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α-Linolenic acid inhibits the migration of human triple-negative breast cancer cells by attenuating Twist1 expression and suppressing Twist1-mediated epithelial-mesenchymal transition

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ABSTRACT

α-Linolenic acid (ALA), an essential fatty acid, has anticancer activity in breast cancer, but the mechanism of its effects in triple-negative breast cancer (TNBC) remains unclear. We investigated the effect of ALA on Twist1, which is required to initiate epithelial-mesenchymal transition (EMT) and promotes tumor metastasis, and Twist1-mediated migration in MDA-MB231, MDA-MB468 and Hs578T cells. Twist1 protein was constitutively expressed in these TNBC cells, particularly MDA-MB-231 cells. Treatment with 100 μM ALA and Twist1 siRNA markedly decreased the Twist1 protein level and cell migration. Moreover, ALA transiently attenuated the nuclear accumulation of STAT3α as well as Twist1 mRNA expression. Treatment with ALA significantly attenuated the phosphorylation of JNK, ERK and Akt and decreased the phosphorylation of Twist1 at serine 68 in MDA-MB-231 cells. ALA accelerated Twist1 degradation in the presence of cycloheximide, whereas the ubiquitination and degradation of Twist1 by ALA was suppressed by MG-132. Pretreatment with ALA mimicked Twist1 siRNA, increased the protein expression of epithelial markers such as E-cadherin, and decreased the protein expression of mesenchymal markers including Twist1, Snail2, N-cadherin, vimentin, and fibronectin. Our findings suggest that ALA can be used not only to abolish EMT but also to suppress Twist1-mediated migration in TNBC cells.

1. Introduction

Breast cancer is a heterogeneous disease and is the most common cancer in women in North America, Europe, Oceania, Latin America, the Caribbean, Africa, and most of Asia [1]. Metastasis remains the leading cause of tumor recurrence and cancer mortality, particularly in basal-like triple-negative breast cancer (TNBC), detecting absence of expression of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu [2]. The epithelial-mesenchymal transition (EMT) plays a crucial role not only in embryogenesis but also in the *trans*-differentiation of stationary epithelial cells into motile mesenchymal cells [3]. The occurrence of EMT is an important key to initiate distant organ metastasis, and it is also the main cause of anti-apoptosis, drug resistance, and cancer stemness of TNBC cells [4,5]. Characteristics of EMT include the loss of the epithelial phenotype, such as a decrease in the expression of E-cadherin, and the display of mesenchymal characteristics, such as an increase in the expression of N-cadherin, fibronectin, and vimentin [6]. EMT-inducing transcription factors, such

Abbreviations: ALA, alpha-linolenic acid; bHLH, basic-helix-loop-helix; CHX, cycloheximide; EMT, epithelial-to-mesenchymal transition; HIF-1α, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; SRC-1, steroid receptor coactivator-1; STAT3, signal transducer and activator of transcription 3; TNBC, triple-negative breast cancer

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as Twist, Snail, Slug, and Zeb, are involved in cancer cell metastasis [7]. Inactivation of Twist remains an attractive target for cancer therapeutics [8].

In mammals, the Twist protein family consists of two subtypes: Twist1 and Twist2. Twist1 is preferentially expressed in mesoderm-derived tissues [9], and Twist2 is mainly found in liver and adipocytes [10]. Twist1 belongs to the basic-helix-loop-helix (bHLH) family of transcription factors, has been identified as an indicator of malignant transformation, and plays a crucial role in tumorigenesis, including EMT, angiogenesis, metastasis, and chemoresistance [11]. Overexpression of Twist1 is frequently observed in different tumor cell lines [12,13]. Induction of Twist1 contributes to the metastasis of various cell lines, including gastric cancer [14], colon cancer [15], and prostate cancer cells [16]. Clinical studies have indicated that higher expression of Twist1 is positively correlated with advanced tumor stage and shorter overall survival time in patients with various types of cancer, including breast cancer [17–19].

