1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol ameliorates EGF-induced MMP-9 expression by promoting receptor desensitization in MDA-MB-231 cells

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Abstract. Activated epidermal growth factor receptors (EGFRs) are crucial for inducing metastasis in cancer cells by promoting matrix metalloproteinase (MMP) expression. The present study was designed to investigate the effects of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) on MMP expression in epidermal growth factor (EGF)-stimulated breast cancer cells *in vitro*. EGF stimulation induced internalization of its cognate receptor, EGFR, for stimulus-desensitization. These internalized receptors, complexed with the ubiquitin ligase c-Cbl and EGFR pathway substrate 15 (EPS15) (for degradation), were evaluated by confocal microscopy at 5-90 min time intervals. During intracellular trafficking of EGFRs, EGF-induced signaling cascades were analyzed by examining EGFR and SHC phosphorylation. Modulation of MMP expression was assessed by evaluating the activity of transcription factor AP-1 using a luciferase assay. PLAG accelerated the assembly of EGFRs with c-Cbl and EPS15 and promoted receptor degradation. This faster intracellular EGFR degradation reduced AP-1-mediated MMP expression. PLAG stimulation upregulated thioredoxin-interacting protein (TXNIP) expression, and this mediated the accelerated receptor internalization. This PLAG-induced increase in EGFR trafficking was blocked in TXNIP-silenced cells. By downregulating MMP expression, PLAG effectively attenuated EGF-induced mobility and invasiveness in these cancer cells. These data suggest that PLAG may be a potential therapeutic agent for blocking metastasis.

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Introduction

Tumor metastasis typically forms secondary tumors in other organs or tissues that originate from the primary tumor, and is responsible for approximately 90% of cancer-related deaths (1). Among epithelial tumors, breast cancer is highly malignant and has a substantial probability of metastasis (2). Degradation of the extracellular matrix (ECM) by cancerous cells is mediated through a variety of proteolytic enzymes, including the matrix metalloproteinases (MMPs). The activity of MMPs in tumor cells contributes to invasion and metastasis (3). MMP-9 is highly expressed in breast cancer cells, and its abundant expression is associated with tumor malignancy (4). MMP-9 secreted from the tumor facilitates intravasation by destroying ECM components in surrounding tissues and the resulting tumor cells in the circulation can spread to distant organs through extravasation (5). Furthermore, in human breast cancer, increased MMP-9 expression is correlated with increased lymph node metastasis and tumor size (6); thus, MMP regulation is considered a therapeutic target for the prevention of metastasis.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK), and it is involved in both physiological and pathological epithelial cell processes (7). Regulating EGFR function is also considered to be the main target for protection against cancer metastasis (8). Ligand binding to EGFRs leads to receptor dimerization and endocytosis (9). Subsequent phosphorylation of tyrosine residues at the carboxyl-terminus of EGFR provides docking sites for proteins with Src homology 2 and phosphotyrosine-binding domains, and triggers signal transduction through Ras-Raf-mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, phosphoinositide 3 kinase, Akt, signal transducer and transcriptions (STATs), phospholipase C γ 1, and other pathways for cell growth, survival, proliferation, and metastasis in mammalian cells (10).

Activated EGFRs are desensitized by promoting receptor endocytosis (11). EGFR endocytosis is directly linked to the decay of intracellular signaling, and to the degradation of the receptor (12). After endocytosis, EGFR complexes can return to the plasma membrane, but they can also be retained in endosomes. Those retained in endosomes are eventually sorted to early/late endosomes and lysosomes for degradation (13), and this degradation leads to signal attenuation (14). Therefore, regulating EGFR endocytosis is a potential therapeutic target for signal termination (15).

α-arrestin is an identified tumor suppressor in metastatic breast cancer (16), and it is known to facilitate direct interactions between modulators of plasma membrane RTKs, such as Grb2, SHP2, and E3 ubiquitin ligase (17,18). Thioredoxin-interacting protein (TXNIP), another α-arrestin family member, is associated with the RTK-Rab5 complex and translocates together with this complex to endosomes after ligand stimulation. These findings suggest that TXNIP modulates RTK internalization and signaling (19).

The lipid 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) is naturally found in deer antler, but its artificially synthesized version has been used to explore its biological functions in neutropenia, oral mucositis, and as an anti-inflammatory agent (20-22). Specifically, PLAG has been shown to help resolve inflammation originating from chemotherapy treatments (21,23), where two common patient complications are neutropenia and oral mucositis. Chemotherapy-induced metastasis remains a serious problem (24), and as described earlier, EGFR modulation is a therapeutic target as activation of these receptors can contribute to tumor metastasis via transcriptional activity of inversion-related genes (25).

In the present study, we investigated the anti-metastatic activity of PLAG in EGF-stimulated cancer cells after successful EGFR activation. The enhanced speed of intracellular EGFR trafficking and its enhanced degradation were examined in PLAG-treated MDA-MB-231 breast cancer cells. Our results suggest that PLAG may be an anti-metastatic agent for attenuating malignancy-related EGFR activation.

Materials and methods

Cell culture and reagents. MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco modified Eagle's medium (DMEM; Welgene) containing 10% fetal bovine serum (FBS; Tissue Culture Biologicals), 100 U/ml penicillin, and 100 μg/ml streptomycin (antibiotic-antimycotic solution; Welgene) at 37˚C in a 5% CO_2 atmosphere. All cells tested mycoplasma-free by polymerase chain reaction (PCR) and were used for experiments within 12 passages after thawing. 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) and 1-palmitoyl 2-linoleoyl 3-hydroxylglycerol (PLH) were produced by and obtained from Enzychem Lifescience Corporation (Jecheon, Korea). Cells were pretreated with PLAH and PLH for 1 h, at doses 10-50 μg/ml.

Transwell cellular assays for invasion and migration. The invasion and migration assays for MDA-MB-231 cells were performed in 24-well Transwell units (8-μm pore size, Corning, Inc.). Transwells were coated with 1 mg/ml Matrigel (Corning). Briefly, MDA-MB-231 cells ($2x10^4$/100 μl) were either placed in the Matrigel-coated Transwells for the invasion assay or in only the upper part of the Transwells for the migration assay. The lower chamber was filled with medium containing 10, 25, or 50 μg/ml PLAG for 1 h and 100 ng/ml EGF (Peprotech). After incubation at 37˚C for 24 h, non-invasive cells that remained on top of the upper chamber were removed using cotton swabs. Cells that invaded to the lower side of the membrane inserts were fixed with formalin at 4˚C for 10 min and then stained with cresyl violet at RT for 5 min. The number of cells that migrated across the membrane to the lower chamber were photographed under a light microscope (Carl Zeiss).

