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# Resveratrol Modulates MED28 (Magicin/EG-1) Expression and Inhibits Epidermal Growth Factor (EGF)-Induced Migration in MDA-MB-231 Human Breast Cancer Cells

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Supporting Information

**ABSTRACT:** Resveratrol and pterostilbene exhibit diverse biological activities. MED28, a subunit of the mammalian Mediator complex for transcription, was also identified as magicin, an actin cytoskeleton Grb2-associated protein, and as endothelial-derived gene (*EG-1*). Several tumors exhibit aberrant MED28 expression, whereas the underlying mechanism is unclear. Triple-negative breast cancers, often expressing epidermal growth factor (EGF) receptor (EGFR), are associated with metastasis and poor survival. The objective of this study is to compare the effect of resveratrol and pterostilbene and to investigate the role of MED28 in EGFR-overexpressing MDA-MB-231 breast cancer cells. Pretreatment of resveratrol, but not pterostlbene, suppressed EGF-mediated migration and expression of MED28 and matrix metalloproteinase (MMP)-9 in MDA-MB-231 cells. Moreover, overexpression of MED28 increased migration, and the addition of EGF further enhanced migration. Our data indicate that resveratrol modulates the effect of MED28 on cellular migration, presumably through the EGFR/phosphatidylinositol 3-kinase (PI3K) signaling pathway, in breast cancer cells.

**KEYWORDS:** magicin, MED28, resveratrol, epidermal growth factor receptor, MDA-MB-231, matrix metalloproteinase

## INTRODUCTION

Some plants produce stilbenes, secondary metabolites, in response to environmental stress, such as fungal infection or ultraviolet irradiation, as part of their defense system.[1] Two examples of phytoalexins, resveratrol (*trans*-3,5,4′-trihydroxystilbene) and pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene, a dimethylated analogue of resveratrol), can be found in grapes and berries, among others.[1,2] Although concerns for the pharmacokinetics and bioavailability of stilbenes have been raised, the majority of the studies have indicated auspicious preventive applications for both resveratrol (RS) and pterostilbene (PS).[3] Ample studies have shown that RS exhibits pleiotropic biological roles, including cardioprotective, antiaging, and anticancer activities.[4−7] For example, RS has been shown to inhibit tumor development in a variety of cell culture systems,[8−10] including breast cancer cells.[7] RS has also been shown to prevent angiogenesis and wound healing of endothelial cells, which make it a good candidate for the prevention of cancer progression.[11,12] Similarly, PS also exhibits potential pharmacological effects, including anticancer, antioxidant, and antiproliferative activities.[3,13,14]

Epidermal growth factor (EGF) receptor (EGFR) family members, including EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4, become activated and dimerized upon EGF and/or related ligand binding.[15] The conformational changes and multiple phosphorylation events of the receptors lead to the coupling of the downstream signal transduction pathways which result in proliferation, migration, or survival.[15,16] Overexpression of the EGFR family has been found to be associated with cancer development,[17,18] including the malignancies in the reproductive systems, such as breast cancer,[18,19] which is one of the leading causes of mortality in women.[20] Specifically, HER2 overexpression has been identified in many breast cancer cases, and clinical intervention with Herceptin (trastuzumab), monoclonal antibodies against HER2, has been approved to treat breast cancer.[15] However, some patients do not respond well or develop resistance to the drug.[21] EGFR, on the other hand, was once thought to play a minor role in breast cancer until recently.[16]

Matrix metalloproteinases (MMPs), including collagenases, gelatinases, and stromelysins, degrade extracellular matrix components according to their substrate specificity.[22] These enzymes are synthesized as zymogen precursors and activated upon proteolytic cleavage.[22] The expression of MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) is associated with tumor migration, invasion, and metastasis in many types of human cancer.[23] MMP-2 is constitutively overexpressed in highly metastatic cancers,[24] whereas MMP-9 can be induced by cytokines or growth factors through the activation of intracellular signal transduction pathways. Both MMP-2 and MMP-9 have been implicated in metastasis gene signature for breast cancer.[25]

Independently identified by several research groups,[26−28] MED28 (magicin; EG-1) displays a number of cellular roles, including interactions with merlin, the neurofibromatosis 2 (NF2)

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tumor suppressor protein, actin cytoskeleton, and Grb2, an adaptor in many signaling pathways[26] and a Mediator subunit (MED28) for transcription.[28] In addition to being an actin cytoskeleton-associated protein and a Mediator subunit, MED28 has been named endothelial-derived gene (*EG-1*), a differentially expressed gene in endothelial cells, whose expression can be upregulated by angiogenic factors.[27] MED28 overexpression may stimulate cell proliferation both *in vivo* and *in vitro*.[29] In contrast, small interfering RNA (siRNA) targeting *MED28* or polyclonal antibodies against MED28 inhibited breast cancer cell growth and xenografts in mice.[30] Moreover, MED28 was found highly expressed in breast, colorectal, and prostate cancers[31] and its expression in breast cancer lesions was higher than that of normal tissue, which predicts a poorer survival rate.[32] Furthermore, MED28 is also associated with the Src-family tyrosine kinases[33,34] where higher Src protein expression and kinase activity are often involved in aberrant growth factor signaling pathways in breast cancer.[35,36] Taken together, the association of MED28 with multiple signaling molecules and the aberrant expression of MED28 in several tumors strongly support a role of MED28 in breast cancer progression.

This study was to investigate the role of MED28 in cellular migration and compare any intervention effect of RS and PS on the role of MED28. MDA-MB-231 was used as a model for EGF-induced cell migration, as indicated by the expression of MMP-9, for breast cancer cells. We found that MED28 appears to potentiate EGF-induced cellular migration, whose effect may be modulated by RS. Our data suggest a novel role of MED28 in cell migration, reinforcing the importance of this multifaceted protein, and raise a possibility of clinical application in the combination of RS supplementation and MED28 suppression in breast cancer.

