

Zerumbone suppresses EGF-induced CD44 expression through the inhibition of STAT3 in breast cancer cells

SANGMIN KIM*, WON HO KIL*, JEONGMIN LEE, SOO-JIN OH, JEONGHUN HAN, MYEONGJIN JEON, TAEWOO JUNG, SE KYUNG LEE, SOO YOUN BAE, HYUN CHUL LEE, JUN HO LEE, HA WOO YI, SEOK WON KIM, SEOK JIN NAM and JEONG EON LEE

Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Gangnam-gu, Seoul 135-710, Republic of Korea

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Abstract. Expression of the CD44 gene is upregulated in breast cancer cells and is correlated with patient survival. Aberrant CD44 expression promotes tumor progression and metastasis. In the present study, we investigated the role of zerumbone (ZER) on regulatory mechanisms of CD44 expression in breast cancer cells. Our results showed that CD44 expression was significantly increased by epidermal growth factor receptor (EGFR) ligands in SKBR3 breast cancer cells. In contrast, EGF-induced CD44 expression was decreased by a MEK1/2 inhibitor, UO126, or STAT3 inhibitor, STAT3 VI, respectively. Notably, ZER downregulated the basal level of CD44 expression in CD44⁺ breast cancer cells. In addition, the induction of CD44 expression by EGFR ligands, EGF or TGF- α , was markedly decreased by ZER treatment. Finally, we investigated the inhibitory mechanism of ZER on EGF-induced CD44 expression. Our results showed that EGF-induced phosphorylation of STAT3 was completely suppressed by ZER. Collectively, ZER suppressed EGF-induced CD44 expression through inhibition of the STAT3 pathway. Therefore, we suggested that ZER may act as a promising therapeutic drug for the treatment of breast cancer.

Introduction

Breast cancer is a highly complex disease with large inter- and intra-tumoral heterogeneity; the basal-like molecular subtype of breast cancer has the poorest clinical outcome (1). Epidermal growth factor receptor (EGFR) is frequently overexpressed in triple-negative breast cancer (TNBC) and

inflammatory breast cancer (IBC) in comparison with other subtypes of breast cancer (2). Highly-expressed EGFR is associated with a number of characteristics including large tumor size, poor differentiation and poor clinical outcome in a variety of cancer cells, including breast and lung cancer cells (3). In addition, elevated EGFR and CK5/14 expression was associated with the status of CD44⁺/CD24⁻ in basal-like subtypes of breast cancer (4). A population of CD44⁺/CD24^{-/low} tumor cells has also been associated with the aggressive basal-like breast cancer (4). In a recent study, we also reported that CD44 expression is regulated through EGFR/ERK-dependent pathways in breast cancer cells (5).

Hyaluronan (HA) is a component of the extracellular matrix and binds to its predominant cell-surface receptor CD44 (6,7). Aberrant expression of HA correlates with poorly differentiated tumors, auxiliary lymph node status and short overall survival time in breast cancer (8). HA/CD44 complex promotes multiple signaling pathways that influence many tumor cell activities, including abnormal growth, migration and invasion (9-11). The standard form of CD44 is generally ubiquitously expressed on epithelial cells and lymphocytes (7). The CD44 gene is composed of at least 20 exons that tissue-specific multiple variant isoforms (CD44v1-v10) is produced by alternate mRNA splicing (12). Indeed, induction of CD44s alone affected the growth characteristics of non-invasive luminal breast cancer cells including the induction of cell proliferation, cell migration and cell invasion *in vitro* (13). In addition, overexpression of CD44v3 isoforms promotes breast tumor cell migration and the attachment of VEGF to the heparin sulfate sites on CD44v3 is responsible for the onset of breast tumor-associated growth (14).

Zerumbone [2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one; (ZER)], a monocyclic sesquiterpene derived from a Southeast Asian ginger, is often used as an anti-inflammatory and antioxidant agent (15-17). ZER has contributed to antitumor activities in a variety of cancers, such as colon and gastric cancer (17). In addition, ZER triggers apoptosis through modulation of the Bax/Bcl-2 ratio in HepG2 liver cancer cells (17). Downregulation of CXCR4 expression by ZER suppressed CXCL12-induced cell invasion through the inhibition of NF- κ B activity in breast and pancreatic cancer cells (18). Despite these studies, however, the regulatory

Correspondence to: Professor Jeong Eon Lee or Professor Seok Jin Nam, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea
E-mail: paojlus@hanmail.net
E-mail: sjnam@skku.edu

*Contributed equally

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mechanisms of ZER on EGFR signaling pathways are not yet fully understood.