Western blot analysis. MDA-MB-231 cells were seeded into 6 wells with a density of $2x10^5$ cells/ml and cultured without serum overnight. For western blot analysis, PLAG- and EGF-stimulated cells were lysed with radioimmunoprecipitation assay buffer (LPS solution, Daejeon, Korea) containing protease inhibitors, and debris was centrifuged at 4˚C for 30 min at 16,609 x g). The supernatant was diluted with 5X sample buffer. Equal protein amounts were separated by sodium dodecyl sulfate-polyacrylamide 10% gel electrophoresis and transferred to polyvinylidine fluoride (PVDF) membranes (Millipore Corp.). The membranes were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h. Membranes were incubated with antibodies to EGFR (cat. no. 4267; Cell Signaling Technology; dilution 1:1,000), phospho-EGFR (Tyr 1068) (cat. no. 3777; Cell Signaling Technology, dilution 1:1,000), SHC (cat. no. 2432; Cell Signaling Technology; dilution 1:500), phospho-SHC (cat. no. 2434; Cell Signaling Technology; 1:1,000), ERK (cat. no. 4693; Cell Signaling Technology; dilution 1:2,000), phospho-ERK (cat. no. 9101; Cell Signaling Technology; dilution 1:3,000), JNK (cat. no. 9252; Cell Signaling Technology; dilution 1:1,000), phospho-JNK (cat. no. 4671, Cell Signaling Technology; dilution 1:2,000), β-actin (cat. no. 3700; Cell Signaling Technology; dilution 1:5,000), TXNIP (cat. no. 14715; Cell Signaling Technology; dilution 1:1,000) and MMP-9 (cat. no. AB19016; Millipore Corp; dilution 1:2,000) for overnight at 4˚C. After three washes in PBST, membranes were stained with peroxidase-conjugated goat anti-rabbit IgG (cat. no. sc-2005; Santa Cruz Biotechnology; dilution 1:5,000) or peroxidase-conjugated goat anti-mouse IgG (cat. no. sc-2004; Santa Cruz Biotechnology; dilution 1:5,000) for 1 h at room temperature. Immobilon Western Chemiluminescent HRP Substrate was used for signal detection (Millipore Corp). Densitometric analysis was performed using ImageJ software (version 1.48; National Institutes of Health).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells with Trizol (Favorgen) according to the manufacturer's protocol. Approximately 500 ng of total RNA was used to prepare cDNA using M-MLV reverse transcriptase (Promega). The following primers (Macrogen) were used: 5'-CGAGAGAGA CTCTACACCCAGGAC-3' and 5'-CACTTCTTGTCGCTG TCAAAGT-3' for MMP-9; 5'-CCATCACCATCTTCCAGG AG-3' and 5'-ACAGTCTTCTGGGTGGCAGT-3' for GAPDH; 5'-GCCACACTTACCTTGCCAAT-3' and 5'-GGAGGAGCT

TCTGGGGTATC-3' for TXNIP. PCR was performed under following conditions: 96˚C for 30 sec, 60˚C (MMP-9, TXNIP) or 58˚C (GAPDH) for 30 sec, and 72˚C for 30 sec, followed by 72˚C for 5 min. PCR products were electrophoresed using 2% agarose gels and stained with ethidium bromide.

Luciferase assay. AP-1 transcriptional activity was measured indirectly using a pGL4-AP-1-luc plasmid-based luciferase reporter assay (Promega) and Attractene transfection reagent (Qiagen) according to the manufacturers' instructions. Cells were seeded into 24-well plates at a density of $5x10^4$/ml and the luciferase-reporter plasmid (1 μg/well) was added for 24 h. Cells were then starved and treated with different concentrations of PLAG for 1 h, followed by stimulation with EGF (100 ng/ml) for 6 h. Transient expression of the reporter gene was quantified using the DualGlo1 luciferase assay system (Promega) in a TD-20/20 Turner luminometer (Promega).

Immunoprecipitation assay. Protein G agarose beads (Roche) were used for immunoprecipitation. Cells ($2x10^5$/ml) were seeded in 6-well plates and starved overnight. After EGF and PLAG treatment, the cells were lysed with lysis buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol). Cell lysates were incubated with anti-EGFR (cat. no. sc-373746, Santa Cruz Biotechnology, dilution 1:50) antibodies by gentle agitation for 6 h at 4˚C. The beads were then added and incubated overnight. After the reaction, the beads were washed using lysis buffer. Target proteins were eluted in 1X sample buffer and analyzed by western blotting using antibodies against EGFR (cat. no. 4267; Cell Signaling Technology; dilution 1:1,000), ubiquitin (cat. no. 3933; Cell Signaling Technology; dilution 1:1,000), c-Cbl (cat. no. 2747; Cell Signaling Technology; dilution 1:1,000), EGFR pathway substrate 15 (EPS15) (cat. no. sc-390259; Santa Cruz Biotechnology; dilution 1:1,000), and TXNIP (cat. no. 14715; Cell Signaling Technology; dilution 1:1,000).

Immunofluorescence staining. Cells ($1x10^5$/ml) were seeded onto cover glasses in 24-well plates and cultured without serum overnight. For visualization of surface EGFRs, cells were fixed with 3.7% formaldehyde for 20 min. For visualization of EGFR-Rab5/Rab7 colocalizations, cells were fixed and then permeabilized with 0.2% Triton X-100 for 20 min. After being washed with PBS twice and blocked with 2% BSA, cells were incubated with anti-EGFR (cat. no. 352904; BioLegend, dilution 1:1,000), anti-Rab5 (cat. no. sc-47792; Santa Cruz Biotechnology; dilution 1:1,000), and anti-Rab7 (cat. no. sc-376362; Santa Cruz Biotechnology; dilution 1:1,000) antibodies overnight at 4˚C. Cells were then washed with PBS twice and incubated with Alexa Fluor 488-conjugated secondary antibodies (cat. no. A32723; Invitrogen; Thermo Fisher Scientific, Inc.; dilution 1:1,000) for 1 h at room temperature. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen; Thermo Fisher Scientific, Inc.). Fluorescence confocal microscopy (Carl Zeiss, Thornwood, NY, USA) was used to assess labeling and its distribution.

Flow cytometry analysis. After PLAG and EGF treatment, cells were collected and placed in a solution of trypsin-EDTA. Collected cells were washed with PBS and then blocked with FACS buffer (0.5% BSA in PBS) for 30 min at 4˚C. Blocked cells were incubated with PE-conjugated anti-human EGFR antibody (cat. no. 352904, BioLegend) for 1 h at 4˚C. Analyses were performed using a BD FACS Verse flow cytometer (BD Biosciences), and the data were processed using FlowJo software (version 10.6; Tree Star, USA).

Transient transfection with small interfering RNA. TXNIP siRNA (cat. no. sc-270490) was purchased from Santa Cruz Biotechnology, Inc. Scrambled siRNA (cat. no. sc-37007) was used as the control. MDA-MB-231 breast cancer cells were transfected with these siRNA duplex targeting constructs (40 nM) and HiPerFect transfection reagent (Qiagen). After 60 h incubations, cells were treated with PLAG (50 μg/ml) for 1 h and EGF (100 ng/ml), and downregulation of target-gene expression was evaluated by RT-PCR.

Statistical analysis. The data are presented as the mean ± SD of at least three independent experiments. For statistical analysis, one-way ANOVA followed by Turkey-Kramer post hoc test was performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). P-value less than 0.05 is considered to indicate statistical significance.

