Regulation of Cell Proliferation and Migration by Keratin19-Induced Nuclear Import of Early Growth Response-1 in Breast Cancer Cells

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Abstract

Purpose: Keratin19 (KRT19) is the smallest known type I intermediate filament and is used as a marker for reverse transcriptase PCR–mediated detection of disseminated tumors. In this study, we investigated the functional analysis of KRT19 in human breast cancer.

Experimental Design: Using a short hairpin RNA system, we silenced KRT19 in breast cancer cells. KRT19 silencing was verified by Western blot analysis and immunocytochemistry. We further examined the effect of KRT19 silencing on breast cancer cells by cell proliferation, migration, invasion, colony formation assay, cell-cycle analysis, immunocytochemistry, immunohistochemistry, and mouse xenograft assay.

Results: Silencing of KRT19 resulted in increased cell proliferation, migration, invasion, and survival. These effects were mediated by upregulation of Akt signaling as a result of reduced PTEN mRNA expression. Silencing of KRT19 decreased the nuclear import of early growth response-1 (Egr1), a transcriptional factor for PTEN transcription, through reduced association between Egr1 and importin-7. We also confirmed that silencing of KRT19 increased tumor formation in a xenograft model.

Conclusions: KRT19 is a potential tumor suppressor that negatively regulates Akt signaling through modulation of Egr1 nuclear localization.

Introduction

Keratins (KRT) are a subgroup of intermediate filament proteins that are present in epithelial tissues (1). There are 2 types of KRTs: low-molecular weight acidic type I KRTs and high-molecular weight basic or neutral type II KRTs (2). One of the most biologically interesting KRTs is KRT19, at 40 kDa the smallest known intermediate filament. KRT19 is used as a marker for reverse transcriptase PCR (RT-PCR)–mediated detection of tumor cells disseminated in lymph nodes, peripheral blood, and bone marrow of patients with breast cancer, and its positivity is considered a prognostic indicator (3–5).

The early growth response-1 (Egr1) transcription factor regulates the expression of several genes including PTEN (6, 7). Egr1 directly binds to the consensus Egr1-binding motif in the PTEN promoter and activates PTEN gene transcription. PTEN is a tumor suppressor in human cancers that functions as a phosphatase to dephosphorylate phosphatidylinositols-trisphosphate (PIP3), the product of phosphoinositide 3-kinase (PI3K), resulting in the formation of PIP2 (8). Increased levels of PIP2 result in recruitment of Akt to the membrane to promote tumorigenesis (9). As a downstream effector of PI3K, Akt regulates cellular survival and metabolism via phosphorylation of many downstream molecules (9).

The S/T-P-S/T nuclear translocation signal (NTS) domain in extracellular signal-regulated kinase-2 (ERK2) was identified and characterized as a novel nuclear localization signal (NLS) domain (10). Importin-7 (Imp7) binds the phosphorylated NTS domain of target molecules and mediates nuclear localization of the cargo as the complex is released from nuclear pore proteins (10). The NLS of Egr1 was previously identified as a zinc finger DNA-binding domain (11). Recently, it was reported that Egr1 also contains a S-P-S NTS domain in its C-terminal region (12). The NTS of Egr1 is required for the nuclear localization of Egr1, which is dependent on association between Egr1 and Imp7. In this study, we showed that KRT19 regulated cell proliferation, migration, invasion, and survival through KRT19-mediated association between Egr1 and Imp7 and subsequent nuclear import of Egr1 in human breast cancer cells.
Materials and Methods