In the present study, we examined the role of ZER on the regulatory mechanism of EGF-induced CD44 expression in breast cancer cells. In particular, we found that ZER has an inhibitory effect on the EGF/STAT3 signaling pathway in breast cancer cells. EGF-induced CD44 expression also was regulated by the STAT3 dependent pathway.

Materials and methods

Reagents and cell culture. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 and the antibiotics were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). UO126 and LY294002 were purchased from Tocris Bioscience (Ellisville, MO, USA). Mouse monoclonal anti-CD44 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). The secondary HRP-conjugated antibodies, as well as the mouse monoclonal anti- β -actin antibody, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit monoclonal phospho- and total-Akt, STAT3 and Erk1/2 antibodies were purchased from Epitomics (Burlingame, CA, USA). ZER was a gift from Dr Murakami (Kyoto University, Kyoto, Japan). EGF and TGF- α were purchased from R&D Systems (Minneapolis, MN, USA). ALDEFluor™ kit was purchased from Stemcell Technologies (Durham, NC, USA).

SKBR3 and MDA-MB468 human breast cancer cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin.

ZER and specific inhibitor treatment. Cells were maintained in culture medium without FBS for 24 h. Then, the culture media was replaced with fresh media without FBS. Cells were pretreated with 5 or 10 μ M concentrations of ZER for 16 h prior to EGF treatment and were then treated with 50 ng/ml EGF for 24 h. In experiments involving specific inhibitors, such as UO126, LY294002 and STAT3 inhibitor, each cell was pretreated with specific inhibitors for 30 min prior to treatment with EGF and they were then treated with EGF for 24 h.

Western blotting. The cell culture media (supernatants) and cell lysates were used in the immunoblot analysis for CD44, EGFR, STAT3, Erk, Akt and β -actin. Cells were lysed on ice for 30 min. The cell lysate was collected into microtubes and samples were centrifuged for 15 min at 12,000 rpm at 4°C. Supernatants were collected and the protein concentrations were measured using the Bio-Rad Protein Assay kit (Hercules, CA, USA). The proteins were boiled for 5 min in Laemmli sample buffer and they were then electrophoresed in 10% SDS-PAGE gels, respectively. The separated proteins were transferred to PVDF membranes and the membranes were then blocked with 10% skim milk in TBS with 0.01% Tween-20 for 15 min. The blots were incubated with anti-CD44, EGFR, STAT3, ERK, AKT and β -actin antibodies in 1% TBS/T buffer (0.01% Tween-20 in TBS) at 4°C overnight. The blots were washed 3 times in TBS with 0.01% Tween-20 and they were subsequently incubated with anti-rabbit peroxidase-conjugated

antibody (1/2,000 dilution) in TBS/T buffer. After 1 h incubation at room temperature (RT), the blots were washed 3 times and ECL Prime reagents were used for development.

Real-time PCR. Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Isolated RNA samples were then used for RT-PCR. Samples (1 μ g of total RNA) were reverse-transcribed into cDNA in 20 μ l reaction volumes using a first-strand cDNA synthesis kit for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

The gene expression was quantified by real-time PCR using a SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA/reaction. The sequences of the primer sets used for this analysis were: human CD44s (forward, 5'-CCA AGA TGA TCA GCC ATT CTG G-3' and reverse, 5'-AAG ACA TCT ACC CCA GCA AC-3') and GAPDH as an internal control (forward, 5'-ATT GTT GCC ATC AAT GAC CC-3' and reverse, 5'-AGT AGA GGC AGG GAT GAT GT-3'). An annealing temperature of 60°C was used for all primers. PCRs were performed in a standard 384-well plate format with an ABI 7900HT Real-Time PCR detection system. For data analysis, the raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to get the Δ C_T. The normalized Δ C_T was then calibrated to the control cell samples to get the $\Delta\Delta$ C_T.

Cell viability. To confirm ZER toxicity, we used treatment with the indicated concentrations of ZER and total cell numbers were evaluated by Quick Cell Proliferation Assay kit II (BioVision, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, CD44-positive cells, MDA-MB468 and SKBR3 human breast cancer cells (5x10⁴/well) were grown in a 96-well plate in 100 μ l/well of culture media in the absence or presence of ZER. After incubating the cells for 24 h, 10 μ l WST reagent was added to each well. Viable cells were quantified photometrically at 480 nm.