Results

PLAG attenuates EGF-induced invasion and migration of MDA-MB-231 cells and effectively downregulates high expression of MMP-9. To evaluate the anti-metastatic effect of PLAG, we investigated the inhibitory activity of PLAG on EGF-induced cell invasiveness and mobility using Transwell assays. EGF-treated MDA-MB-231 cells (100 ng/ml) showed high invasiveness and mobility, whereas PLAG-treated cells (10, 25, or 50 μg/ml) exhibited significantly reduced invasiveness and mobility in a dose-dependent manner (Fig. 1A-C). As expected, MMP-9 suppression alone by siMMP-9 reduced the invasiveness and migration of EGF-treated cells (data not shown). PLAG treatment was also investigated for modulation of invasion-associated MMP-9 expression. MMP-9 expression was examined in EGF-treated cells using RT-PCR and western blotting analysis. Similar to the mobility results, MMP-9 expression was high after EGF treatment alone, but PLAG-treated cells showed significantly lower MMP-9 mRNA and protein expressions (Fig. 1D and E). These data indicate that PLAG affected EGF-induced tumor cell motility by modulating the EGFR signaling pathway and its downstream influences on gene expression (e.g., on MMPs).

PLAG promotes endocytosis and ubiquitination of ligand-bound EGFR. EGF treatment induces MMPs through its cognate receptor, EGFR. A PLAG-induced decrease in MMP expression results from possible modifications to the EGFR signaling pathway, including receptor internalization. We assessed EGFR internalization by examining plasma membrane-localized EGFRs using flow cytometry and confocal microscopy. EGF treatment reduced the number of cell-surface EGFRs on MDA-MB-231 cells. These EGFRs were internalized 5 min after EGF stimulation, and most EGFRs were also internalized within 5 min in the PLAG-treated cells. EGFR internalization induced by PLAG treatment was assessed by flow cytometry

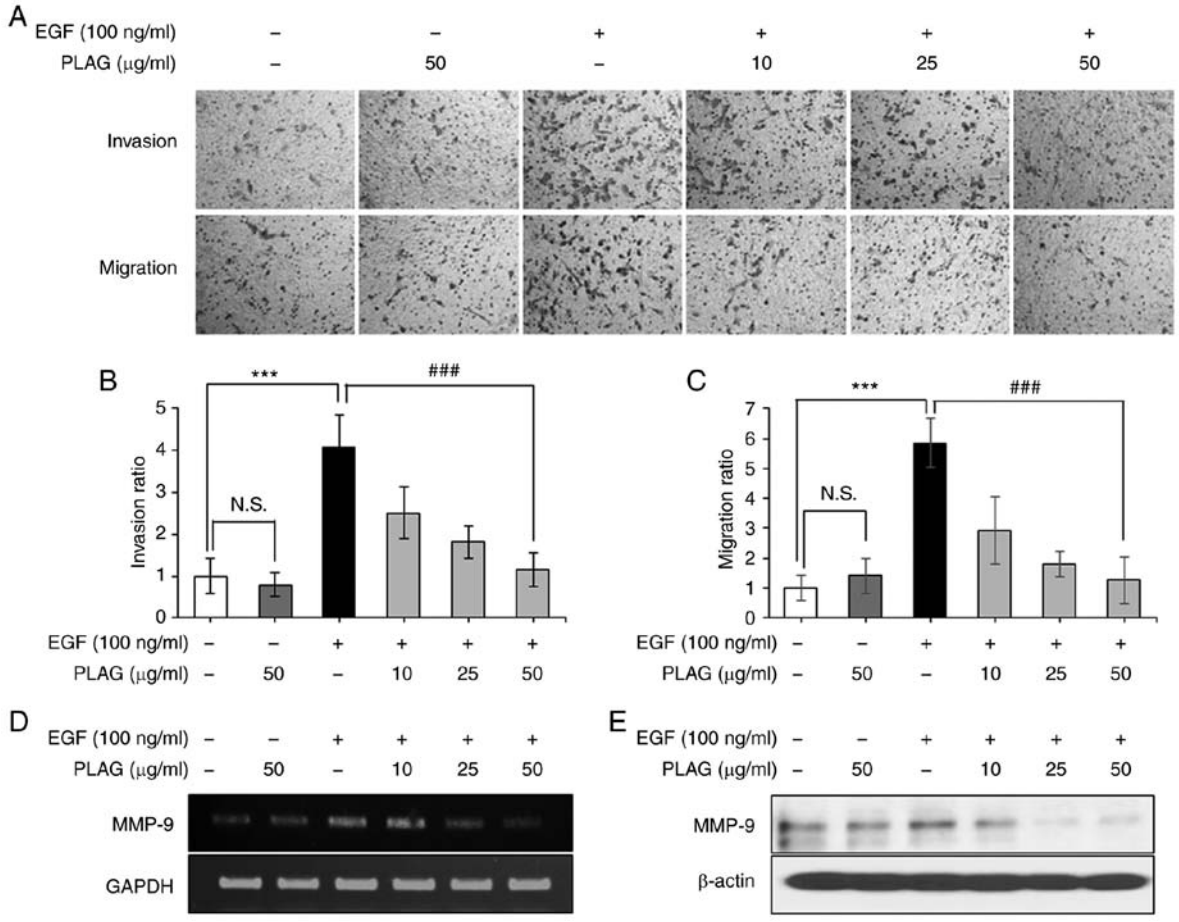

Figure 1. PLAG attenuates EGF-induced invasiveness and migration via MMP-9 downregulation. (A-C) PLAG inhibits migration and invasiveness in EGF-treated cells. Invasive and migrating cells were assessed by counting using a light microscope at x200. MMP-9 expression was attenuated by PLAG. MMP-9 expression was analyzed by (D) RT-PCR at 6 h, and by (E) western blotting at 24 h, after stimulation. Statistical significance was determined by ANOVA (Tukey's test). ***P<0.005, compared with the untreated group; $^{###}$P<0.005, compared with the EGF only treated group. N.S., not significant; PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; MMP-9, matrix metalloproteinase 9.

at different time intervals (Fig. 2A-C) and by confocal microscope analysis (Fig. 2D). Internalized surface EGFRs did not return to the plasma membrane (data not shown). Degradation of these internalized EGFRs was also examined using an assay for ubiquitination. After EGF stimulation, ubiquitinated EGFRs were observed at 30 min, and were sustained for 60 min in the immunoprecipitation assay. In PLAG-treated cells, ubiquitinated EGFRs were observed 5 min after EGF treatment and sustained for 30 min. Complexes with ubiquitin ligase, c-Cbl, and EPS15 were also detected. These dissociated at earlier time points in the PLAG-treated cells than in EGF-only treated cells, suggesting that PLAG accelerates ligand-stimulated EGFR internalization and degradation (Fig. 2E). These activities eventually led to EGFR desensitization and contributed to preventing the mobility and invasiveness mediated by EGFR activation.

PLAG accelerates intracellular trafficking and degradation of EGFRs. Degradation of the internalized EGFRs was further investigated using a colocalization assay with Rab5 and Rab7. Rab5 is required for intracellular trafficking of EGFR to endosomal compartments, leading to EGFR degradation (26). Rab7 regulates membrane trafficking at the late endosome-lysosome stage (27).