Cell lines, antibodies, reagents, and plasmids

T47D, MCF7 vec, MCF7 HER2, BT474, MDA-MB-231, and SKBR3 human breast cancer cells and 293T human kidney cells were grown and routinely maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were incubated at 37°C in 5% CO₂ and 95% humidified air. KRT19-silenced cells were generated by transfection with KRT19 short hairpin RNA (shRNA; Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). The following antibodies were used: p-HER2, p-PI3K, p-Akt, Akt, p-GSK 3β, caspase-3, Egr1, and c-Jun antibodies (Cell Signaling Technology); p-PI3K (300×), p-PI3K (100×), p-ERK1/2, p-ERK1/2, p-PI3K, p-Akt, p-GSK 3β, GSK 3β, p-IκBα, IKKα, IKKβ, p-IκBα, IkBα, p-NFκB, NFkB, p-c-Raf, c-Raf, p-MEK1/2, MEK1/2, p-ERK1/2, ERK1/2, p-Elk, Elk, actin, HA, caspase-3, Egr1, and c-Jun antibodies (Cell Signaling Technology); PTEN, Egr1, IκBα, β-tubulin, and normal rabbit or mouse immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology); KRT19 antibody (Chemicon); HER2 (HER2/neu) antibody (Lab Vision); PARP antibody (BD Pharmingen); anti-mouse IgG-Cy3 (Zymed); and anti-Mouse IgG-PE antibody (Abcam). The following reagents were used: Alexa Fluor 488 phalloidin (Invitrogen); doxorubicin (Calbiochem); cycloheximde and MG132 (Sigma-Aldrich); and 70% ethanol, with two 5-minute incubations in each step. Immunofluorescence was monitored with an Olympus upright fluorescence microscope (BX50F).

Immunohistochemistry of tumor tissue sections

Tumor tissues were fixed in Bouin’s solution (Sigma). Tissues were dehydrated, washed in 70% ethanol, embedded in paraffin, and prepared as 5-μm thick sections. Paraffin sections were mounted on poly-L-lysine-coated slides and incubated in a dry oven at 60°C for 1 hour followed by deparaffinization in xylene. The slides were rehydrated by sequential incubation in 100%, 90%, 80%, and 70% ethanol, with two 5-minute incubations in each solution. The slides were then incubated with primary antibodies in PBS with 3% skim milk in PBS for 1 hour, followed by incubation with primary antibody diluted in 1% skim milk in PBS for 1 hour. After washing with PBS, the samples were treated with anti-mouse IgG-Cy3. For DNA staining, samples were incubated with Hoechst 33342 (1 μg/mL) for an additional 10 minutes. Immunofluorescence was monitored with an Olympus upright fluorescence microscope (BX50F).

Immunoprecipitation

Cells grown in 12-well plates were transfected with 0.2 μg of reporter constructs and 0.002 μg of pCMV-RI as an internal control using Lipofectamine 2000 (Invitrogen). At 48-hour post-transfection, cell lysates (1 mg) were precleared by addition of 30 μL of Protein A or G Sepharose (Invitrogen) for 2 hours at 4°C. After centrifugation at 1,000 g, the supernatant was incubated with either primary antibodies or normal rabbit/mouse IgG overnight at 4°C. Protein A or G Sepharose (30 μL) was added and incubated for 3 hours at 4°C. After centrifugation, the pellet was washed 3 times with cell lysis buffer. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Western blot analyses

Cells were lysed in lysis buffer containing 50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, and protease inhibitor cocktail (Roche). Samples were separated on SDS-PAGE and transferred to nitrocellulose membranes (Whatman). After blocking with 5% skim milk in TBS-T, membranes were incubated with the appropriate primary antibodies overnight, followed by 2 hours incubation with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized with the WEST ZOL plus System (iNtRON).

Translational Relevance

Keratin19 (KRT19) is used as a marker for reverse transcriptase PCR–mediated detection of tumor cells, and its positivity is considered as a prognostic indicator. On the contrary, we found that silencing of KRT19 increased cell proliferation, migration, invasion, and survival in human breast cancer cells. We also confirmed that silencing of KRT19 increased tumor formation in a xenograft model. These effects were mediated by upregulation of Akt signaling as a result of reduced PTEN mRNA expression. Silencing of KRT19 decreased nuclear import of early growth response-1 (Egr1), a transcriptional factor for PTEN transcription, through reduced association between Egr1 and importin-7. To our knowledge, this is the first report on KRT19-mediated tumor suppression and this result may indicate that the potential of KRT19 as a prognostic marker should be carefully reevaluated.
posttransfection, dual luciferase assays were conducted according to the manufacturer’s protocol (Promega).

Cycloheximide decay assay

Cycloheximide (100 μg/mL, Sigma) was added to block further protein synthesis. After treatment, cells were harvested at each of the indicated time points and subjected to Western blot analyses.