Twist1 is transactivated by a variety of transcription factors in cancer cells. These include signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor 1 (HIF-1α), nuclear factor κB (NF-κB), and steroid receptor coactivator-1 (SRC-1) [20–22]. Several signal transduction pathways, including Akt, STAT3, mitogen-activated protein kinase (MAPK), and Wnt signaling, are involved in upregulation of Twist1 [8]. Phosphorylation of Ser68 of Twist1 by activation of MAPKs contributes to Twist1 stabilization and promotes EMT and metastasis in breast cancer cells [23].

Alpha-linolenic acid (C18:3n-3, ALA) is an essential fatty acid with a variety of benefits, encompassing elevated insulin sensitivity [24], antihypertensive effects [25], and anti-inflammatory effects [26]. Previous studies showed that ALA is a potential nutrient in the prevention and treatment of breast cancer [27,28]. Although ALA has been demonstrated to have an inverse association with the risk of breast cancer [29], the underlying mechanism of the anti-metastasis effect of ALA in breast cancer cells remains unclear. In this study, therefore, we investigated the effect of ALA on Twist1 expression and Twist1-mediated cell migration in MDA-MB231 and Hs578T TNBC cells and the possible mechanisms involved.

2. Materials and methods

2.1. Reagents

Albumin (essentially fatty acid–free BSA), aprotinin, butylated hydroxytoluene, chloroform, crystal violet, cycloheximide, dithiothreitol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, EDTA, leupeptin, LY294002, MG-132, Nonidet P-40, PMSF, sodium bicarbonate, sodium orthovanadate, and α-tocopheryl succinate were from Sigma–Aldrich (St. Louis, MO); DMEM, FBS, 25% trypsin-EDTA, and penicillin–streptomycin solution were from GIBCO/BRL (Grand Island, NY); SP600125, PD98059, and SB203580 were from TOCRIS (Ellisville, MO); WP1066, isopropanol from Merck Chemical co. (Dermstadt, Germany); ALA was from Cayman Chemical (Ann Arbor, MI); TRIzol reagent, Opti-MEM and Lipofectamine RNAi MAX Transfection Reagent were from Invitrogen (Carlsbad, CA); antibodies against Twist1/2 (GTX127310), E-cadherin (GTX100443), N-cadherin (GTX127345), vimentin (GTX132608) and fibronectin (GTX20299) were from GeneTex Inc. (Irvine, CA); antibody against p-Twist1 (Ser68; ab187008) was from Abcam (Cambridge, UK); antibodies against JNK1/2 (#9252), p-JNK1/2 (#4668S), Akt (05-591), p-Akt (Ser473; #9271), p38 (#9212), p-p38 (#9211), and p-STAT3α (Tyr705; #9138) were from Cell Signaling Technology (Beverly, MA); antibodies against ERK1/2 (#05-1152), p-ERK1/2 (#05-797R), and Snail2 (#ABE993) were from MILLIPORE (Billerica, MA); antibody against ubiquitin (sc-9133) was from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against p-Akt (Thr308; #05-802R), STAT3α (06–596), and β-actin (#MAB1501) were from Merck Chemical co. (Dermstadt, Germany). KAPA™ SYBR® FAST qPCR Kit was from KapaBiosystems (Woburn, MA); SuperScript™III reverse transcription kit was from Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Cell culture

The human breast cancer cell lines MDA-MB-231 and Hs578T were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured and maintained in DMEM (pH 7.2) supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator. For MDA-MB-468 cells, the DMEM culture medium was additionally added with 2 mM glutamine and 1 mM sodium pyruvate.

2.3. Fatty acid preparation

ALA was freshly prepared and complexed with fatty acid–free BSA at a 6:1 M ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.4. Cell viability assay

Cell viability was assessed by using MTT assay, performed according to a previous study [30]. The MTT assay measures the ability of viable cells to reduce a yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan by mitochondrial succinate dehydrogenase. MDA-MB231 and Hs578T cells were grown to 70% to 80% confluence and were then treated with or without various concentrations of ALA for 24 h. Afterwards, the medium was removed, and the cells were washed with cold phosphate-buffered saline (PBS). The cells were then incubated with MTT (0.5 mg/mL) in DMEM medium at 37 °C for an additional 3 h. The medium was removed, and 2-propanol was added to dissolve the formazan. After centrifugation at 10000 × *g* for 5 min, the supernatant of each sample was transferred to 96-well plates, and absorbance was read at 595 nm in an ELISA reader. The absorbance in the control group was regarded as 100% cell viability.