## MATERIALS AND METHODS

**Chemicals and Reagents.** All chemicals and reagents were obtained from Sigma, Inc. (St. Louis, MO), and antibodies were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise indicated. RS was synthesized using 4-methoxybenzyl alcohol and 3,5-dimethoxybenzaldehyde as the starting materials, and PS was prepared using a similar strategy as described before.[37] Both compounds were kindly provided by Dr. Chi-Tang Ho (Rutgers University, NJ). LY-294002, a PI3K inhibitor, was purchased from Calbiochem (EMD Chemical, San Diego, CA). MED28 antibodies were purchased from Abcam (San Francisco, CA). Human breast cancer cell lines including MDA-MB-231, BT-474, and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA).

**Cell Culture.** All cell culture reagents and materials were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. Human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 containing 10% fetal bovine serum (FBS), 100 mg/L streptomycin, and 100,000 units/L penicillin in a 37 °C humidified atmosphere with 5% $CO_2$.

**Cell Viability Assay.** Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (MTT) assay. MDA-MB-231 cells were subcultured into 96-well culture plates at a density of $5 \times 10^4$ per well in 100 $\mu$L of DMEM medium. The next day, the medium was changed and EGF (100 ng/mL), with or without various concentrations of RS or PS, was added for another 24 h. Control cells were treated with dimethyl sulfoxide (DMSO) to yield a final concentration of 0.05% (v/v). At the conclusion of the incubation, 150 $\mu$L of MTT was added to each well, and the plates were incubated at 37 °C for 3 h. The absorbance of the oxidized MTT was detected at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA). All experiments were performed in triplicate.

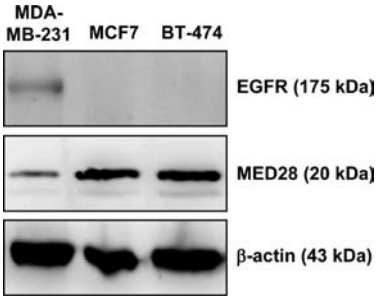


**Figure 1.** EGFR and MED28 expression in MDA-MB-231, MCF7, and BT-474 human breast cancer cells. MDA-MB-231, MCF7, and BT-474 cells were cultured as described under Materials and Methods. Fifty micrograms of total lysates from each cell line was analyzed for the expression of EGFR and MED28 with respective antibodies, where $\beta$-actin serves as a loading control.

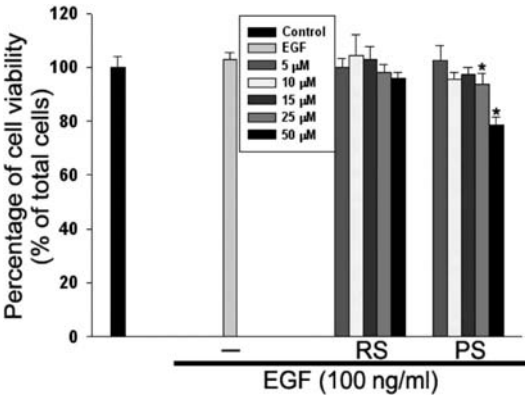


**Figure 2.** Effect of RS and PS on cell viability. MDA-MB-231 cells were subcultured into 96-well plates at a density of $5 \times 10^4$ per well. After 12 h of incubation, EGF (100 ng/mL), with or without various concentrations of RS or PS, was added. Control cells were treated with DMSO to yield a final concentration of 0.05% (v/v). After another 24 h, the cells were washed with PBS, and 150 $\mu$L of 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (MTT) was added to each well and incubated ar 37 °C for 3 h. The oxidized MTT solution was detected at 570 nm by an ELISA reader (*, $p < 0.05$ compared with the EGF treatment alone).

**Wound-Healing Assay.** MDA-MB-231 cells were grown to 90% confluence in a 6-well plate in a 37 °C, 5% $CO_2$ incubator. A wound was created by scratching cells with a sterile 200 $\mu$L pipet tip. The cells were washed twice with phosphate-buffered saline (PBS) to remove the floating cells, and then a serum-free medium containing EGF (100 ng/mL) was added, with or without various concentrations of RS or PS, for 24 h. For the experiments with MED28 overexpression, cells were first transiently transfected with a control plasmid or HA-MED28 plasmid (MED+), followed by the EGF treatment. For the experiments with LY-294002, cells were pretreated with 10 $\mu$M or 20 $\mu$M LY-294002 for 30 min, and then EGF was added for 24 h. Photos of the wound were taken under 40× magnification.

**Transwell Migration Assay.** The migratory ability of MDA-MB-231 cells was also determined using Transwell migration chamber with 6.5 mm diameter polycarbonate filters (8 $\mu$m pore size, Millipore Corp., Bedford, MA). The upper compartment contained $5 \times 10^4$ cells in 200 $\mu$L of serum-free medium with or without RS or PS, EGF (positive control),