Cell fractionation

Cells were washed with ice-cold PBS and harvested in cytoplasmic extraction buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonylfluoride; PMSF) with agitation for 10 minutes at 4°C. After addition of NP-40 (final 0.5%), the samples were further agitated for 10 minutes at 4°C. Contents were subjected to centrifugation at 13,000 rpm in a microcentrifuge (Eppendorf) for 5 minutes. The supernatant was collected as the cytosolic fraction. The nuclear pellets were washed twice with cold PBS and resuspended in a nuclear extraction buffer (20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L PMSF). The nuclear extracts were agitated for 10 minutes at 4°C, centrifuged at 13,000 rpm at 4°C, and the supernatants were collected as the nuclear fraction.

Reverse transcriptase PCR

Expression of transcripts was assessed using the following primers: PTEN, Fwd 5'-aaa gct gga aag gga cga ac-3' and Rev 5'-cag gta acg gct gag gga ac-3'; Egr1, Fwd 5'-tca ggc gga cac ggg cga gc-3' and Rev 5'-tca ggc gta cag ggg tgg gc-3'. Quantification of actin expression was conducted as an internal control using an RT-PCR primer and control primer set (Invitrogen).

Proliferation assays

Cells were seeded at 1 × 10⁵ cells/well in 12-well dishes, trypsinized, and counted with a hemocytometer in triplicate every 24 hours for 5 days.

Cell-cycle analyses by flow cytometry

Cells were harvested with 0.25% trypsin and washed once with PBS. After centrifugation, cells were fixed in 100% ice-cold methanol overnight at −20°C. Fixed cells were incubated with 50 μg/mL propidium iodide in PBS and 1 mg/mL RNAse for 30 minutes. Cell-cycle analyses were conducted on a BD FACS (Becton & Dickinson Biosciences).
Soft agar and Matrigel colony formation assays
As a base agar, 0.7% low melting point agar gel (Bio-Rad) containing 20% FBS in 2× DMEM was added to 12-well dishes. Cells (1×10^4/well) were plated in 0.4% low melting point agar gel with 20% FBS in 2× DMEM and allowed to grow for 4, 8, and 12 days. Matrigel colony forming assays were conducted according to the manufacturer’s protocol (BD Bioscience).

Cell migration and invasion assays
Cell migration assays were conducted using 8-μm pore size Transwell chambers (Corning). The lower chamber was filled with medium containing 10% FBS. Cells were suspended in DMEM with 1% FBS and plated into the upper chamber. After 6, 12, and 24 hours, the number of cells on the bottom surface of the polycarbonate membranes was counted visually using crystal violet dye and a light microscope. Cell invasion assays were conducted using the same procedure except that the upper chamber was coated with Matrigel.

Xenografts
SKBR3 Cont sh and KRT19 sh cells were passaged in 8-week-old BALB/C nude mouse (Orient Bio) by subcutaneous injection of 1×10^6 or 5×10^6 cells into the lower flanks of mice. The mice were monitored for tumor formation and growth. Tumor weight was determined after sacrificing all the mice together at 39 days post-tumor formation. The mice were cared for and treated in compliance with institutional guidelines (HY-IACUC-10-048).

Statistical analyses
All of the results were confirmed in at least 3 independent experiments. Data are presented as mean ± SD. Comparison of results from treated versus control cells was conducted using t tests. A value of P < 0.05 was considered statistically significant.

Results
Silencing of KRT19 expression increases proliferation of breast cancer cells
We established KRT19-silenced cells (KRT19 shRNA cells) and control cells (Cont shRNA cells) using BT474, SKBR3, T47D, MCF7 vec, and MCF7 HER2 human breast cancer cell lines and a shRNA transfection system. Western blot and immunocytochemical analyses confirmed the downregulation of KRT19 in KRT19 shRNA cells (Fig. 1A and quantified in Supplementary Fig. S6A; Supplementary Fig. S1A and S1B). We examined the cell morphology and rearrangement of the actin filament cytoskeleton following silencing of KRT19 using F-actin staining and found that KRT19 silencing did not significantly affect cell morphology and actin rearrangement (Supplementary Fig. S1B). First, we compared the cell proliferation rate of KRT19 shRNA cells with that of Cont shRNA cells. As shown in Fig. 1B and Supplementary Fig. S1C, the growth rates of five KRT19 shRNA cell lines were upregulated compared with those of Cont shRNA cell lines, suggesting that KRT19 silencing induced cellular event(s) associated with increased cell proliferation. To confirm the effect of KRT19 on cell proliferation, MTT assays were conducted in KRT19-negative/low 293T and MDA-MB-231 cells using KRT19-overexpressing system (Supplementary Fig. S1E). The proliferation rates of KRT19-overexpressing cells were downregulated compared with that of control cells (Supplementary Fig. S1F). When the cell-cycle profiles of KRT19 shRNA and