ALDEFLUOR assay. The ALDEFLUOR kit (Stem Cell Technologies, Grenoble, France) was used for the immunofluorescent detection of intracellular ALDH enzyme activity, using a FACS-vantage (Becton-Dickinson, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, cells were incubated in ALDEFLUOR assay buffer containing ALDH substrate (1 mM/l per 13,106 cells). In each experiment, a sample of cells was incubated, under identical conditions, with 50 mM/l of diethylaminobenzaldehyde, a specific ALDH inhibitor, as a negative control.

Statistical analysis. Statistical significance was determined using the Student's t-test. Data are presented as means \pm SEM. All quoted P-values are two-tailed and P-values <0.05 were considered to indicate statistically significant differences. Microsoft Excel was used for statistical analyses.

Results

EGF augments the basal level of CD44 mRNA and protein expression in the SKBR3 breast cancer cells. In the present

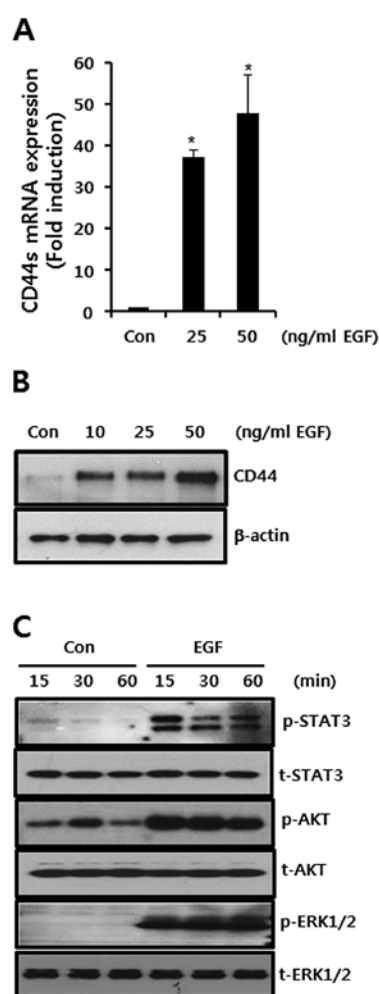


Figure 1. EGF augments the basal level of CD44 mRNA and protein expression in the SKBR3 breast cancer cells. (A and B) After serum-starvation for 24 h, SKBR3 cells were treated with EGF at the indicated doses for 24 h. The levels of CD44 mRNA (A) and protein (B) expression were analyzed by real-time PCR and western blotting, respectively. (C) After serum-starvation for 24 h, SKBR3 cells were treated with 50 ng/ml EGF for the indicated times. The phosphorylation levels of EGFR, STAT3, Akt and Erk were analyzed by western blotting. The results are representative of 3 independent experiments. The values shown are the means \pm SEM. * $P < 0.05$ vs. control. Con, control.

study, we sought to verify the effect of EGF on CD44 mRNA and protein expression. As a result, we treated the CD44 with EGF for 24 h at the indicated concentrations. As shown in Fig. 1A and B, the levels of CD44 mRNA and protein expression were increased by EGF treatment in a dose-dependent manner. After 50 ng/ml EGF treatment, the levels of the CD44 mRNA expression were significantly increased to 48.1-fold that of the control level (Fig. 1A).

We also examined EGF-induced the phosphorylation of STAT-3, ERK and AKT which are the downstream signaling molecules of EGF. The levels of STAT-3, ERK and AKT phosphorylation were significantly increased by 50 ng/ml EGF treatment in SKBR3 breast cancer cells (Fig. 1C).

EGF ligand-induced CD44 mRNA and protein expression levels are suppressed by MEK1/2 or STAT-3 inhibitors in SKBR3 breast cancer cells. To verify the regulatory mecha-