2.5. Cytosolic and nuclear extracts and whole cell protein preparation

After each experiment, cells were washed twice with cold PBS and were then scraped from the dishes with PBS. Cell homogenates were centrifuged at 2000 × *g* for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 350 μL of hypotonic extraction buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF. After centrifugation at 7000 × *g* for 15 min, the resulting supernatant was used as the cytosolic fraction for western blotting and the pellets containing nuclei were extracted by gentle mixing with 50 μL of hypertonic extraction buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 10% glycerol, and 0.2 mM PMSF at 4 °C for 30 min. The samples were then centrifuged at 10000 × *g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at −80 °C until western blotting was performed. For whole cell protein preparations, cells were washed twice with cold PBS and were harvested in 100 μL of 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant was used as whole cell proteins for western blotting.

2.6. Western blotting

The protein content in each sample was quantified by use of the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company,

Rockford, IL). Protein aliquots were denatured and separated on 7.5–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes (New Life Science Product, Inc., Boston, MA). The nonspecific binding sites in the membranes were blocked with 5% nonfat dry milk in 15 mM Tris-150 mM NaCl buffer (pH 7.4) at 4 ˚C overnight. After blocking, the membranes were hybridized with respective primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immunereactive bands were developed by use of the Western Lightning™ Plus-ECL kit (PerkinElmer, Waltham, MA).

2.7. RNA isolation and real-time PCR

Total RNA was isolated from MDA-MB231 and Hs578T cells by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Amounts of 1 μg of total RNA were used to synthesize complementary DNA by use of SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The conditions of the reverse transcription reactions were as follows: 5 min at 65 ˚C, followed by 50 min at 50 ˚C, and 15 min at 70 ˚C. Real-time RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System using the KAPA™ SYBR® FAST qPCR kit (KAPA™). Oligonucleotide primers for real-time PCR analysis were as follows: Twist1 (forward, 5′-GGACAAGCTGAGC AAGAT-3′; reverse, 5′-CTCTGGAGGACCTGGTAG-3′) and β-actin (forward, 5′-CGGCATCGTCACCAACTG-3′; reverse, 5′-TCTCAAACATGATC TGGGTCATCT-3′). The conditions of the PCR reaction were as described in our previous study [31]. β-Actin was used as an internal standard gene and the threshold cycle (Ct) of a test sample to a control sample (ΔΔCt method) was used for relative quantification of target gene expression.

2.8. Plasmid construction and transfection

The human Twist1 promoter sequence contains putative three STAT3 binding site located at −95 to −102, −106 to −114, and −190 to −197 bp from the transcription start site, predicted by that of a previous study [32]. The template clone of Twist1 promoter wild (+95 to −371) was obtained from Protech Technology Enterprise (Huntsville, AL, USA). The PCR product was digested by KpnI and HindIII restriction enzymes (NEB), and then the product was ligated into the same sites of pGL4.10 expression vector. The pGL4.10 vector containing the wild-type Twist1 promoter was transfected into MDA-MB-231 cells by using TransIT®-2020 transfection reagent, according to the manufacturer's instructions (Mirus Bio, Inc., Madison, WI, USA). Cells were transiently transfected with the 2.5 μg of pGL4.10 plasmid and 1.0 μg of pSV-β-galactosidase (β-gal) plasmid by using 7.5 μL of TransIT®-2020 in Opti-MEM medium. After 12 h of transfection, the transfection medium was replaced with normal medium and incubation for an additional 16 h. The cells were then treated with or without ALA for an additional 4 h. Cells were then washed twice with cold PBS, scraped with lysis buffer and centrifuged at 14,000*g* for 3 min. Supernatants of the cell lysates were applied to measure the luciferase and β-gal activities by using a Luciferase Assay system (Promega, Madison, WI) and β-Galactosidase Enzyme Assay system (Promega, Madison, WI). The luciferase activity of each sample was corrected on the basis of β-gal activity.