Figure 2. KRT19 inhibition upregulates cell migration, invasion, and survival. BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells were plated on Transwell plates for (A) migration assay or (B) invasion assay. The number of cells on the bottom of the membrane was counted at 6, 12, and 24 hours ( P < 0.05; ** P < 0.005). C, at 24 hours after cell seeding, BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells were treated with serum-free medium containing doxorubicin (500 nmol/L) or Herceptin (20 μg/mL). After a further 24 hours, the cells were counted with a hemocytometer. Cell lysates were analyzed by Western blotting using antibodies against p-Akt, caspase-3, and PARP, and anti-actin antibody as a loading control.
Cont shRNA cells were compared, we found that the S phase fractions were significantly increased in KRT19 shRNA cells with a concomitant reduction in G1 phase (Fig. 1C and Supplementary Fig. S1D). We also investigated the effect of KRT19 silencing on three-dimensional colony formation. The results of both soft agar and Matrigel colony formation assays suggest that KRT19 silencing resulted in a significantly increased rate of colony formation (Fig. 1D and Supplementary Fig. S2A).

**KRT19 silencing upregulates cell migration, invasion, and survival**

Next, we examined the effect of KRT19 silencing on cell migration and invasion. As shown in Fig. 2A and B and Supplementary Fig. S2B and S2C, the migration and invasion rates of KRT19 shRNA cells were upregulated compared with those of Cont shRNA cells. To determine the effects of KRT19 silencing on cell survival and apoptosis, the cells were exposed to stress conditions including serum starvation, treatment with the DNA-damaging agent, doxorubicin (500 nmol/L), or treatment with Herceptin (20 μg/mL), a therapeutic antibody against HER2 for 48 hours. Herceptin was chosen for this study because both BT474 and SKBR3 cell lines express robust levels of HER2. The cell death induced by these treatments was attenuated in KRT19 shRNA cells compared with that of Cont shRNA cells (Fig. 2C, top).

We also showed efficient downregulation of the apoptotic markers of proteolytic cleavage of caspase-3 and PARP after treatment with doxorubicin or Herceptin in KRT19 shRNA cells compared with Cont shRNA cells (Fig. 2C, bottom). Conversely, we could observe a sensitizing effect of KRT19 overexpression on cytotoxicity of doxorubicin (Supplementary Fig. S1G). These results indicate that KRT19 silencing might confer resistance to apoptosis. Together, the results of Figs. 1 and 2 suggest that KRT19 might act as a tumor suppressor in human breast cancer through the regulation of cancer cell proliferation and survival.

**KRT19 silencing induces Akt activity through downregulation of PTEN mRNA expression**

To investigate the mechanisms responsible for KRT19-silencing–mediated upregulation of cell proliferation, migration, invasion, and survival, we examined the status of intracellular signaling molecules. HER2 activates a number of intracellular signal cascades including the PI3K/Akt pathway (16). We found that silencing of KRT19 induced downregulation of HER2 (Fig. 3A), possibly via blockade of proteosomal-mediated degradation of HER2 (Ju and colleagues, unpublished data). Interestingly, despite the downregulation of HER2 by KRT19 silencing, we found that phosphorylation of PI3K, Akt, and Akt downstream factors including GSK3β and IκB kinase (IKK)/IκB/NFκB was upregulated in all five KRT19 shRNA cell lines (Fig. 3A and quantified in Supplementary Fig. S6B; Supplementary Fig. S3A).