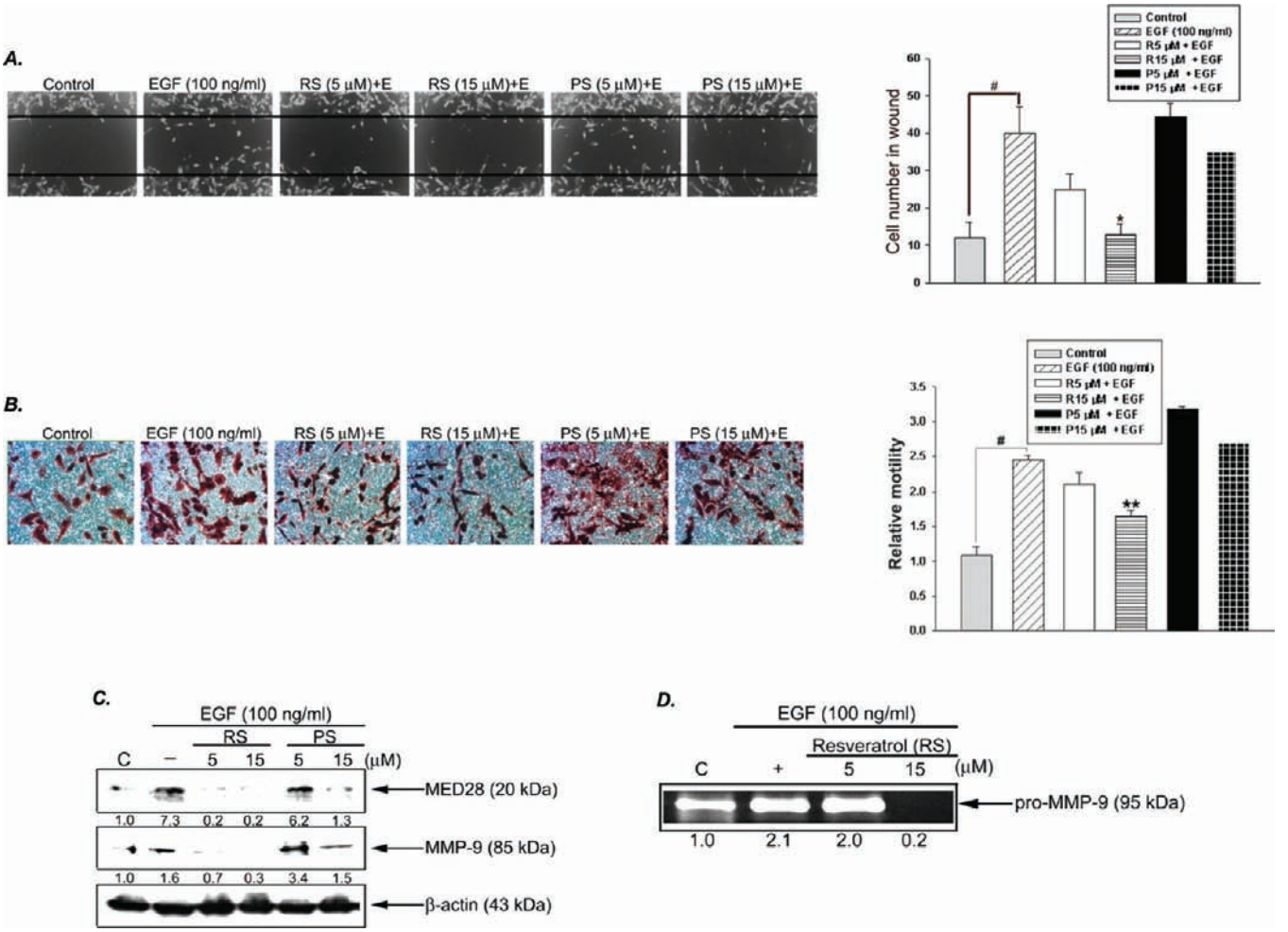


**Figure 3.** Effect of RS and PS on cell migration. (*A*) MDA-MB-231 cells were grown to 90% confluence in a 6-well plate in a 37 °C, 5% $CO_2$ incubator. Cells were scratched to create a wound with a sterile 200 $\mu$L pipet tip and washed twice with PBS to remove the floating cells. Serum-free medium (control), EGF, or EGF with 5 or 15 $\mu$M RS or PS was then added as indicated. Photos of the wound were taken under 40× magnification. Cell numbers in wound for each treatment were quantified. #, $p < 0.05$ as compared with control. ∗, $p < 0.05$ as compared with the EGF treatment (100 ng/mL). (*B*) MDA-MB-231 cells were applied onto a Transwell migration chamber (Millipore). The upper compartment contained $5 \times 10^4$ cells in 200 $\mu$L of serum-free medium, in the presence or absence of RS, PS, or EGF; the lower compartment contained culture medium with 10% FBS. After 24 h, nonmigrating cells were scraped from the upper surface of the membrane with a cotton swab, and the cells remaining on the bottom surface were counted after staining with crystal violet. Photos of the migrated cells were taken under 100× magnification. #, $p < 0.01$ as compared with control. ∗∗, $p < 0.01$ as compared with the EGF treatment. (*C*) After 12 h of incubation, serum-free medium (control) or EGF (100 ng/mL), with or without RS or PS was added. After another 24 h, total cell lysates were harvested, quantified, and probed with anti-MMP-9 or MED28 antibodies by Western blotting. $\beta$-Actin serves as a loading control. (*D*) Serum-free medium (control) or EGF (100 ng/mL), with or without RS, was added for 24 h. The medium was collected and measured for MMP-9 secretion by zymography as described under Materials and Methods.

or LY-294002; the lower compartment contained culture medium with 10% FBS. After 24 h, nonmigrating cells were scraped from the upper surface of the membrane with a cotton swab, and the cells remaining on the bottom surface were counted after staining with crystal violet. Photos of the migrated cells were taken under 100× magnification. For the experiments with MED28 overexpression, cells were first transiently transfected with a control plasmid or HA-MED28 plasmid (MED+), followed by the EGF treatment. The experiments were performed in triplicate and repeated three times.

**Gelatin Zymography.** MDA-MB-231 cells were incubated in serum-free medium in the presence of EGF (100 ng/mL), with or without RS or LY-294002, for 24 h. The conditioned medium was collected and separated on 10% sodium dodecyl sulfate−polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 in distilled water for 30 min twice at room temperature and then incubated in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% $NaN_3$ and 1% Triton X-100, pH 8.0) at 37 °C for 18 h. Bands corresponding to the secreted gelatinases were visualized by negative staining using 0.3% Coomassie blue in 50% methanol and 10% acetic acid. The secretion was detected as a clear band against dark blue background.