2.9. RNA interference by small interfering RNA of Twist1

Oligonucleotides of small interfering RNAs (siRNAs) for Twist1 (sense siRNA sequence: 5′-GGUGUCUAAAUGCAUUCAUtt-3′) were predicted and synthesized by MDbio Inc. (Taipei, Taiwan). The cells were grown to 60–70% confluence in 35-mm plates and were transfected with Twist1 siRNA or nontargeting siRNA (negative control) by use of Lipofectamine RNAi MAX Transfection Reagent. Twist1 siRNA or non-targeting control (NTC) siRNA was diluted in 50 μL Opti-MEM medium, respectively, and mixed with 2 μL of transfection reagent diluted in 98 μL of Opti-MEM medium. NTC was used as a negative control in this study. After incubation for 20 min at room temperature, the mixture was added to 800 μL of Opti-MEM medium and applied to the cells (1 mL/plate). After 8 h of transfection, the transfection reagent-containing medium was replaced with 10% FBS-containing DMEM for another 24 h, and then the cells were treated as indicated in the experimental design.

2.10. Wound-healing assay

For the cell migration assay, an IBIDI culture insert (iBIDiGmbH Inc., Martinsried, Germany) was placed into a 35-mm culture dish and slightly pressed on top to ensure tight adhesion. An equal number of untreated and Twist1-silenced cells (70 μL; 5×10^5 cells/mL) were seeded into the two reservoirs of the same insert and incubated at 37 ˚C in a 5% CO_2 humidified incubator. After 24 h, the insert was gently removed, creating a gap of about 500 μm. The cells were then incubated in DMEM with or without ALA for another 24 h. Cells were then photographed (100 × magnification) to monitor cell migration into the wounded area, and the width of the cell-free zone (distance between the edges of the injured monolayer) was calculated.

2.11. Transwell cell migration assay

The activity of cell migration was performed by using Transwell (Corning Life Science, Corning, NY; pore size, 8 mm) in 24-well dishes and assayed as described previously [33]. Before performing the migration, 1×10^5 cells in 300 mL of serum-free medium were placed in the upper chamber, and 600 mL of medium without 10% FBS was added to the lower chamber. The plates were incubated at 37 ˚C in a 5% CO_2 humidified incubator. After 2 h, the cells were then treated with various concentrations of ALA for 24 h in the presence or absence of MG-132. Cells were fixed in 4% paraformaldehyde for 30 min and were then washed with PBS 2 times. The cells were stained with 1% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Migration were quantified by counting cells on the lower surface of the filter, and each experiment was repeated at least 3 times.

2.12. Immunoprecipitation

MDA-MB-231 cells were incubated with or without 100 μM ALA for 24 h in the presence of MG-132. Total cell lysates diluted to 1 μg/μL with IP buffer (40 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/μL aprotinin, 1 μg/μL lupeptin, 20 mM NaF and 1 mM sodium orthovanadate), incubated with 1.5 μL anti-Twist1 antibody (#46702, Cell Signaling Technology) for 16 h at 4 ˚C, mixed with protein A-sepharose™ CL-4B (0.1 mg/mL) (Piscataway, NJ) and incubated at 4 ˚C for an additional 1 h. Immunoprecipitates were collected by centrifugation at 15,900 × *g* for 1 min. The pellet was washed with 250 μL of IP buffer three times and then subjected to western blotting.

2.13. Statistical analysis

Data were analyzed by using one-way ANOVA (version 10.0; SPSS, Chicago, IL). The significance of the difference among group means was determined by Tukey's test, and the difference between mean values was determined by Student's *t*-test. P values < 0.05 were taken to be statistically significant.