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**Figure 3.** KRT19 inhibition induces Akt activity through downregulation of PTEN expression. A, total cell lysates were prepared from BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells and subjected to Western blot analyses with the indicated antibodies. B and D, BT474 KRT19 sh, BT474 Cont sh, SKBR3 KRT19 sh, and SKBR3 Cont sh cells were transfected with NF-κB, FOXO or PTEN-Luc reporter constructs, harvested after 48 hours, and analyzed by dual luciferase assays (*, P < 0.05; **, P < 0.005; ***, P < 0.0005). C, mRNA from each cell line was analyzed by RT-PCR using primers specific for PTEN and actin (as a loading control). Total cell lysates were prepared from each cell line and subjected to Western blot analyses with PTEN and actin antibodies.
Accordingly, NF-kB-dependent transcriptional activities were significantly increased in KRT19 shRNA cells (Fig. 3B, left). To further assess the effect of KRT19 on activity of Akt and its downstream signaling, we reintroduced pSuper shRNA-resistant KRT19 mutant in BT474 pSuper KRT19 shRNA clone (Supplementary Fig. S3E). We confirmed that KRT19 shRNA exclusively regulates its level and Akt signaling. We also examined the nuclear localization and transcriptional activity of FOXO, a transcription factor downstream of Akt1 that is negatively regulated by Akt activity (17). Basal levels of FOXO-dependent transcriptional activities were effectively decreased by KRT19 silencing (Fig. 3B, right). Localization of ectopic FOXO3a in nucleus was also reduced in KRT19 shRNA cells, as revealed by cell fractionation assays (Supplementary Fig. S3B). The results in Fig. 3A and B collectively suggest that silencing of KRT19 efficiently increased PI3K/Akt downstream signaling.

To investigate the mechanism by which KRT19 silencing upregulated the Akt pathway despite downregulation of HER2 levels, we considered the possibility that KRT19 silencing regulates PTEN, a negative modulator of the PI3K/Akt pathway. We found that mRNA and protein levels of PTEN were significantly downregulated in KRT19 shRNA cells compared with those of Cont shRNA cells (Fig. 3C and quantified in Supplementary Fig. S6C; Supplementary Fig. S3C and S3E). Transcriptional activity of a reporter construct containing the promoter region of PTEN (−500/+273 bp) was also decreased by KRT19 silencing (Fig. 3D). Accordingly, cotransfection of KRT19 increased PTEN promoter activity in 293T cells (Supplementary Fig. S3D). To determine whether KRT19 directly affects the stability of PTEN protein, we measured PTEN protein level after treatment with MG132, a 26S proteasome inhibitor. MG132 treatment resulted in a slight increase in PTEN protein level (Supplementary Fig. S4A). PTEN protein level is known to be regulated by NEDD4-1 E3 ligase via ubiquitination-mediated proteosomal degradation (18). Although the decreased basal expression level of PTEN in KRT19 shRNA cells was restored by treatment with MG132, the levels were still lower than the PTEN level in Cont shRNA cells after MG132 treatment (Supplementary Fig. S4A, lane 2 vs. 4 and lane 6 vs. 8). These results suggest that silencing of KRT19 did not downregulate PTEN protein level through a decrease in protein stability of PTEN. In cycloheximide decay assays, the decay slopes of PTEN were similar between KRT19 and Cont shRNA cells (Supplementary Fig. S4B). These findings confirmed that transcriptional control of PTEN mRNA is the major molecular mechanism responsible for KRT19-silencing–induced downregulation of PTEN.

KRT19 regulates nuclear localization of Egr1 transcriptional factor

We next focused on transcription factors that might be responsible for the regulation of PTEN transcription. Egr1 is a well-known transcription factor that upregulates PTEN transcription (6). We investigated the effect of Egr1...
Figure 5. KRT19 mediates association between Egr1 and Imp7. A and B, cell lysates were immunoprecipitated with KRT19, Egr1, Imp7, or normal IgG antibodies and Protein A or G Sepharose. The immunoprecipitates were analyzed by Western blotting with KRT19, Egr1, and Imp7 antibodies. C, full length Egr1 (WT), NLS domain deletion mutant (ΔNLS), NTS alanine substitution mutant (APA), and double mutant (ΔNLS/APA) were subcloned into pcDNA3-Flag vector between the BamH I and Xho I restriction sites. Egr1 constructs were cotransfected with KRT19 into 293T cells, and the lysates were divided into nuclear and cytosolic fractions. Each fraction was subjected to Western blot analyses with the indicated Flag, KRT19, c-Jun (as nucleus marker), and β-tubulin (as cytosol marker) antibodies. Actin antibody was used to verify equal loading. D, the lysates were immunoprecipitated with Flag, KRT19, Imp7 antibodies, or normal IgG and Protein A or G Sepharose. The immunoprecipitates were analyzed by Western blotting with Flag, KRT19, or Imp7 antibodies.
knockout on PTEN expression in vivo using an Egr1-deficient mouse model. As depicted in Fig. 4A, Western blot analyses revealed that PTEN protein level was downregulated in the mammary glands of Egr1-deficient mice compared with their wild-type (WT) littermates. Phosphorylation-deactivated Akt was upregulated in Egr1-deficient mice as a result of reduced PTEN expression.