In confocal microscope images, the overlap of red PE-conjugated EGFRs with green Alexa 488-bound Rab5 appeared yellow, indicating colocalization of EGFRs and Rab5. Yellow EGFR and Rab5 complex assemblies appeared 10 min after EGF stimulation and were sustained for 60 min. The same yellow EGFR and Rab5 complexes in PLAG-treated cells were detected within 5 min and dissociated by 30 min (Fig. 3A). Colocalization of EGFR and Rab5 was quantified by measuring fluorescence intensity in the images (Fig. 3B). These data indicate that PLAG accelerates not only the assembly, but also the degradation, of EGFR complexes.

Using the same assay, the colocalization EGFR and Rab7, a late endosome marker, was observed at 15 min and disappeared at 120 min in EGF-only stimulated cells. In contrast, PLAG-treated cells exhibited EGFR-Rab7 colocalization within 5 min and dissociated by 90 min (Fig. 3C). This colocalization of EGFR and Rab7 was also verified by measuring fluorescence intensity in the confocal images (Fig. 3D). These colocalization assay results show that PLAG may accelerate ligand-bound EGFR intracellular trafficking and EGFR degradation via lysosome sorting.

Signals originating from EGFR activation are attenuated in PLAG-treated cells. The data presented above

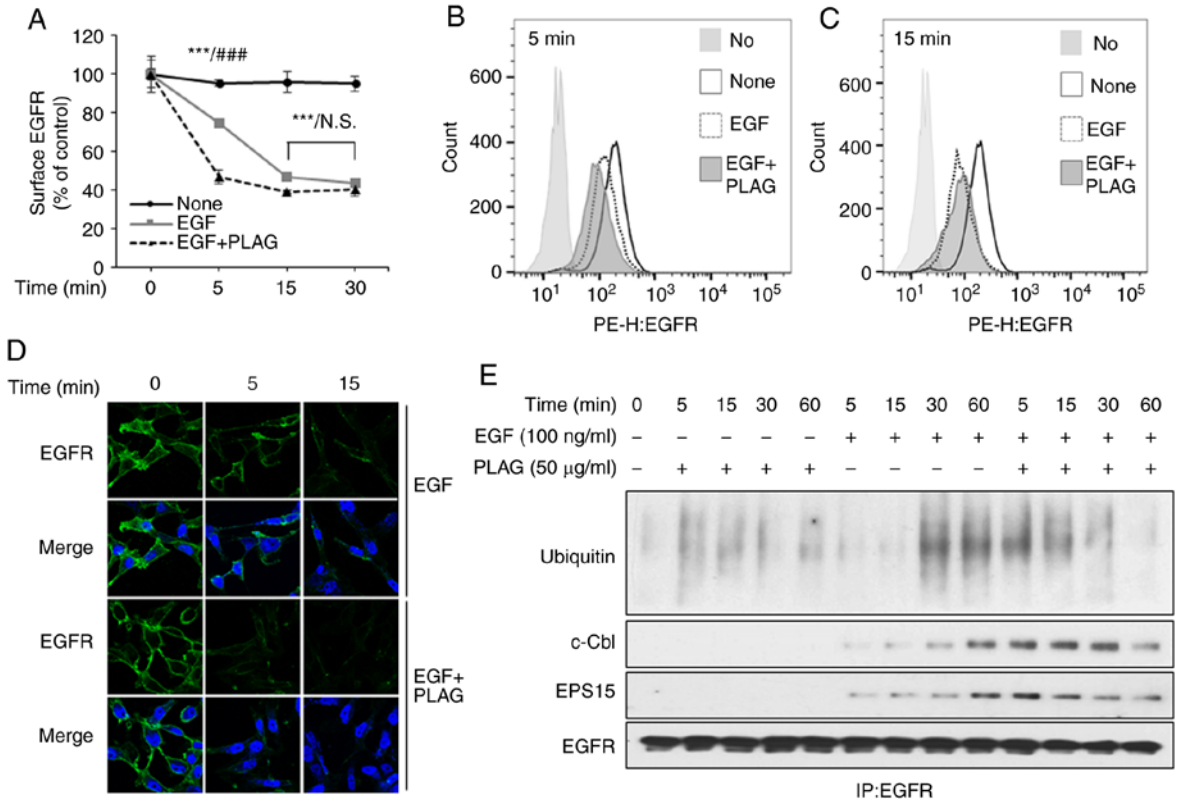

Figure 2. Accelerated EGFR endocytosis and ubiquitination in PLAG-treated cells. (A-D) MDA-MB-231 cells were pretreated with PLAG (10, 25, or 50 μg/ml) for 1 h and then treated with EGF (100 ng/ml). (A-C) Surface EGFRs were analyzed by flow cytometry, and the data are represented by means ± SD. (D) Surface EGFRs were detected by fluorescence confocal microscopy at x1,000. (E) EGFR-binding protein and ubiquitination were confirmed by western blotting via co-immunoprecipitation. Statistical significance was determined by ANOVA (Tukey's test). ***P<0.005, compared with the None group; $^{###}$P<0.005, compared with the EGF only treated group. N.S., not significant; PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPS15, EGFR pathway substrate 15.

demonstrated that EGFR degradation occurs later (at 90 min) in EGF-treated cells than in PLAG-treated cells (at 60 min). Western blot analysis was used to assess whether this accelerated EGFR degradation influenced EGFR-dependent signaling. EGFR degradation was observed at 120 min, and the decay of phosphorylated EGFRs was detected at 60 min in PLAG-treated cells; earlier than the degradation and decay times for EGF-only treated cells. In addition, phosphorylated SHC, ERK, and JNK induced by EGF stimulation was sustained for 120 min. In contrast, for PLAG-treated cells, phosphorylated SHC, ERK, and JNK were dephosphorylated at 60 min (Fig. 4A). The proportions of degraded EGFR (Fig. 4B) and phosphorylated EGFR (Fig. 4C), as well as those of phosphorylated SHC, ERK, and JNK, were all monitored over time (Fig. 4D-F). Similar studies have reported that herbacetin accelerates the internalization and degradation of EGFR, and subsequently suppressed the activation of the downstream kinase, ERK (28). In addition, the adaptor protein SHC has an essential role in the integration of EGFR signaling (29). Similar to the EGFR degradation findings, kinase-associated EGFR signaling for MMP-9 expression was also terminated earlier in PLAG-treated cells. Based on PLAG's unique mechanism for attenuating EGFR signaling, we further characterized kinase-activated AP-1, a major transcription factor regulating MMP-9 expression, using a luciferase assay. AP-1 activity induced by EGF treatment was reduced in PLAG-treated cells in a dose-dependent manner (Fig. 4G). These results provide further evidence for the potential role of PLAG in blocking metastasis-inducing EGFR activation.