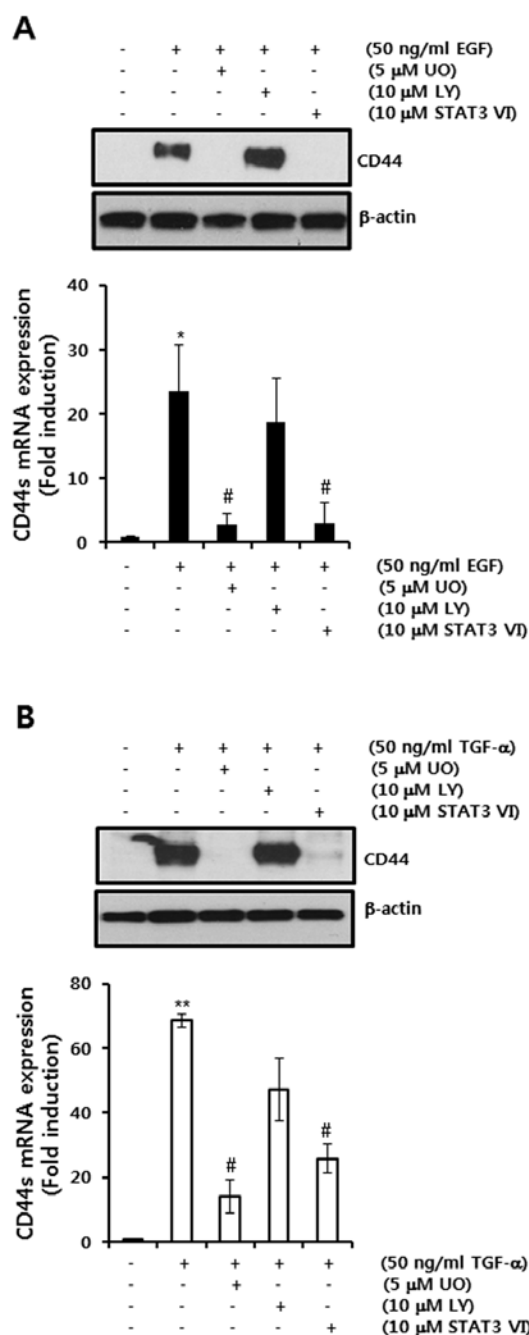


Figure 2. EGF ligand-induced CD44 mRNA and protein expression levels are suppressed by a MEK1/2 inhibitor or STAT3 inhibitor, respectively. (A and B) After serum-starvation for 24 h, SKBR3 cells were pretreated with 10 μM UO, LY and STAT3 VI, respectively, for 30 min and were then treated with 50 ng/ml EGF (A) or TGF-α (B) for 24 h, respectively. The levels of CD44 and β-actin protein expression were analyzed by western blotting. The levels of CD44s mRNA were analyzed by real-time PCR. The results are representative of 3 independent experiments. The values shown are the means \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. control. # $P < 0.05$ vs. EGF-treated cells. Con, control; UO, UO126; LY, LY294002; STAT3 VI, STAT3 inhibitor VI.

nism of EGF-induced CD44 mRNA and protein expression, we pretreated cells with the specific inhibitors, including UO, a MEK1/2 inhibitor; LY, a PI3K inhibitor; and STAT3 IV, a STAT-3 inhibitor, respectively, for 30 min prior to 50 ng/ml EGF treatment. Our results showed that EGF-induced CD44 protein and mRNA expression was decreased by UO or STAT-3 IV inhibitors, respectively (Fig. 2A).