On the basis of these in vivo observations, we investigated whether the KRT19-silencing–induced downregulation of PTEN is mediated by Egr1. Interestingly, we observed that both mRNA and protein expression levels of Egr1 were decreased by KRT19 silencing in HER2-positive MCF7, HER2, BT474, and SKBR3 cells, but not in HER2-negative/low T47D and MCF7 vec cells (Fig. 4B and C and Supplementary Fig. S5A and S5B). To explain the differential effect of KRT19 silencing on Egr1 expression between HER2-positive and -negative/low cells, we examined the possible modulation of ERK signaling by KRT19 silencing because Egr1 transcription is regulated by the ERK/E twenty-six (ETS)-like transcription factor (Elk) (19, 20). Our results indicate that activities of ERK signaling after KRT19 silencing were vastly different between HER2-positive and -negative/low cells (Supplementary Fig. S5C). In HER2-positive MCF7 HER2, BT474, and SKBR3 cells, the activities of c-Raf/mitogen-activated protein kinase (MAPK)–ERK kinase (MEK), ERK, and Elk were downregulated by KRT19 silencing, determined by their phosphorylation status, whereas activities of c-Raf/MEK/ERK/Elk in HER2-negative/low T47D, and MCF7 vec cells were not significantly changed after KRT19 silencing. Needless to say, activation of the ERK pathway in HER2-negative/low cells was achieved by a HER2-independent mechanism, whereas that of HER2-positive cells was HER2 dependent (21, 22). Downregulation of HER2 by KRT19 silencing (Fig. 3A) decreased the activity of the c-Raf/MEK/ERK/Elk cascade, culminating in inhibition of Egr1 expression. These results suggest that downregulation of Egr1 expression by KRT19 silencing was a HER2-dependent phenomenon present only in HER2-positive cells.

We also evaluated the possible modulation of Egr1 nuclear translocation by KRT19 silencing because Egr1 acts as a transcription factor within the nucleus. KRT19 silencing decreased nuclear Egr1 levels, as determined by cell fractionation assay and immunocytochemistry, regardless of HER2 expression status (Fig. 4C and D and Supplementary Fig. S5B). We confirmed KRT19-mediated nuclear translocation of Egr1 using transient transfection experiments with SKBR3 cells (Supplementary Fig. S5D). As a consequence of Egr1 nuclear translocation by KRT19, PTEN expression increased and Akt activity decreased. These results indicate that upregulated cell proliferation, migration, invasion, and survival caused by KRT19 silencing (Figs. 1 and 2) were most likely attributable to modulation of Egr1 nuclear translocation and subsequent changes in PTEN expression.

KRT19 mediates association between Egr1 and Imp7

The phosphorylated S/T-P-S/T NTS domain regulates nuclear localization of molecules through interaction with Imp7 (10). Egr1 contains a NTS domain that binds Imp7 for nuclear localization (12). On the basis of these findings, we were interested in whether KRT19 silencing affected the association between Egr1 and Imp7. Coimmunoprecipitation studies (Fig. 5A) revealed that KRT19, Egr1, and Imp7 formed a ternary complex in all 5 cell lines. Control immunoprecipitates with normal IgG did not contain KRT19, Egr1, or Imp7. In KRT19 shRNA cells, the Egr1–Imp7 association was significantly impaired compared with control cells (Fig. 5B). Therefore KRT19 might facilitate association between Egr1 and Imp7 thereby inducing nuclear targeting of Egr1.