PLAG accelerates EGFR internalization via TXNIP production. Our results demonstrated that PLAG reduces MMP-9 expression in EGF-stimulated cells. The data suggest that ligand-bound EGFRs affect intracellular trafficking and activate signals for MMP-9 expression, and that PLAG accelerates EGFR internalization and degradation, resulting in a reduced MMP-9 signal. Therefore, we focused on molecules modulated by PLAG treatment that are involved in receptor trafficking. In PLAG-treated cells, TXNIP mRNA and protein expression were elevated (Fig. 5A). Immunoprecipitation using the EGFR antibody revealed that elevated TXNIP was complexed with EGFR in the PLAG-treated cells (Fig. 5B). These assembly results were corroborated using RT-PCR in TXNIP-silenced cells, where TXNIP knockdown was detected (Fig. 5C). In addition, the accelerated internalization of surface EGFRs was no longer observed in the TXNIP-silenced cells (Fig. 5D). In the Transwell invasion and migration assays, TXNIP-silenced cells did not show PLAG-induced reductions in invasiveness and mobility (Fig. 5E-G). Downregulation of MMP-9 expression in PLAG-treated cells was also not observed after TXNIP-silencing (Fig. 5H). These results indicate that PLAG promotes the internalization of receptors by increasing the expression of TXNIP and that increased EGFR degradation reduces MMP-9 expression.

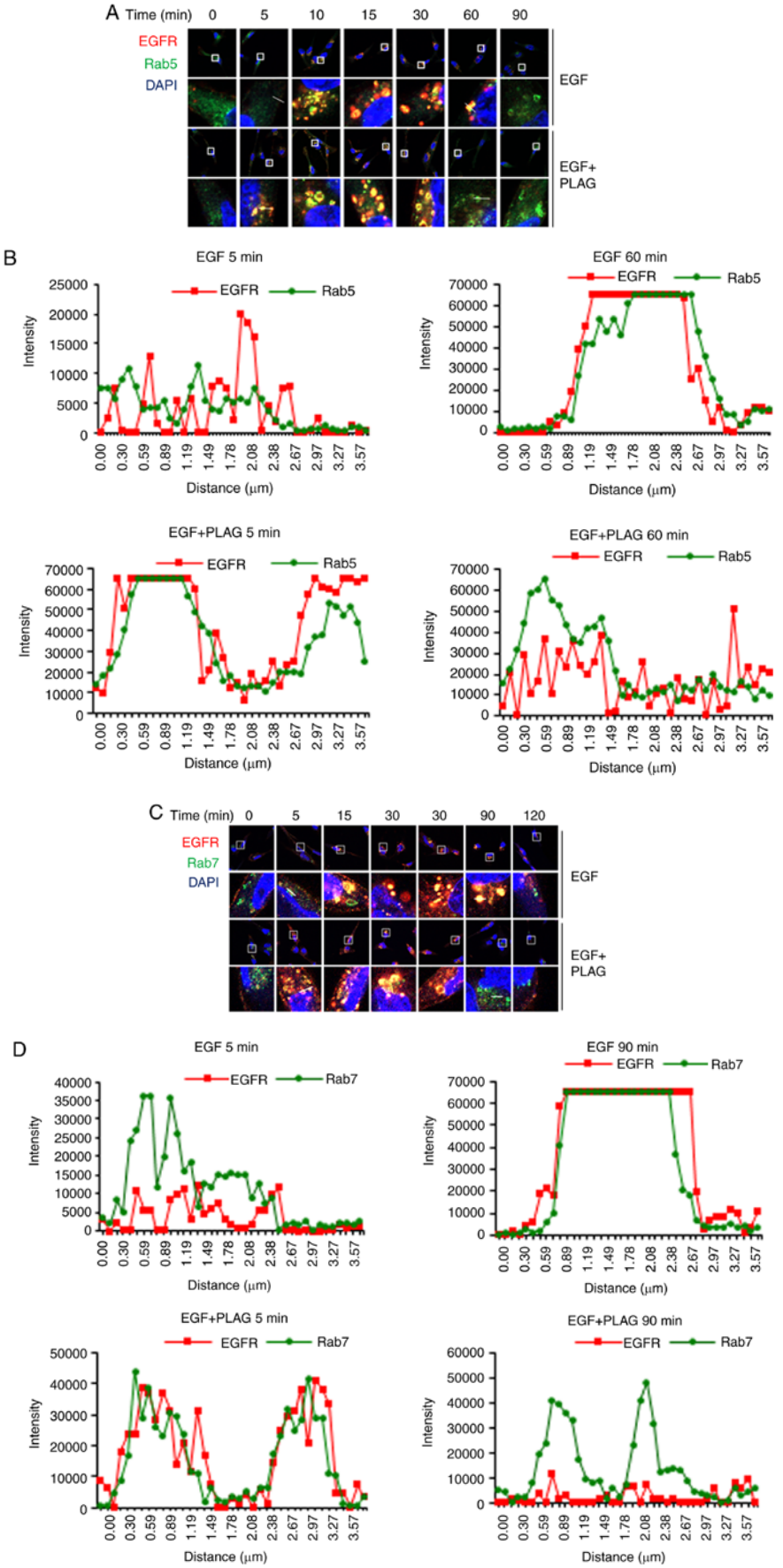

Figure 3. Colocalization analysis of EGFR intracellular trafficking. (A) Colocalization of EGFR (red) and Rab5 (green) was detected by immunofluorescence staining. (B) Graphs represent fluorescence intensity profiles calculated from white line in the image obtained from ZEN for EGFR and Rab5 (C) Colocalization of EGFR (red) and Rab7 (green) was detected by immunofluorescence staining. EGFR, Rab5, and Rab7 were imaged by fluorescence confocal microscopy at x630. (D) Graphs represent fluorescence intensity profiles calculated from white line in the image obtained from ZEN for EGFR and Rab7. The cells were counterstained with DAPI (blue) for visualization of nuclear morphology. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