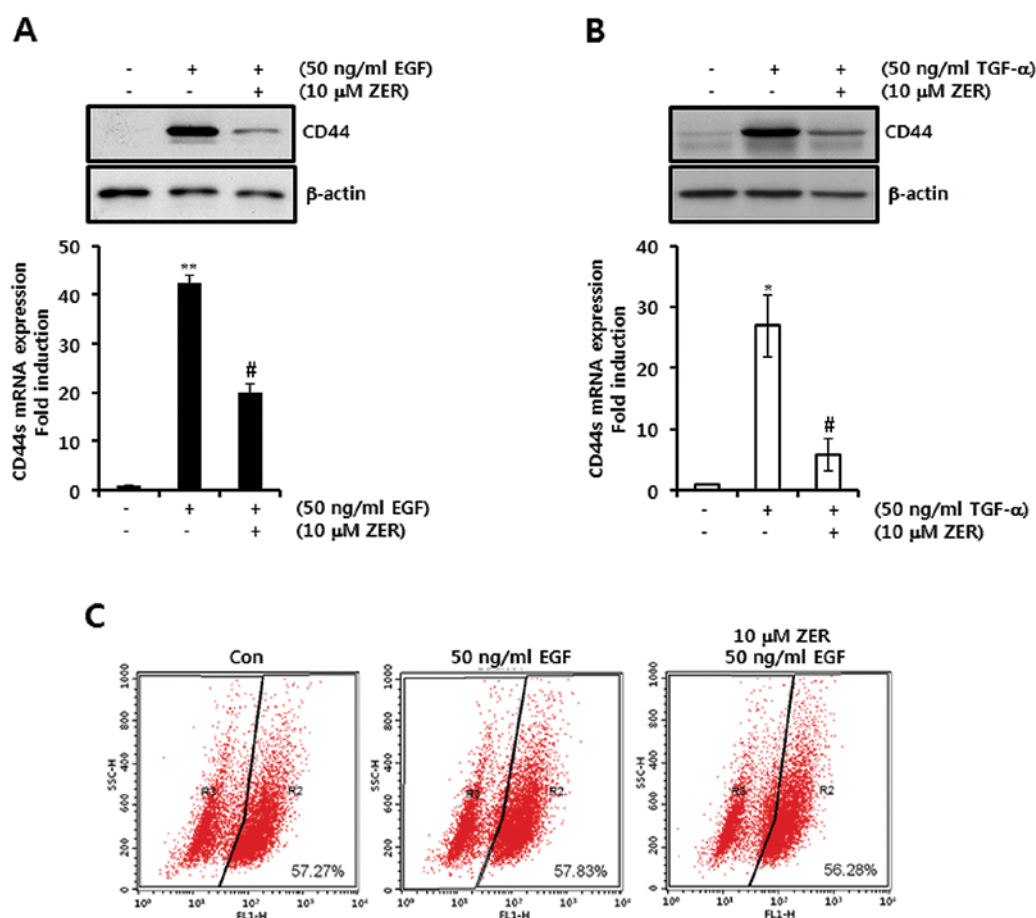


Figure 5. EGF ligand-induced CD44 mRNA and protein expression levels are suppressed by ZER in SKBR3 breast cancer cells. (A-C) After serum-starvation for 24 h, SKBR3 cells were pretreated with 10 μ M ZER for 16 h, and were then treated with 50 ng/ml EGF (A) or TGF- α (B) for 24 h, respectively. The levels of CD44 and β -actin protein expression were analyzed by western blotting. The levels of CD44s mRNA were analyzed by real-time PCR. (C) ALDH enzyme activity was detected as described in Materials and methods. The results are representative of 3 independent experiments. The values shown are the means \pm SEM. * P <0.05, ** P <0.01 vs. control. # P <0.05 vs. EGF-treated cells. Con, control; UO, UO126; LY, LY294002; STAT3 VI, STAT3 inhibitor VI; ZER, zerumbone.

10 μ M ZER for 16 h prior to 50 ng/ml EGF treatment. Our results showed that the levels of CD44 protein and mRNA expression by EGF were significantly decreased after ZER treatment (Fig. 5A). The levels of EGF-induced CD44 mRNA expression were suppressed by 19.8-fold of control level by 10 μ M ZER treatment (Fig. 5A). Under the same conditions, we investigated the effect of ZER on TGF- α -induced CD44 protein and mRNA expression. As expected, our results showed that the induction of CD44 protein and mRNA expression in response to TGF- α was decreased by ZER treatment (Fig. 5B). The levels of TGF- α -induced CD44 mRNA expression were decreased by 5.9-fold of control level by 10 μ M ZER treatment (Fig. 5B).

Next, we examined the correlation between CD44 expression and ALDH enzyme activity. As shown in Fig. 5C, the ALDH⁺ population was not changed; however, CD44 expression was altered by EGF and/or ZER treatment. Therefore, we demonstrated that ZER may act as a promising inhibitor of the EGF signaling pathway in breast cancer cells.

EGF-induced phosphorylation of STAT3 is suppressed by ZER treatment in SKBR3 breast cancer cells. We investigated whether ZER regulates the phosphorylation of EGF/EGFR

downstream signaling molecules in breast cancer cells. We pretreated cells with 10 μ M ZER for 16 h and then 50 ng/ml EGF for 30 min. We observed that EGF-induced STAT3 phosphorylation was markedly decreased by ZER but not ERK1/2 and AKT phosphorylation (Fig. 6). Therefore, we demonstrated that ZER may suppress EGF-induced CD44 expression through the inhibition of the STAT-3 dependent pathway in breast cancer cells.