In addition to the NTS domain, classical NLS domain in the zinc finger domain regulates nuclear localization of Egr1 (11). If KRT19-mediated increased association between Egr1 and Imp7 only occurs through potentiation of Imp7 binding to NTS domain of Egr1, the classical NLS-mediated nuclear localization of Egr1 may still happen in KRT19-silenced cells. So we further hypothesized that Egr1 nuclear localization by classical NLS domain was also achieved by a KRT19-dependent mechanism. Because NLS domain of Egr1 plays a dominant role in Egr1 nuclear localization as compared with NTS domain (12), it was indeed less likely to conclude that KRT19-silencing–induced perturbation in nuclear targeting of Egr1 is mainly dependent on decreased binding of Imp7 to NTS domain of Egr1. To precisely determine the interaction motifs in Egr1-KRT19-Imp7 ternary structure, we cloned full length Egr1 (WT), NLS domain deletion mutant (ΔNLS), NTS alanine substitution mutant (APA), and NLS/NTS double mutant (ΔNLS/APA). Egr1 constructs were transiently cotransfected with KRT19 WT construct into 293T cells, and the cell lysates were divided into nuclear and cytosolic fractions. Our results showed that KRT19 could effectively translocate Egr1-WT, ΔNLS, and APA from cytosol to nucleus, but not double mutant (Fig. 5C). In addition, Egr1-WT and Egr1-APA could effectively bind KRT19, whereas Egr1-ΔNLS and double mutant exhibited reduced affinity toward KRT19 (Fig. 5D, first and second, lane 3, 7 vs. 5, 9). These data indicated that the binding of Egr1 to KRT19 is primarily mediated by the interaction between NLS domain of Egr1 and KRT19. When the interaction between Imp7 and Egr1 was examined, both...
Egr1-WT and Egr1-ΔNLS could effectively bind Imp7 and this association between Imp7 and Egr1 was enhanced by KRT19 coexpression (Fig. 5D, third, lane 2 vs. 3 and 4 vs. 5). However, both Egr1-APA and double mutant could not bind Imp7 (Jae 6–9). Interestingly, we found that the association between KRT19 and Imp7 was Egr1-independent phenomena because KRT19 could bind Imp7 even in the presence of Egr1 double mutant in which the interaction site between Egr1 (NLS) and KRT19 and the site between Egr1 (NTS) and Imp7 were deleted (Fig. 5D, fourth, lane 9). Taken together, the data in Fig. 5 collectively suggest that KRT19 is a necessary requirement for the association between Imp7 and NTS domain of Egr1.

**KRT19 regulates in vivo tumorigenesis via modulation of Egr1 subcellular localization in mouse xenograft model**

We next examined the effect of KRT19 silencing on in vivo tumor formation using a xenograft mouse model (Fig. 6A). When 1 × 10^6 SKBR3 KRT19 shRNA and Cont shRNA cells were inoculated subcutaneously into both sides of the lower flank of BALB/C nude mice, none of the mice examined developed tumors (Fig. 6A and B). When 5 × 10^6 SKBR3 KRT19 shRNA cells were inoculated, all mice developed xenograft tumors. In contrast, tumors developed at only 2 out of 10 injection sites inoculated with 5 × 10^6 SKBR3 Cont shRNA cells. In addition, the weights of SKBR3 KRT19 tumors were approximately 6-fold higher than those of control tumors (Fig. 6A and B). Immunohistochemistry experiments revealed that SKBR3 KRT19 tumors exhibited upregulation of phosphorylated Akt and downregulation of PTEN compared with control cell tumors (Fig. 6D). The KRT19 level was still efficiently downregulated in SKBR3 KRT19 shRNA tumors. These results are in accordance with those obtained using in vitro cultured cells in Fig. 3. We also confirmed that Egr1 nuclear localization was significantly impaired in SKBR3 KRT19 shRNA tumors (Fig. 6E) compared with control tumors, whereas the subcellular distribution of Imp7 seemed to be similar in the 2 types of tumor.

**Discussion**

This study uncovers a new mechanism by which KRT19-mediated association between Egr1 and Imp7 downregulates breast cancer cell proliferation, migration, invasion, and survival through upregulated transcription of PTEN and subsequent inhibition of Akt by promoting nuclear localization of Egr1 both in vitro and in vivo. Egr1 is a crucial transcription factor that is induced by stress, injury, mitogens, and differentiation signals (7, 23, 24). Egr1 upregulates the expression of multiple genes, including PTEN and several tumor suppressor genes. Transcription of Egr1 was previously shown to be induced by MAPK pathways, especially by ERK signaling through phosphorylation and activation of Elk1 using MEK inhibitors or the natural anticancer compound curcumin, in various types of tumors (25–28). In addition to the transcriptional regulation of Egr1 by ERK, we show here that KRT19 plays a role in regulating the nuclear localization of Egr1 by modulating its association with Imp7.