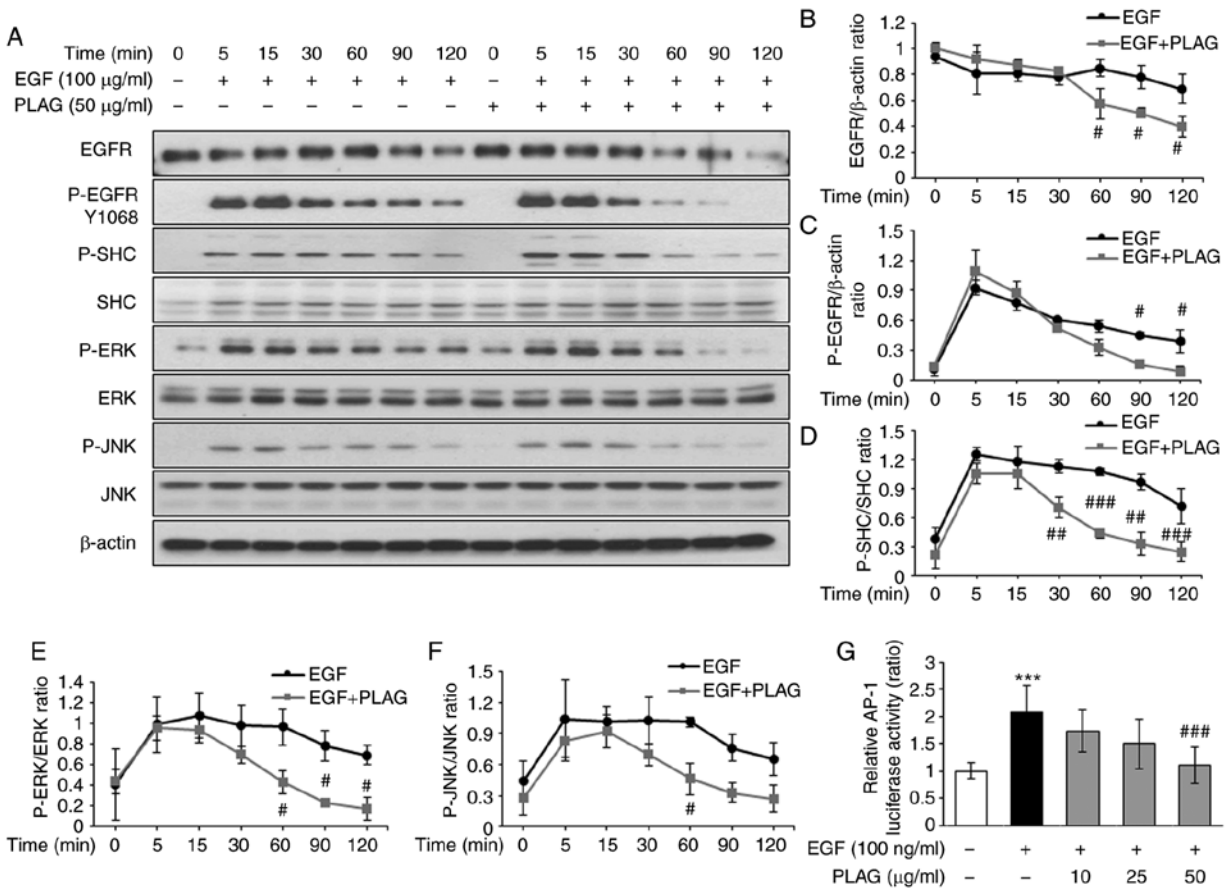

Figure 4. Attenuation of EGF-signaling by PLAG. (A-F) Western blot analysis of EGFR degradation and downstream phosphorylation. (G) To assess luciferase activity, MDA-MB-231 cells were transfected with constructs containing the AP-1 promoter. Cells treated with PLAG and EGF were cultured for 6 h, and luciferase activity was determined. Statistical significance was determined by ANOVA (Tukey's test). ***P<0.005, compared with the untreated group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.005, compared with the EGF group. N.S., not significant; PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; p-, phosphorylated.

PLAG has specificity for blocking cell migration and invasion. PLAG's specificity for inhibiting EGF-induced invasiveness and high mobility was verified by direct comparison with another diacylglycerol, 1-palmitoyl 2-linoleoyl 3-hydroxylglycerol (PLH) (23). In the Transwell invasion and migration assays, EGF-only treated cancer cells (100 ng/ml) showed high invasiveness and mobility, whereas PLAG-treated cells (25 or 50 μg/ml) showed a significant reduction in invasiveness and mobility in a dose-dependent manner; this was not seen in the PLH-treated cells (25 or 50 μg/ml) (Fig. 6A-C). PLAG and PLH treatments were further compared for the modulation of MMP-9 expression. Whereas PLAG-treatment (50 μg/ml) of EGF-treated cells reduced MMP-9 expression, PLH treatment (50 μg/ml) did not (Fig. 6D). We also compared TXNIP expression and complexing with EGFR using the two treatment conditions. In PLAG-treated cells, TXNIP mRNA and protein expression was enhanced, but in PLH-treated cells TXNIP mRNA and protein expression was not (Fig. 6E). TXNIP and EGFR complexes were enhanced in PLAG-treated cells, but in PLH-treated cells, they were not (Fig. 6F). These data indicate that PLAG shows specificity for inhibiting EGF-induced tumor cell mobility and suggest that the acetylated 3-position of the molecule is important for recognition of its cognate receptor and for activation of related signaling pathways.

Discussion

EGFR ubiquitination induces receptor degradation and consequently attenuates EGFR signaling (30). Upon EGF stimulation, several endocytic accessory factors are also ubiquitinated and cotraffic with EGFR along the endocytic pathway (15,31). Recruitment of the E3 ubiquitin ligase, c-Cbl, to activated EGFRs is a key event for receptor ubiquitination and is also associated with EGFR degradation (32). Trafficking ubiquitinated receptors into the endosome requires multiple proteins, one of which is the scaffolding protein EPS15 (33). EPS15 has a ubiquitin-interacting motif that mediates the early steps of EGFR endocytosis (34,35). Rab5, an early endosome marker, is also a checkpoint protein that plays a role in the endocytic pathway, designating whether or not an endocytosed receptor is sorted to the late endosome/lysosome compartment or recycled back to the plasma membrane (36). For early and late endosomes, Rab7 complexes are essential for endocytic trafficking and lysosomal degradation (37).

Our results have demonstrated that PLAG accelerates endocytosis, ubiquitination, and lysosomal degradation of ligand-bound EGFRs. The complexing of c-Cbl and EPS15 with EGFR was verified by immunoprecipitation with anti-EGFR antibody, and EGFR localization to early/late endosomes was assessed by examining complexes using anti-Rab5 and