Discussion

The EGFR and its ligands activate several mechanisms underlying tumor progression, including cell proliferation, survival and tumor invasion (19,20). In particular, aberrant EGFR activation by frequent overexpression or constitutive activation can promote tumor processes including angiogenesis and metastasis; it is also associated with poor prognosis in many human malignancies (21). The major signaling pathways activated by EGFR are mediated by different intracellular signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)/Akt, the Raf/MEK/ERK and the signal transducer and activator of transcription (STAT) pathways (22,23). In accordance with these previous studies, our results showed that the

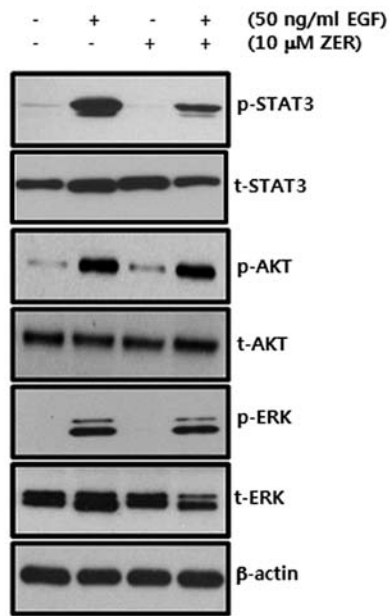


Figure 6. EGF-induced phosphorylation of STAT3 is suppressed by ZER treatment in SKBR3 breast cancer cells. After serum-starvation for 24 h, SKBR3 cells were pretreated with 10 μ M ZER for 16 h, and were then treated with 50 ng/ml EGF for 30 min. The levels of phospho- and total-STAT3, Akt and ERK were analyzed by western blotting. The results are representative of 3 independent experiments. ZER, zerumbone.

phosphorylation of STAT3, Akt and ERK were significantly increased by EGF treatment in breast cancer cells. Although we did not observe data, MMP-9 expression, which is a drive gene of tumor invasion and metastasis, also markedly increased under the same conditions.

The EGF/EGFR signaling pathway stimulates cellular interactions with ECM components, HA in particular, by upregulation of CD44 expression in murine NR6 cells (24,25). Overexpression of CD44 is involved in tumor progression, including induction of tumor cell motility and enhancement of tumor growth and metastasis (26). The promoter region of CD44 contains AP-1 binding sites and EGF-induced AP-1 transcription activity mediates cell invasion through induction of CD44 (27). We also reported that the transcriptional activity of CD44 is regulated by an EGFR/ERK-dependent pathway in breast cancer cells (5). Consistent with these reports, our results showed that EGFR ligands, EGF and TGF- α augment the level of CD44 expression in a dose-dependent manner. EGF-induced CD44 expression was also decreased by a MEK1/2 specific inhibitor, UO126. In particular, EGFR ligand-induced CD44 expression was markedly decreased by STAT-3 specific inhibitor, STAT3 VI. Therefore, we demonstrated that the transcriptional activity of STAT-3 plays a key role in the induction of CD44 in breast cancer cells.

Zerumbone (ZER), a sesquiterpene, is an anti-inflammatory agent (17); it has apoptotic effects against a wide variety of tumor cells, including colon cancer and leukemia cells (17,28). In addition, ZER suppresses CXCL12-mediated invasion of breast and pancreatic tumor cells through the downregulation of CXCR4 (18). The suppression of NF- κ B activity by ZER inhibits the secretion of angiogenic factors, such as VEGF and IL-8, in pancreatic cancer cells (29). We also found that ZER abolishes the basal levels of CD44 mRNA expression in

CD44-positive breast cancer cells. Furthermore, ZER significantly decreased EGFR ligand-induced CD44 expression in SKBR3 breast cancer cells. In the present study, we found for the first time that ZER significantly suppresses EGF-induced phosphorylation of STAT3. Therefore, we demonstrated that ZER inhibits EGFR ligand-induced CD44 expression through the suppression of STAT3 activity.

To date, a variety of cancer stem/progenitor cells from different tissues, including breast and colorectal cancer, have illustrated highly expressed CD44 molecules (30,31). In a recent study, Herishanu *et al* reported that anti-CD44 mAbs can also inhibit proliferation and induce apoptosis of leukemia stem cells (32). However, our results showed that the reduction of CD44 expression by ZER treatment did not lead to the alteration of ALDH⁺ population. Therefore, we demonstrated that ZER is not directly involved with the stemness of breast cancer cells.

In conclusion, we clearly demonstrated the regulatory mechanism of ZER on EGF-induced CD44 expression in breast cancer cells. Elevated STAT3 activity in response to EGF ligands, including EGF and TGF- α , directly regulated the levels of CD44 expression. On the other hand, EGF-induced CD44 expression was suppressed by a STAT3 specific inhibitor, STAT3 VI. In particular, EGF-induced STAT3 phosphorylation and CD44 expression were significantly decreased by ZER treatment. Based on these findings, we suggest that ZER may be a promising therapeutic drug for the treatment of breast cancer through the blockage of the EGFR signaling pathway.

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