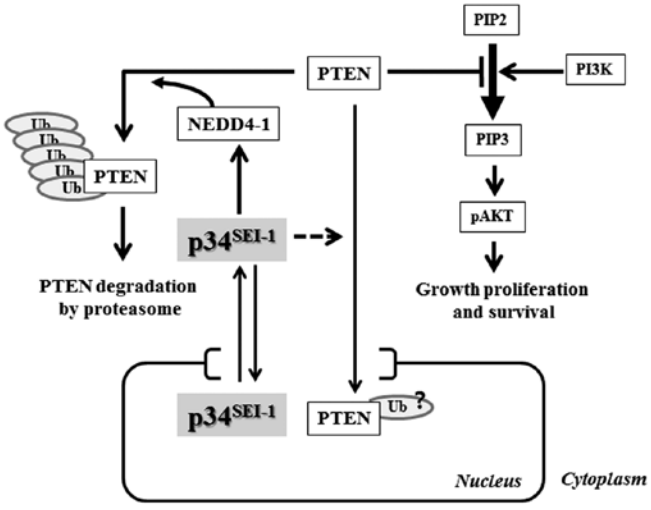

Figure 6. Summary model. Cellular function of p34^{SEI-1} onco-protein in NEDD4-1-mediated ubiquitination/degradation of PTEN tumor suppressor protein and activation of the PI3K/AKT pathway.

These data confirm that p34^{SEI-1} has a positive effect on the NEDD4-1 expression in cancer cells. This is consistent with the findings that both p34^{SEI-1} and NEDD4-1 have oncogenic potential and their expression levels are increased as tumor invasiveness progresses. Taken together, our data strongly suggest that p34^{SEI-1} and NEDD4-1 are significantly over-expressed in breast cancer tissues.

PTEN subcellular localization in response to p34^{SEI-1}. It has been suggested that PTEN is located in the nucleus as well as the cytosol. Trotman *et al* suggested that PTEN localization is affected by NEDD4-1, which can both mono- and poly-ubiquitinate (31). PTEN nuclear localization is mediated by its mono-ubiquitination. We thus far have shown that p34^{SEI-1} induces NEDD4-1-mediated PTEN poly-ubiquitination. Considering p34 has an oncogenic potential working with NEDD4-1 E3 ligase, it was expected that p34^{SEI-1} might affect PTEN nuclear localization. To test this hypothesis, we analyzed PTEN nuclear localization in response to p34^{SEI-1} by using immunofluorescence, as mentioned in Materials and methods. In normal conditions, endogenous PTEN is found both in the nucleus and cytoplasm of MCF7 cells. However, when p34^{SEI-1} is overexpressed as EGFP fusion, our IF data revealed a different distribution of PTEN in nucleus in p34^{SEI-1} overexpressing and control cells. PTEN accumulated much more inside the nucleus after transfection within 16 h and lasted until 48 h (Fig. 5). In contrast, the PTEN protein level was slightly reduced in cytosolic accumulation compared to non-transfected cells (Fig. 5). This suggests that p34^{SEI-1} positively affects PTEN nuclear import. Taken together, the

results indicate that p34^{SEI-1} affects subcellular localization of PTEN, in which p34^{SEI-1} overexpression induces PTEN nuclear localization.

Discussion

The PI3K signaling pathway plays important roles in cells by controlling many different cellular functions, in which AKT is the key regulator of the PI3K pathway to promote signal transduction related with tumor malignancies. AKT is phosphorylated and activated by either PI3K activation or PTEN inactivation. In the present study, we suggest a mechanism of how p34^{SEI-1} has oncogenic potential to promote carcinogenesis. Our data show that p34^{SEI-1} overexpression stabilizes NEDD4-1 and in turn NEDD4-1 induces poly-ubiquitination/degradation of a PTEN tumor suppressor, and subsequently promotes tumorigenesis by positively regulating the PI3K/AKT pathway as summarized in Fig. 6.

We previously showed that p34^{SEI-1} directly binds and stabilizes the X-linked inhibitor of apoptosis protein (XIAP) leading to an anti-apoptotic effect (26). Interestingly, the group of Van Themsche (36) suggested that XIAP can act as an E3 ligase for PTEN and therefore directly induces PTEN ubiquitination. Considering all these results, it is likely that p34^{SEI-1} may have an indirect effect on PTEN ubiquitination/degradation through XIAP. Thus, we also tested the effect of p34^{SEI-1} on XIAP mediated PTEN ubiquitination/degradation. However, our data revealed XIAP-independent PTEN ubiquitination/degradation (data not shown). Similarly, it needs to be considered that p34^{SEI-1} may affect different types of PTEN ubiquitin ligases that are responsible for PTEN ubiquitination/degradation.

Our data revealed that p34^{SEI-1} affects PTEN subcellular localization as well as its protein expression. It has been suggested that NEDD4-1 can mediate both mono- and poly-ubiquitination of PTEN and it can cause PTEN nuclear transport by catalyzing PTEN mono-ubiquitination (31). Considering the positive effect of p34^{SEI-1} on NEDD4-1 expression, it is suspected that p34^{SEI-1} may induce mono-ubiquitination as well as poly-ubiquitination of PTEN. Therefore, p34^{SEI-1} seems to positively control nuclear import of PTEN by upregulating NEDD4-1 expression as shown in Fig. 5. However, our immunofluorescence data were unexpected in the PTEN's subcellular distribution in response to p34^{SEI-1} overexpression. The reason is that PTEN nuclear localization has been suggested to be essential to suppress tumorigenesis and is mediated by its mono-ubiquitination (31). Considering that p34^{SEI-1} is an oncoprotein, which normally activate tumorigenesis, it was expected that p34^{SEI-1} overexpression would inhibit PTEN nuclear import. We thought that p34^{SEI-1} might exert a negative effect on PTEN in the nucleus as in the case of cytosol, even though p34^{SEI-1} induced PTEN nuclear import. However, the mechanism by which p34^{SEI-1} exerts a positive effect on PTEN nuclear localization remains unclear. Further research is needed to determine the effects of p34^{SEI-1} on PTEN nuclear localization.

In summary, our previous and current results suggest that p34^{SEI-1} is highly expressed in human breast cancer cells acting as an oncoprotein. In this process, p34^{SEI-1} appears to cause tumorigenesis by inducing NEDD4-1-mediated PTEN down-regulation and positively regulating the PI3K/AKT pathway. Thus, therapeutic strategies that interfere with the function of p34^{SEI-1} are expected to be promising targets for new anticancer drugs for breast cancer treatment.

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