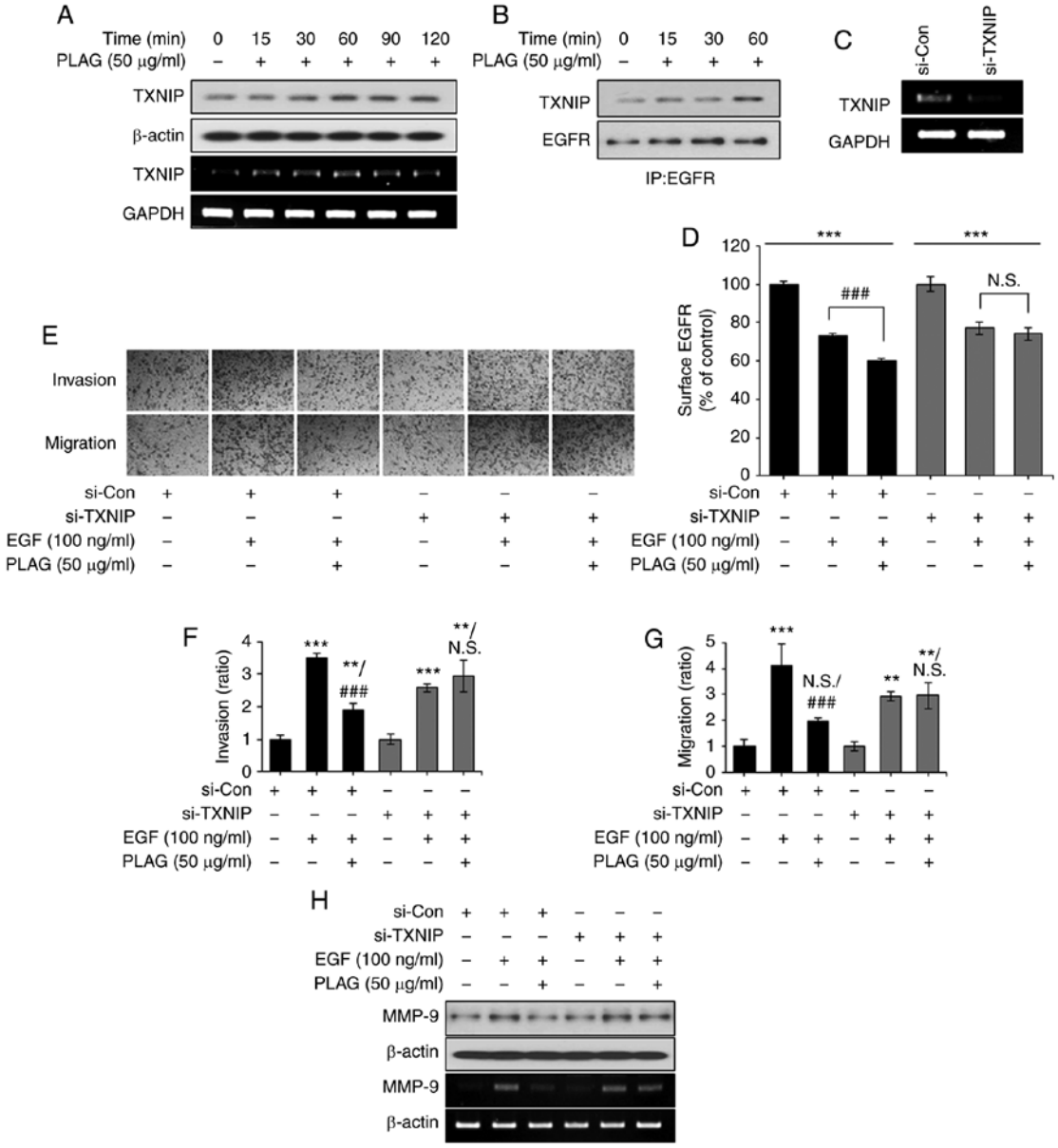

Figure 5. TXNIP induction by PLAG treatment. (A) TXNIP mRNA and protein expression was analyzed by RT-PCR and western blotting, respectively. (B) EGFR-bound TXNIP was confirmed by western blotting via co-immunoprecipitation in PLAG-only treated cells. (C) TXNIP knockdown was confirmed by RT-PCR. (D) After treatment with PLAG and EGF, EGFR internalization was analyzed by flow cytometry. (E-G) PLAG did not reduce EGF-induced cell migration and invasion in TXNIP-silenced cells. Invasive and migrating cells were counted in the assay at x200. (H) MMP-9 expression was not modified in the si-TXNIP treated cells. MMP-9 expression was analyzed by RT-PCR 6 h, and by western blotting 24 h, after stimulation. Statistical significance was determined by ANOVA (Tukey's test). **P<0.01 and ***P<0.005, compared with the untreated group; $^{###}$P<0.005, compared with the EGF group. N.S., not significant; PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MMP-9, matrix metalloproteinase 9; TXNIP, thioredoxin-interacting protein; si-Con, scrambled siRNA transfected MDA-MB-231 breast cancer cells; si-TXNIP, TXNIP siRNA transfected MDA-MB-231 breast cancer cells.

anti-Rab7 antibodies. PLAG-only treatment did not induce EGFR endocytosis, which means that PLAG does not activate EGFR on its own. PLAG-cotreated cells showed similar surface EGFR levels compared with EGF-only treated cells. However, EGFR endocytosis was increased the most by PLAG pretreatment when compared with other groups. These results demonstrate that PLAG does not induce EGFR endocytosis by itself, and it requires about 60 min to exhibit its effect in EGFR endocytosis (Fig. S1). PLAG induced weak ubiquitination, but c-Cbl and EPS15 were not complexed with EGFR. EGFR ubiquitination can be induced by other factors such as calcium and oxidative stress (12,38). In the present study, PLAG increased calcium inflex (data not shown). PLAG induced weak ubiquitination through other factors, not through EGF-mediated ubiquitin ligase molecules. This result indicates that PLAG has no effect itself in the EGF-mediated process. Formation of these complexes and EGFR localization to endosomes was accelerated in PLAG-treated cells, suggesting that PLAG promoted EGFR degradation.

Modulation of EGFR signaling pathways was investigated in PLAG-treated cells. The EGF-bound receptor is known to activate multiple signaling pathways, including MAPK, phosphatidylinositol 3-kinase (PIK3/Akt, and nuclear factor (NF)-κB (39,40). Of interest here, the expression of MMP-9 is