**Western Blotting.** Fifty micrograms of total cell lysates or cytosolic and membrane fractions were separated by SDS−PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were blocked using a blocking solution (20 mM Tris-HCl, pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). The membrane was then further incubated with specific antibodies against ERK, pERK1/2, p38, p-p38, JNK, pJNK, PI3K, p-PI3K, Akt, pAkt, MED28, EGFR, pEGFR (Y845, Y992, Y1045, and Y1068) or MMP-9 overnight at 4 °C. The membranes were then

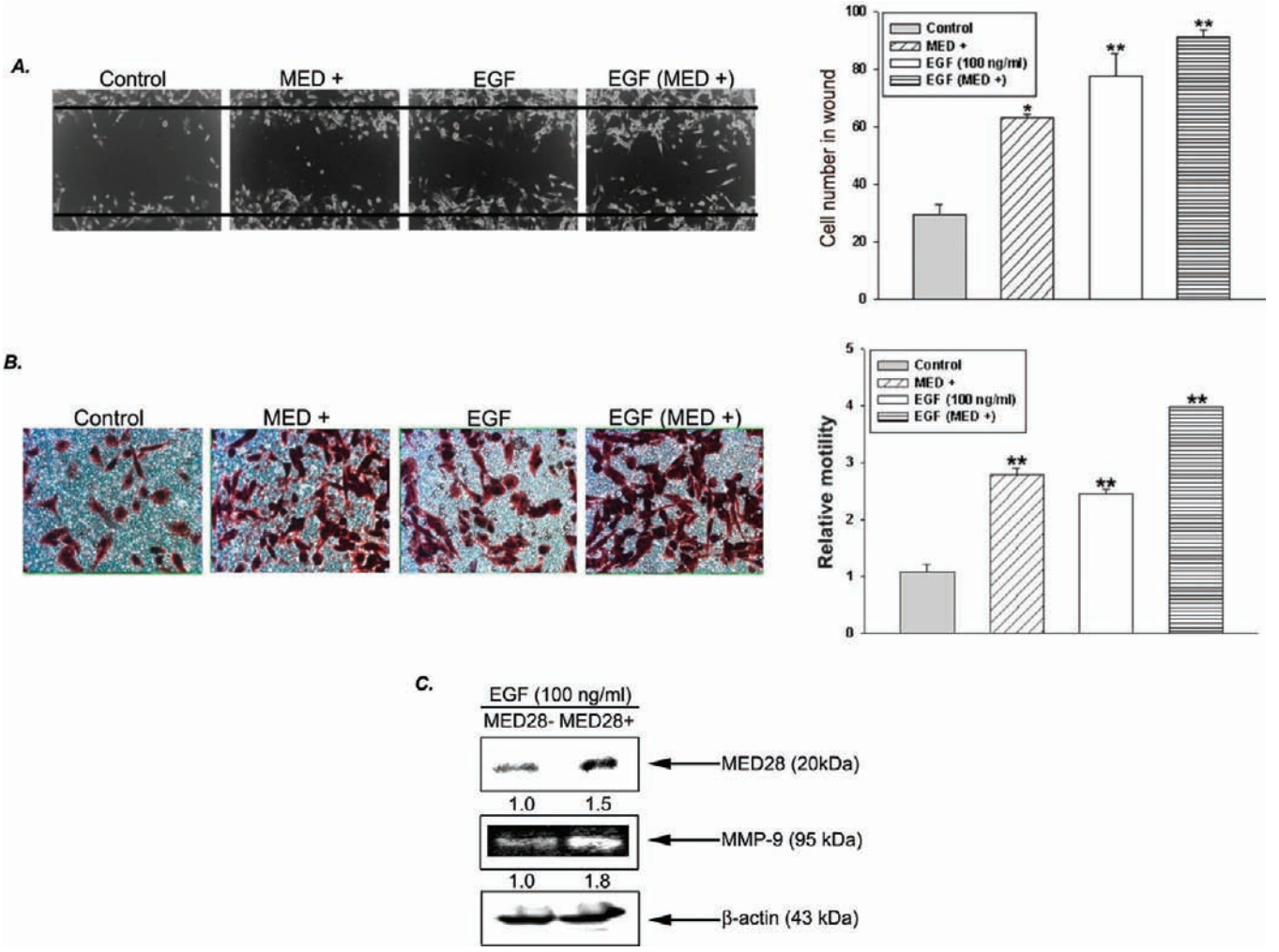


**Figure 4.** Effect of MED28 on cell migration. MDA-MB-231 cells were grown to 90% confluence. Cells were treated with serum-free medium (control) or EGF, with or without the transfection of the HA-MED28 plasmid (MED+). (*A*) A wound was created with a sterile 200 $\mu$L pipet tip and allowed to heal for 24 h. Photos of the wound were taken under 40× magnification. Cell numbers in wound for each treatment were quantified. *, $p < 0.05$, **, $p < 0.01$, compared with control. (*B*) Cells were applied onto a Transwell migration chamber (Millipore). The upper compartment contained $5 \times 10^4$ cells in 200 $\mu$L of serum-free medium with each respective treatment; the lower compartment contained culture medium with 10% FBS. After 24 h, nonmigrating cells were scraped from the upper surface of the membrane with a cotton swab, and the cells remaining on the bottom surface were counted after staining with crystal violet. Photos of the migrated cells were taken under 100× magnification. **, $p < 0.01$, as compared with control. (*C*) Cells were treated with EGF, with or without the transfection of the HA-MED28 plasmid (MED+). After 24 h, total cell lysates were harvested, quantified, and probed with anti-MED28 antibodies by Western blotting. $\beta$-Actin serves as a loading control. The medium was collected and measured for MMP-9 secretion by zymography as described under Materials and Methods.

probed with appropriate anti-mouse, anti-rabbit or anti-goat secondary antibodies, conjugated with horseradish peroxidase (Transduction Laboratories, Lexington, KY), and detection was achieved by measuring the chemiluminescence of the blotting agent, ECL (Amersham Corp., Arlington Heights, IL). The densities of the bands were quantified with a computerized densitometer (AlphaImager 2200 System, Alpha Innotech Corporation, San Leandro, CA). The total proteins were extracted via the addition of 200 $\mu$L of gold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1% NP-40, and 10 mg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 12000*g* for 30 min at 4 °C. The cytosolic fractions were extracted via the addition of 100 $\mu$L of hypotonic buffer (50 mM NaCl, 0.3 mM Na-orthovanadate, 50 mM NaF, 10 $\mu$g/mL leupeptin, and 5 $\mu$g/mL aprotinin) on ice for 30 min, followed by overnight at −80 °C and ice-out on room temperature recycle thrice, and then centrifugation at 12000*g* for 2 h at 4 °C. The pellets were combined with 100 $\mu$L of lysis buffer (25 mM Tris base, 250 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1 mM PMSF, 50 $\mu$g/mL leupeptin and 50 $\mu$g/mL aprotinin) on ice for 30 min, followed by centrifugation at 12000*g* for 2 h at 4 °C.