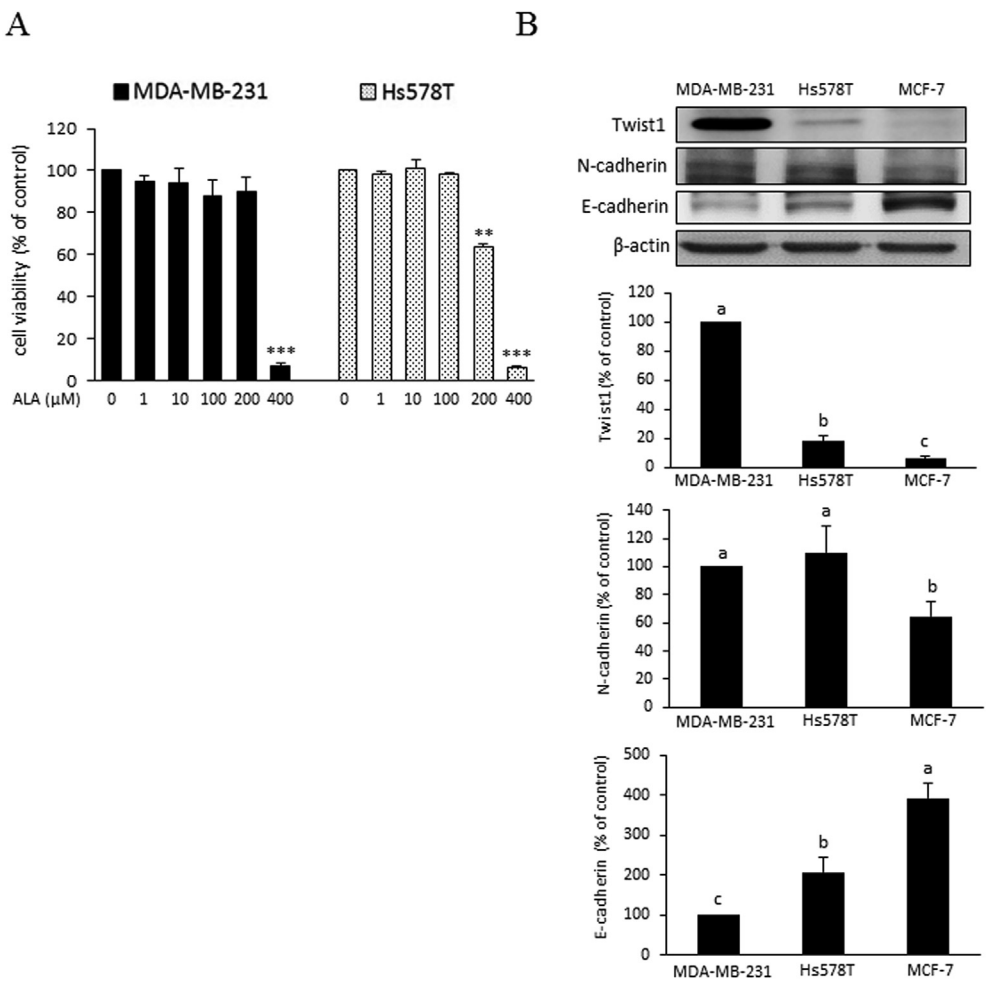

Fig. 1. Effects of ALA on cell viability and basal expression of EMT markers in breast cancer cell lines. Breast cancer cells were treated with or without 1, 10, 100, 200 and 400 μM ALA (A) MTT assay. (B) Cell cultures of the three cell lines (MDA-MB-231, Hs578T, and MCF-7) were harvested when 70–80% confluent by scraping into ice-cold PBS. Aliquots of whole cell lysates corresponding to 20 μg of total protein per lane were prepared for each cell line. Basal levels of Twist1, N-cadherin, and E-cadherin protein were analyzed by western blotting. Band intensities were measured by densitometry and are presented in the lower panel. Values are mean ± SD (n = 3). [abc]Values not sharing the same letter are significantly different ($P < .05$). The asterisk indicates significant difference between ALA treatment and control at the same time based on Student's *t*-test (**$P < .01$, ***$P < .001$).

3. Results

3.1. Twist1 knockdown and ALA reduce MDA-MB-231 and Hs578T cell migration

As measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the viability of MDA-MB-231 cells treated with 1, 10, 100, 200, and 400 μM ALA was 95 ± 2.5%, 94 ± 6.7%, 88.0 ± 7.2%, 90 ± 6.8%, and 7.0 ± 1.4%, respectively, compared with the untreated controls (100%). The viability of Hs578T cells treated with 1, 10, 100, 200, and 400 μM ALA was 98 ± 1.6%, 101 ± 4.0%, 98 ± 1.0%, 64 ± 0.9%, and 6 ± 0.9%, respectively, compared with the untreated controls (100%) (Fig. 1A). These results indicated that there were no adverse effects on cell growth up to a concentration of 100 μM ALA. Therefore, the highest concentration of ALA was set at 100 μM, which was used to suppress the expression of Twist1 in the following experiments.