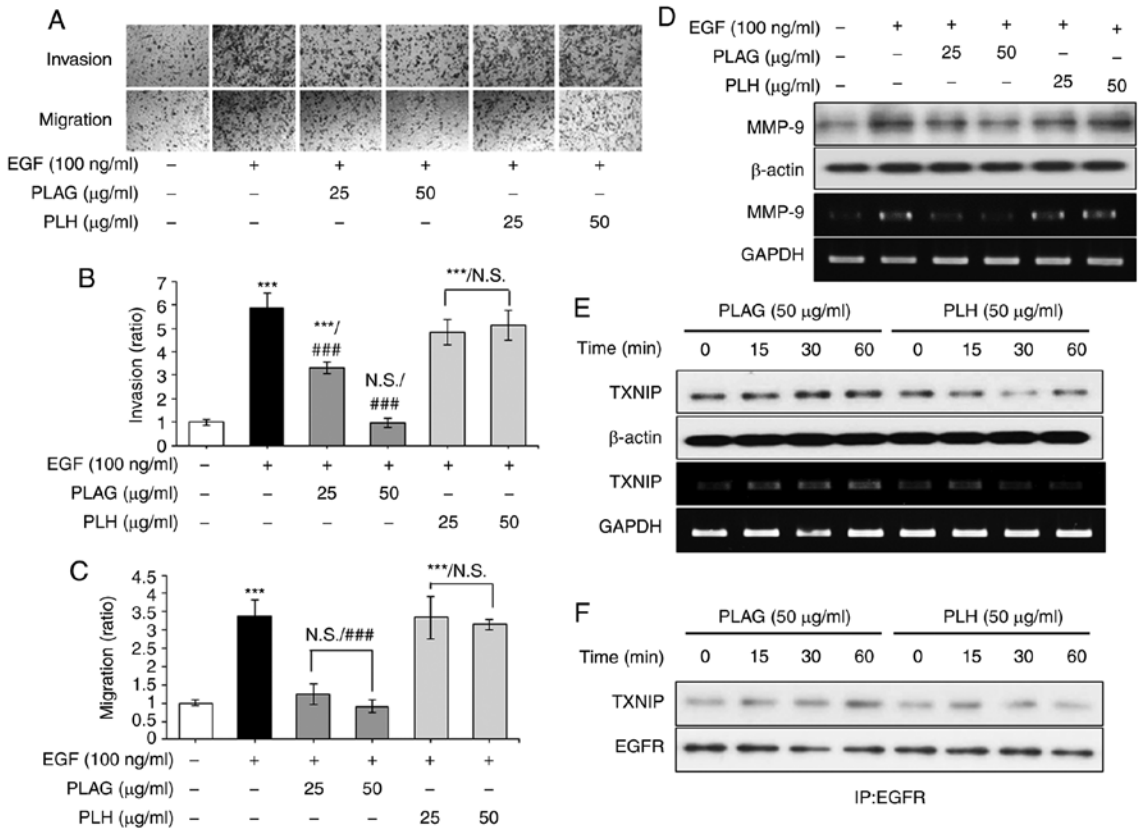

Figure 6. PLAG-specific treatment induces TXNIP. (A-C) PLH did not inhibit EGF-induced cell migration and invasion. Cells were counted in the invasion and migration cell assays. (D) MMP-9 expression was not modified in the PLH-treated cells. MMP-9 expression was analyzed by RT-PCR at 6 h, and by western blotting at 24 h, after stimulation. (E) TXNIP mRNA and protein expression was analyzed by RT-PCR and western blotting, respectively. (F) EGFR and TXNIP complexing was confirmed by western blotting via co-immunoprecipitation in PLAG- and PLH-treated cells. Statistical significance was determined by ANOVA (Tukey's test). ***P<0.005, compared with the untreated group; $^{###}$P<0.005, compared with the EGF group. N.S., not significant; PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TXNIP, thioredoxin-interacting protein; PLH, 1-palmitoyl 2-linoleoyl 3-hydroxylglycerol.

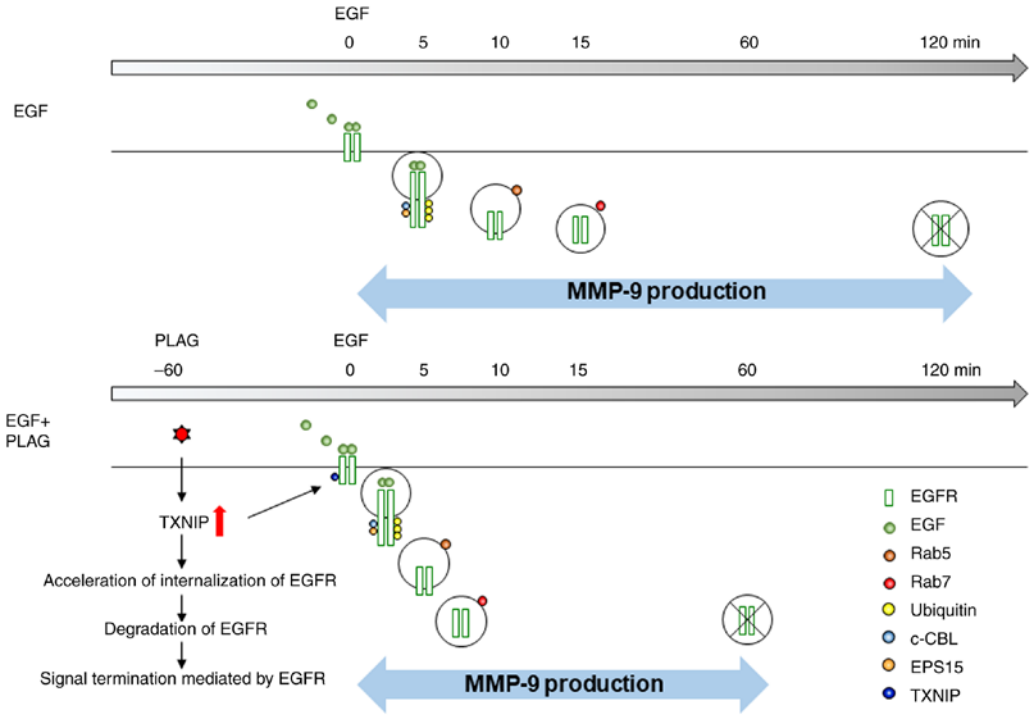

Figure 7. Model of PLAG-induced TXNIP and MMP-9 expression in breast cancer cells. PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TXNIP, thioredoxin-interacting protein; MMP-9, matrix metalloproteinase 9; EPS15, EGFR pathway substrate 15.

associated with the metastasis of breast cancer cells (41,42). MMP expression in breast cancer cells is mediated by the MAPK signaling pathway (43). As intermediary signaling molecules for the process of EGFR-mediated MMP-9 expression, the phosphorylation states of SHC, ERK, and JNK were investigated. EGFR, SHC, ERK, and JNK phosphorylation was detected at 5 min and was sustained for 120 min in EGF-treated cells, but most of these signals disappeared by 60 min in 50 μg/ml PLAG-treated cells, suggesting that enhanced EGFR degradation in PLAG-treated cells limited downstream MMP-9 expression. As part of this downstream regulation, the transcription factor AP-1 (an MMP-9 promoter) was activated in EGF-treated cells, and PLAG treatment attenuated its enhanced activity in a dose-dependent manner.

As the results demonstrated, signaling molecules, including SHC and c-Cbl, were complexed with EGFRs and co-internalized into endosomes, resulting in degradation. This process was accelerated by PLAG treatment. The EGFR-dependent signaling for MMP-9 expression was reduced, and the invasiveness of these breast cancer cells was consequently attenuated by PLAG treatment. PLAG-only treatment exhibited no effect on invasion and migration and MMP-9 expression. This decreased MMP-9 expression by PLAG was initiated during EGFR internalization by the induction of thioredoxin-interacting protein (TXNIP) expression, and enhanced TXNIP expression is associated with trafficking to the endosome/lysosome, supporting the interaction with ubiquitin ligases (44). Therefore, in PLAG-treated cells, increased TXNIP expression promoted EGFR internalization and subsequent degradation.

PLAG specificity was verified by treating cells with a closely related diacylglycerol, PLH, and then comparing the two sets of results from the EGF-induced invasion and migration assays. In these assays, PLAG significantly reduced the invasiveness and migration of cells. But in PLH-treated cells, even though PLH has a similar lipid structure, these reductions were not observed. These PLAG results were regulated through the expression of TXNIP, and PLH did not alter TXNIP expression.

PLAG therapy has been shown to mitigate the effects of various diseases. Recent reports have documented that PLAG controlled neutrophil recruitment by regulating the trafficking of Toll-like receptors and enhanced efferocytosis through membrane redistribution of G protein-coupled receptors (45,46). We believe that these published studies have shown the ability of PLAG to modulate the movement of receptors, thus we hypothesized that PLAG may also modulate the trafficking of receptor tyrosine kinases (RTKs). We demonstrated that PLAG accelerates EGFR trafficking via TXNIP and that it reduces cancer cell invasiveness and mobility (Fig. 7). These results suggest that PLAG could be a specific agent for blocking metastasis in breast cancer via TXNIP regulation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author by reasonable request.

Authors' contributions

JWK made substantial contributions to the concept and design of the study. KHY acquired, analyzed and interpreted the data. SC made substantial contributions to the manuscript and critically revised it for important intellectual content. GTK assisted with technical support. SYY analyzed the data and prepared the figures. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the research to ensure that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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