**Luciferase Assay.** The luciferase assay was performed as described previously.[38] Briefly, MDA-MB-231 cells were grown in a 10 cm culture dish, subconfluent cells were replaced with serum-free Opti-MEM (Invitrogen), and cells were transfected with the pNF-$\kappa$B-Luc luciferase reporter construct (Stratagene, La Jolla, CA) using LipofectAMINE reagent (Invitrogen). After transfection, the medium was replaced, and cells were cultured for 12 h. Cells were then plated in 24-well plates, serum-starved, and treated with EGF (100 ng/mL), with or without various concentrations of RS or PS, or LY-294002 for 24 h. Luciferase activity was measured by a luminometer using the Dual-luciferse reporter Assay (Promega, Madison, WI) according to the manufacturer's instruction.

**Statistical Analysis.** Data are presented as mean ± SE for at least three replicates. A one-way Student's *t* test was used to assess the

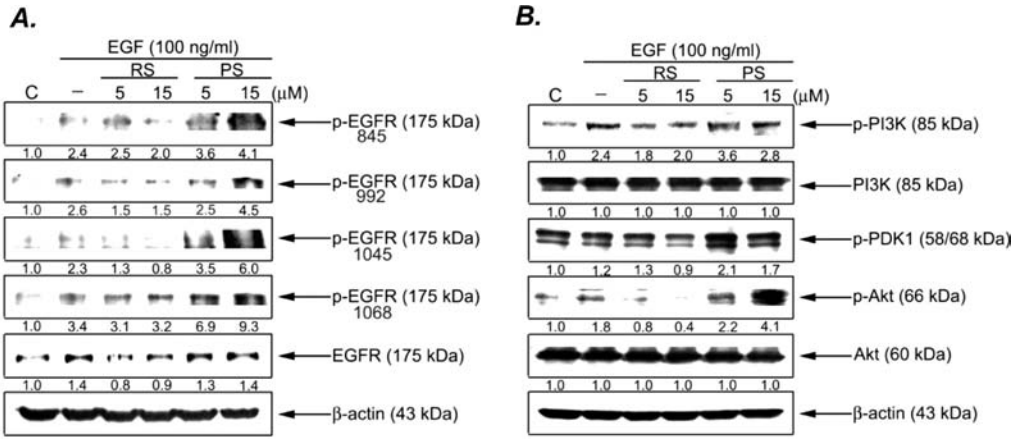


**Figure 5.** Effect of RS and PS on EGFR/PI3K signaling. MDA-MB-231 cells were grown to 90% confluence in a 6-well plate in a 37 °C, 5% $CO_2$ incubator. After 12 h, cells were incubated with serum-free medium (control) or EGF (100 ng/mL), with or without various concentrations of RS or PS. After another 24 h, total cell lysates were harvested, quantified, and probed with various anti-p-EGFR, EGFR (*A*), p-PI3K, PI3K, p-PDK1, pAkt, or Akt (*B*) antibodies by Western blotting. $\beta$-Actin serves as a loading control.

statistical significance between the EGF group and the other treatments. A $p$-value <0.05 was considered statistically significant.

## RESULTS

**EGFR and MED28 Expression in MDA-MB-231, MCF7, and BT-474 Human Breast Cancer Cells.** Three human breast cancer cell lines, MDA-MB-231, MCF7, and BT-474 cells, were analyzed for their expression of EGFR and MED28. As shown in Figure 1, only MDA-MB-231 cells express an appreciable amount of EGFR, whereas MCF7 and BT-474 cells do not express EGFR. On the other hand, MED28 is highly expressed in both MCF7 and BT-474 cells, but much less in MDA-MB-231 cells (Figure 1). Since our focus was to investigate the stimulatory effect of EGF, we chose MDA-MB-231 as our cellular model to study the role of MED28 in EGFR signaling.

**Effect of RS and PS on Cell Viability.** The MTT assay was employed to study cell viability upon RS or PS treatment. There was no statistical difference in cell viability among various doses (5−50 $\mu$M) of RS as compared with the EGF treatement (100 ng/mL), whereas both 25 and 50 $\mu$M of PS treatments resulted in cell death (mean cell viability 93.7% and 78.5%, respectively) in MDA-MB-231 human breast cancer cells (Figure 2). Therefore, two doses, 5 and 15 $\mu$M, of RS and PS were used in the subsequent experiments.

**Effect of RS and PS on Cell Migration.** Stepwise cancer development involves metastatic migration as well as uncontrolled cell growth. We used EGF as a promoter of cellular migration and compared the effect of RS and PS on cellular migration in MDA-MB-231 cells. As shown in Figure 3A,B, neither 5 $\mu$M nor 15 $\mu$M PS inhibited EGF-induced migration to any significant level, whereas 15 $\mu$M RS did. We also investigated the protein expression and secretion of MMP-9, an indicator of migratory ability, by Western blotting and zymography, respectively. MMP-9 protein and secretion were induced by EGF, but reduced upon the addition of 15 $\mu$M of RS treatment (Figure 3C,D). In contrast, PS did not suppress MMP-9 expression (Figure 3C). Interestingly, MED28 expression was also induced by EGF and decreased correspondingly with the addition of RS, but not as profoundly upon PS treatment (Figure 3C). The induction of MED28 and MMP-9 upon EGF stimulation and the concomitant reduction of MED28 and MMP-9 by RS suggest a potential link between RS, MED28, MMP-9, and cellular migration.