Twist1 has been identified an indicator of metastasis in several types of cancer, and elevated levels of Twist1 protein have a strong association with advanced tumor stage and poor prognosis in breast carcinoma cells [19]. MDA-MB-231 and Hs578T cells are identical to the mesenchymal-like subtype of breast cancer cell lines that belong to most malignant cells [34]. In an animal study, an ALA-rich diet was shown to suppress mammary tumor growth and metastasis [28]. In the present study, MDA-MB-231 and Hs578T cells expressed a higher level of Twist1 and N-cadherin protein than did MCF-7 cells (Fig. 1B). Thus, MDA-MB-231 and Hs578T cells were used to distinguish whether the Twist1-mediated migration of breast cancer cells was suppressed by ALA.

As shown in Fig. 2A (left panel), ALA suppressed cell migration in a dose-dependent manner, and a similar effect of ALA on MDA-MB-231 and MDA-MB-468 cell migration was observed by using transwell cell migration assay (data not shown). After knockdown of Twist1 expression by siRNA transfection, the basal level of Twist1 and MDA-MB-231 cell migration were suppressed (Fig. 2A, right panel). A similar effect of ALA and Twist1 siRNA on cell migration was found in Hs578T cells (Fig. 2B).

3.2. ALA down-regulates Twist1 expression as well as nuclear STAT3α accumulation and STAT3-luciferase reporter activity in MDA-MB-231 and Hs578T cells

To demonstrate the effect of ALA on Twist1 gene expression, we treated MDA-MB-231 and Hs578T cells with 1, 10, and 100 μM ALA for 24 h. As shown in Fig. 3A, treatment with 100 μM ALA for 24 h significantly decreased the Twist1 protein level. ALA caused a transient decrease in the Twist1 mRNA level at 4 h, whereas this transient inhibition returned to basal levels at 12 h in MDA-MB-231 and Hs578T cells (Fig. 3B). Nuclear accumulation of STAT3α was attenuated by treatment with ALA for 1 h, whereas this transient inhibition returned to basal levels at 8 h in MDA-MB-231 cells (Fig. 3C). Afterward, we used cells transfected with luciferase reporter vectors carrying the STAT3 sequence of Twist1 to ascertain the effect of ALA for this inhibition. ALA decreased the STAT3-driven luciferase activity in MDA-MB-231 cells (Fig. 3D). These results indicated that ALA treatment down-regulated Twist1 mRNA expression within a short period of time, whereas the protein level of Twist1 was significantly decreased after ALA treatment for 24 h in TNBC cells.