KRTs are dynamically regulated intermediate filaments that are fully integrated within the cellular framework and interact with a range of cellular proteins (1). Many of these proteins, including various kinases, receptors, adaptors, and other types of effector, function in signaling networks that regulate the cell-cycle, apoptosis, and the cellular response to stress (1). It has been shown that KRT8 and KRT18 bind TNF receptor-2, reflecting their ability to specifically interact with other signaling proteins (29). This interaction affects the TNF-dependent activation of downstream effectors such as c-jun-NH2-kinase and NF-κB. KRT17 upregulates protein synthesis and cell growth via the Akt/mTOR pathway by binding to the adaptor protein 14-3-3 (30). One of the most interesting keratins is KRT19, the smallest known intermediate filament protein (31). KRT19 has a highly conserved α-helical central domain that is essential for filament formation but lacks the C-terminal non-helical tail domain present in other acidic KRTs (32). A previous study showed that absence of KRT19 induced skeletal myopathy in a KRT19 knockout mouse model (33). However, compared with other KRT isoforms, KRT19-mediated intracellular signaling has not been widely studied.

It was previously reported that KRT19 expression is coupled with HER2 expression in breast cancer (34). Our in silico analysis also revealed that KRT19 expression is positively correlated with HER2 (Supplementary Fig. S7). In our experiments, when KRT19 was knocked down, HER2 expression was dramatically downregulated. Tumorigenicity of HER2-amplified breast cancer cells such as SKBR3 and BT474 is presumably dependent on HER2 (35, 36). Because a HER2-targeted therapy would be used for a HER2-over-expressing tumor, this may suggest that any intracellular genetic event(s), which completely silences KRT19 levels, could lead to resistance to HER2-targeted therapies. However, it is also possible that a modest decrease in HER2 level by attenuation of KRT19 would lead to a decrease in tumorigenic potential of HER2 by inhibition of HER2 downstream signaling.

It was reported that Egr1 nuclear localization and transcriptional activity are regulated by α-tubulin cytoskeleton protein in benign prostate cells but not in malignant prostate cells (37). The authors showed that Egr1 associates with α-tubulin in benign prostate cells but not in prostate cancer cells using immunofluorescence confocal analyses and communoprecipitation experiments. Furthermore, α-tubulin-mediated Egr1 localization in benign prostate cells is unique among the Egr family because other related proteins including Egr2, Egr3, Sp1, or WT1 do not exhibit such behavior. Interestingly, they also showed that KRT colocalized with Egr1 in the perinuclear region of DU-145 prostate cancer cells using pan-KRT antibody and immunofluorescence confocal analyses. We speculate that microtubules and KRTs may compete with each other for binding with Egr1 or may together constitute molecular cross-talk to regulate Egr1 nuclear targeting. The phosphorylated S/T-P-S/T NTS domain is a newly identified NTS that induces
nuclear localization of ERK2, SMAD3, and MEK1 by binding to Imp7 (10). Recently, Chen and colleagues reported that Imp7 regulates Egr1 nuclear localization through binding to the S-P-S NTS domain in the C-terminal region of Egr1 (12). They also showed that Imp7-dependent nuclear localization of Egr1 is not solely dependent on the previously identified classical NLS.

On the basis of these previous studies, we investigated the possibility that KRT19 might mediate nuclear localization of Egr1 by facilitating Egr1–Imp7 complex formation in breast cancer cells. KRT19 was indeed required for proper Egr1 nuclear localization via formation of a KRT19–Egr1–Imp7 ternary complex. Upon nuclear localization of Egr1, PTEN was expressed through transcriptional activity of Egr1, resulting in downregulation of PI3K/Akt activity in breast cancer cells. The PI3K/Akt pathway is centrally involved in cell proliferation, migration, invasion, and survival (9, 38).

As summarized in Fig. 6F, KRT19 silencing upregulated the activity of Akt, and subsequently increased cell proliferation, migration, invasion, and survival. It was recently reported that activation of Akt could be subject to negative feedback regulation by PTEN (7). Akt-mediated phosphorylation of Egr1 induces SUMOylation of Egr1 by PI14ARF, which is required for Egr1-mediated transcription of its target genes including PTEN (7). We also identified KRT19 as a necessary component of the negative feedback loop that downregulates PI3K/Akt pathway. Although KRT19-silenced cells exhibit high Akt activity, the absence of KRT19 fails to establish negative feedback on PI3K/Akt pathway due to impaired nuclear import of Egr1. The lack of negative feedback regulation of Akt activity may account for the dramatic rise in Akt activity as well as the tumorigenic potential of KRT19-silenced cells in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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