**Effect of MED28 on Cell Migration.** We next investigated any potential effect of MED28 on migration. Overexpression of MED28 alone significantly increased migration in MDA-MB-231 cells, as comparable with the addition of EGF, and the combination of EGF and MED28 overexpression enhanced migration even further (Figure 4). These data strongly suggest a role of MED28 in promoting cellular migration such that MED28 may by itself induce migration and the stimulation of EGF further amplifies the induction.

**Effect of RS and PS on EGFR/PI3K Signaling.** The potential downstream effectors of the EGFR signaling include the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK (p38), and phosphatidylinositol 3-kinase (PI3K). Therefore, we examined the activation of these putative targets upon EGF stimulation in MDA-MB-231 cells. Time-course (0−180 min) experiments were conducted to follow the activation status of these signaling molecules upon EGF stimulation. Neither ERK and JNK nor p38 was activated in our experimental setting, whereas the PI3K pathway was activated (Supplemental Figure 1 in the Supporting Information).

EGF can induce cellular migration, whereas RS and, to a much less extent, PS suppress the induction (Figure 3); these observations prompted us to investigate the changes of the downstream components associated with EGF-induced migration. Several tyrosine residues, Y845, Y992, Y1045, and Y1068, of EGFR were phosphorylated, and the expression of EGFR was increased upon EGF stimulation (Figure 5A). The EGFR expression and some phosphorylation events appeared reduced upon RS treatment, but not with the addition of PS (Figure 5A), indicating an inhibitory effect of RS on EGFR signaling. Since the MAPK family did not appear to be activated upon EGF induction in our study (Supplemental Figure 1 in the Supporting Information), we next focused on the effects of RS and PS on the PI3K pathway. As shown in Figure 5B, PI3K, PDK1, and Akt were phosphorylated and activated upon EGF stimulation. The addition of RS inhibited the EGF activation of the components in the PI3K pathway, whereas no suppression or even some activation was observed

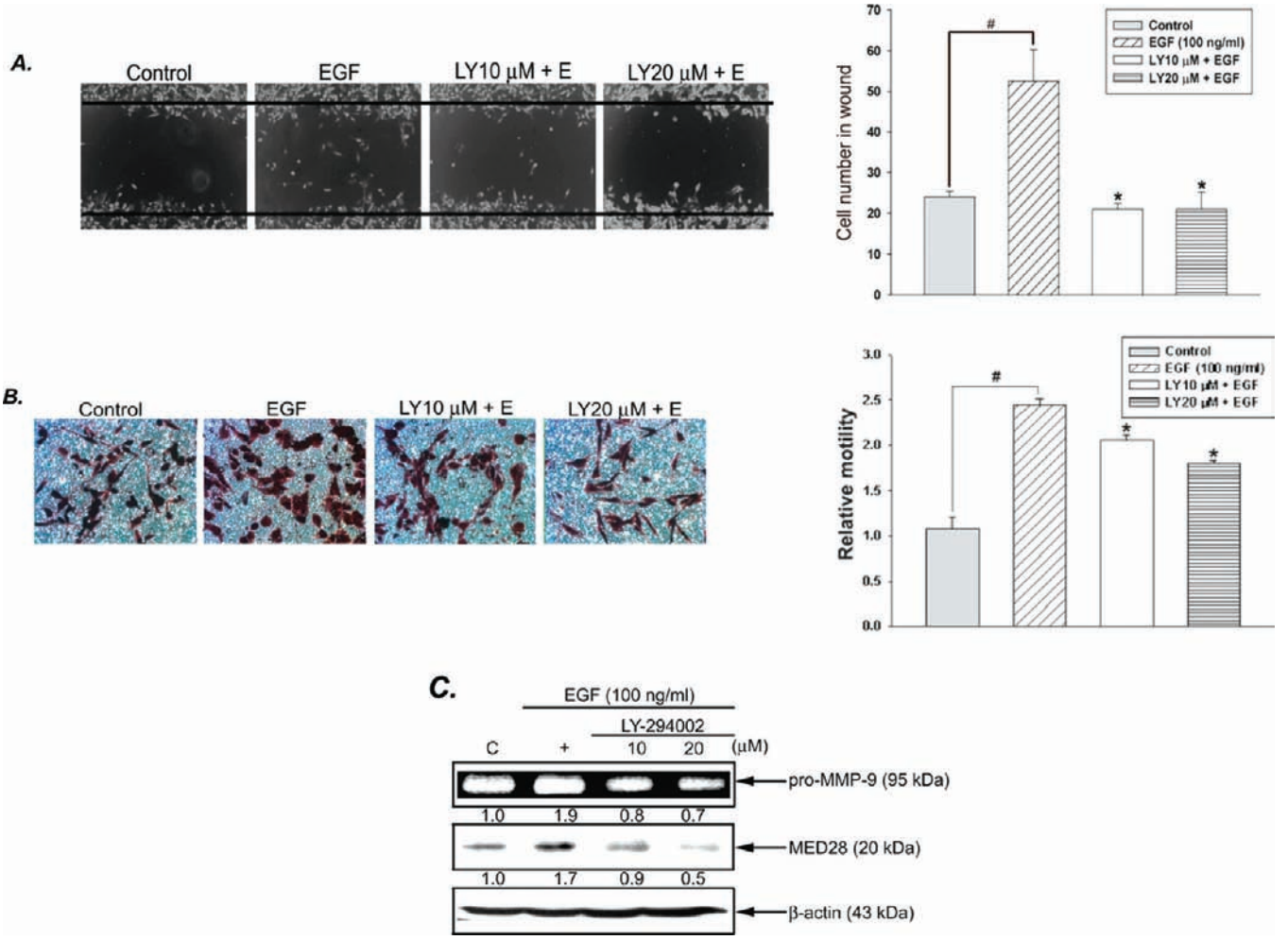


**Figure 6.** Effect of the PI3K inhibitor, LY-294002 on EGF-induced cell migration. MDA-MB-231 cells were grown to 90% confluence in a 6-well plate in a 37 °C, 5% $CO_2$ incubator. (*A*) Cells were scratched to create a wound with a sterile 200 $\mu$L pipet tip and washed twice with PBS to remove the floating cells. Serum-free medium (control), EGF (100 ng/mL), or EGF with 10 or 20 $\mu$M LY-294002 was then added as indicated. Photos of the wound were taken under 40× magnification. Cell numbers in wound for each treatment were quantified. #, $p < 0.05$ as compared with control. ∗, $p < 0.05$ as compared with the EGF treatment (100 ng/mL). (*B*) MDA-MB-231 cells were applied onto a Transwell migration chamber (Millipore). The upper compartment contained $5 \times 10^4$ cells in 200 $\mu$L of serum-free medium, in the presence or absence of LY-294002 or EGF; the lower compartment contained culture medium with 10% FBS. After 24 h, nonmigrating cells were scraped from the upper surface of the membrane with a cotton swab; the cells remaining on the bottom surface were counted after staining with crystal violet. Photos of the migrated cells were taken under 100× magnification. #, $p < 0.01$ as compared with control. ∗, $p < 0.05$ as compared with the EGF treatment (100 ng/mL). (*C*) Cells were incubated with serum-free medium (control), EGF (100 ng/mL), or EGF with 10 or 20 $\mu$M LY-294002 for 24 h. The medium was collected and measured for MMP-9 secretion by zymography as described under Materials and Methods. Total cell lysates were harvested, quantified, and probed with anti-MED28 antibodies by Western blotting. $\beta$-Actin serves as a loading control.

upon PS treatment (Figure 5B). These data suggest that RS has a stronger inhibitory effect than that of PS on EGFR/PI3K activation in MDA-MB-231 human breast cancer cells.

**Effect of the PI3K Inhibitor, LY-294002 on EGF-Induced Cell Migration.** In order to confirm the involvement of the PI3K pathway in cellular migration by EGF, we also included LY-294002, a PI3K inhibitor, to study its effect on EGF-stimulated cellular migration. As shown in Figure 6A,B, the addition of LY-294002 inhibited EGF-induced migration. In addition, we also observed a concomitant reduction of MMP-9 secretion and MED28 expression upon the addition of the PI3K inhibitor (Figure 6C), suggesting that both MED28 and MMP-9 involve in the PI3K-mediated cellular migration and function downstream of PI3K.

**Effect of RS, PS, and LY-294002 on NF-κB DNA-Binding Activity.** The EGF induction of cellular migration corresponds to an increase of the secreted MMP-9 levels, which is a target of NF-κB activation and correlated with the NF-κB binding activity. Therefore, we investigated the effect of RS, PS, and LY-294002 on NF-κB DNA-binding activity, which can be translated into the expression of MMP-9 and, subsequently, cellular migration. As shown in Figure 7A, the addition of either RS or PS significantly reduced the EGF-induced NF-κB binding activity; 5 $\mu$M or 15 $\mu$M of RS showed a similar, dramatic suppression effect, whereas a comparable reduction was only observed in the higher dose, 15 $\mu$M, of PS. The addition of the PI3K inhibitor, LY-294002, strongly inhibited the EGF activation of NF-κB DNA binding activity (Figure 7B). These data indicate that Akt is involved in the EGF-induced NF-κB activation events and RS and PS can inhibit EGF-induced NF-κB activation.

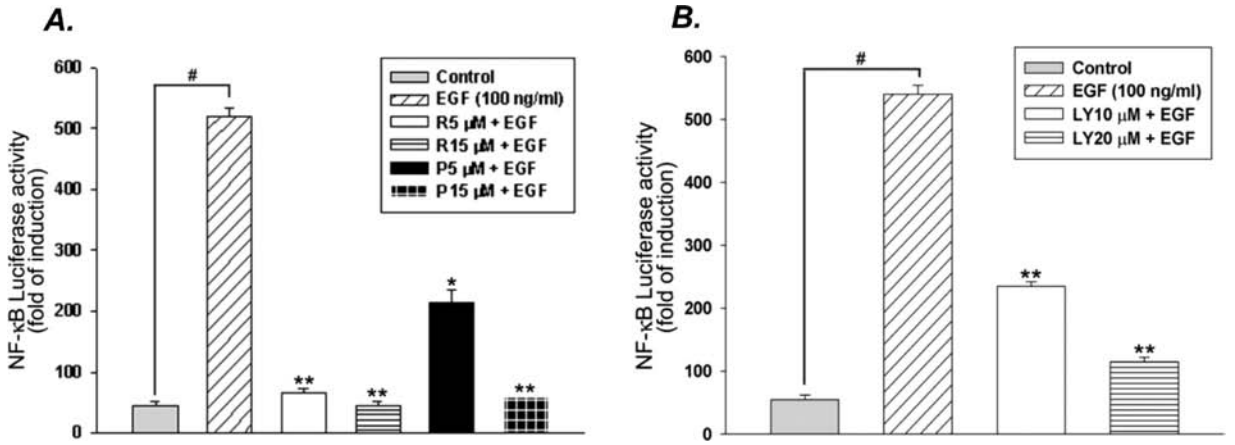


**Figure 7.** Effect of RS, PS, and LY-294002 on NF-$\kappa$B DNA-binding activity. MDA-MB-231 cells were transiently transfected with 2.5 $\mu$g of pNF-$\kappa$B-Luc reporter plasmid and then treated with various doses of RS or PS (*A*) or LY-294002 (*B*) in the presence of EGF (100 ng/mL) for 24 h. Cellular protein was extracted for measuring luciferase activity. #, $p < 0.001$ as compared with control. *, $p < 0.05$, **, $p < 0.001$ indicate statistically significant differences from the EGF-treated group.

## DISCUSSION

MED28 (magicin/EG-1) is a multifaceted protein, and several cellular roles have been identified thus far.[26−28,39] It interacts with merlin, the neurofibromatosis 2 (NF2) tumor suppressor protein, actin cytoskeleton, and Grb2, an adaptor in many signaling pathways.[26] MED28 also mediates transcriptional activation as a Mediator subunit,[28] which might be responsible for the enhancement of magicin in breast cancer cell proliferation.[31] In addition to these multiple roles, our data suggest that MED28 is also involved in cellular migration in breast cancer cells.

Stepwise cancer development involves uncontrolled tumor cell growth and metastatic migration. Several growth factors, such as EGF, can promote cellular proliferation and migration.[40] Cancers overexpressing EGFR family members are often associated with high metastatic potential.[17,18] For example, MDA-MB-231 and BT-474 cells, two breast cancer cell lines, exhibit invasive ability, expressing EGFR and HER2, respectively. Since HER2 does not have a cognate ligand identified and recent work has proposed an overlooked role of EGFR overexpression in breast cancer,[16] we used MDA-MB-231 as a model to investigate the effect of EGF stimulation on cellular migration and its underlying mechanism. It appears that the activation of EGFR initiates the downstream PI3K pathway and increases cellular migration (Figure 8). EGF activates EGFR, which, in turn, triggers the downstream effector, PI3K, signaling. Activated Akt induces the expression of MED28 and MMP-9, which further increases cellular migration. Activation of NF-$\kappa$B induced MMP-9 gene expression,[41] which is associated with the invasion and metastasis of cancer cells by inflammatory cytokines, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), or growth factors such as EGF.[38,42,43] The induction effect of EGF on MED28 may activate subsequent MMP-9 expression by enhancing the transcription of the MMP-9 upstream effector, NF-$\kappa$B, through the role of Mediator subunit. It is noteworthy that MED28 expression can be upregulated by angiogenic factors,[27] which coincides with the increasing migratory potential upon EGF treatment in MDA-MB-231 cells in our study. Both estrogen receptor (ER)-positive breast cancer cell lines, MCF7 and BT-474, highly express MED28. In contrast, ER-negative MDA-MB-231 cells express much less MED28. Whether ER expression plays a role in the differential expression of MED28 in the breast cancer cell lines is unclear at present. Although MDA-MB-231 cells do not highly express MED28, EGFR activation upon EGF stimulation leads to activation of the PI3K pathway and induction of MED28, which could be an effector of Akt to relay the downstream migration event.

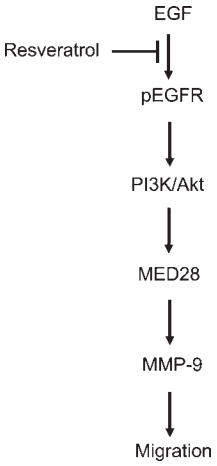


**Figure 8.** Model of RS and MED28 in EGF-induced cellular migration. RS modulates EGF-induced migration by inhibiting the transduction of the EGFR signaling pathway through PI3K/Akt/MED28 activation of MMP-9 expression.

Treatment of RS (10−100 $\mu$M) for 48 h has been found to inhibit cell growth by inducing apoptosis in MDA-MB-231 breast cancer cells, and the inhibition is associated with MAPK signaling and protein translation.[44] In the current study, we did not observe a growth-inhibitory effect using 5−50 $\mu$M RS for 24 h in MDA-MB-231 cells. However, our data indicated that RS could suppress cellular migration by inhibiting the PI3K pathway. The discrepancy may be due to RS doses and durations such that higher doses of RS for a longer period induced apoptosis and inhibited cell growth and lower doses of RS for a shorter period suppressed cellular migration. Therefore, our finding suggests another potential advantage of RS in the intervention of breast cancer. It has been found that RS

inhibited invasion of HER2-expression breast cancer cells by suppressing MMP-9 expression.[45] It has also been reported that RS inhibited cell invasion in MDA-MB-231 cells using EGF as chemoattractant.[40] Our study further identified that RS inhibited EGF-induced migration with a concomitant decrease in the expression of EGFR, activation cascade of EGFR and PI3K, expression of MMP-9 and MED28, and NF-$\kappa$B DNA-binding activity, suggesting that the MMP-9 suppression by RS might be through a putative EGFR/PI3K/Akt/MED28/NF-$\kappa$B pathway. In this study, RS reduced the expression of EGFR that might be responsible for the suppression of the downstream activation events. In contrast, PS induced relatively higher phosphorylation status of EGFR than that of RS treatment, which may be partially due to invariable EGFR expression. RS also appears to inhibit cellular migration by altering the actin cytoskeleton structure.[40,46] To this end, MED28 was found to be associated with actin-cytoskeleton, Grb2,[26] and Src family members.[33,34] It is possible that MED28 contributes to the reorganization of actin cytoskeleton through Src and Grb2 to relay the growth factor signaling and cellular migration pathway. Therefore, MED28 could participate in EGF-induced migration as a downstream effector of Akt to activate MMP-9 and a binding partner of Grb2 and/or Src to modulate cytoskeleton structure, whereas RS could inhibit the EGFR signaling thereby inhibiting MED28 expression and subsequent cellular migration.

Triple-negative breast cancers, in the absence of estrogen receptor, progesterone receptor, and HER2, but often expressing EGFR, are associated with metastasis and poor survival.[16,47,48] Several mechanisms, including activation of other signaling cascades such as the insulin-like growth factor (IGF)/PI3K and Notch-1 pathways, have been proposed to be responsible for the resistance to the available therapeutic strategies.[49] Ample studies have supported that RS inhibits cellular proliferation and migration in cancer cells. Several lines of evidence have indicated the important cellular roles of MED28. Together with existing literature, our study further suggests a possible translational application of the combination of RS supplementation and MED28 inhibition as an accessory to anti-EGFR therapy in breast cancer.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Figure depicting time-course study of the EGFR signaling molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; *EG-1*, endothelial-derived gene; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase; magicin, merlin and Grb2 interacting cytoskeletal protein; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MMP-2, gelatinase-A; MMP-9, gelatinase-B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide; NF2, neurofibromatosis 2; p38, p38 MAPK; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PS, pterostilbene; RS, resveratrol; siRNA, small interfering RNA; SMC, smooth muscle cell; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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