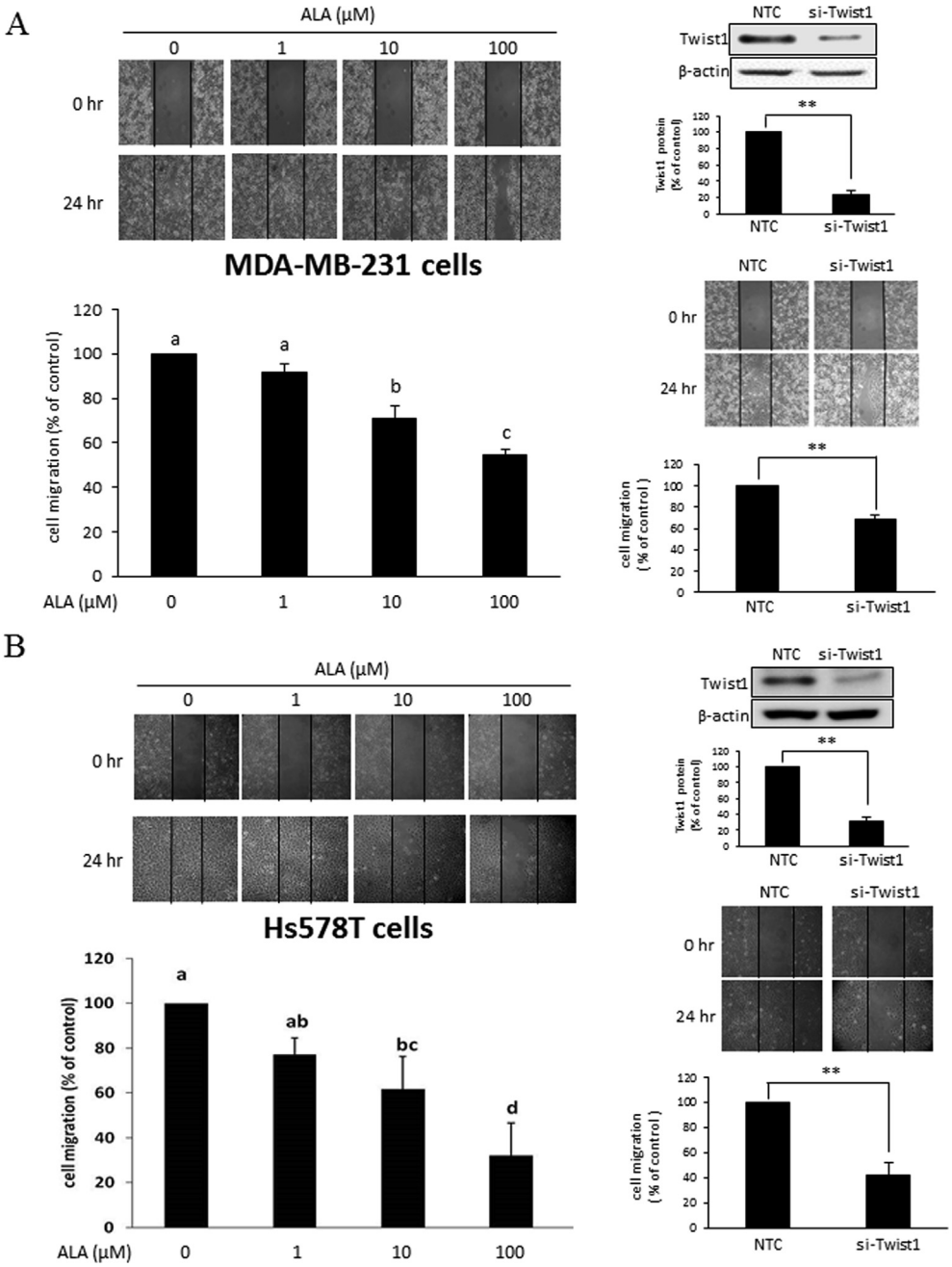

Fig. 2. ALA and si-Twist1 suppress TNBC cell migration. Untreated or Twist1-silenced cells were transferred to the IBIDI culture insert and then incubated for 24 h. (A) MDA-MB-231 and (B) Hs578T cells were then treated with or without 0, 1, 10, or 100 μM ALA for 24 h. Migration was observed by using a phase-contrast microscope at 100 × magnification. Values are mean ± SD (n = 3). [abcd]Values not sharing the same letter are significantly different ($P < .05$). Difference between nontargeting control (NTC) and si-Twist1 group means was compared by Student's *t*-test (**$P < .01$).

3.3. ALA down-regulates Twist1 expression via MAPK, PI3K/Akt, and STAT3α signaling pathways in MDA-MB-231 and Hs578T cells

It has been shown that induction of the MAPKs, phosphoinositide 3-kinase/Akt (PI3K/Akt), and STAT3 signaling pathways is necessary for Twist1 expression [35] and protein stability [23]. To investigate whether these signaling pathways are involved in the suppression of Twist1 expression by ALA, we used pharmacologic inhibitors of MAPKs, PI3K, and STAT3, such as SP600125 (JNK1/2 inhibitor), PD98059 (ERK inhibitor), SB203580 (P38 inhibitor), LY294002 (PI3K inhibitor), and WP1066 (STAT3 inhibitor). The phosphorylation of JNK1/2, ERK1/2 and Akt in MDA-MB-231 cells was reduced by treatment with ALA for 30 or 60 min (Fig. 4A, left panel). A similar inhibition of these MAPKs and Akt phosphorylation by ALA at 15 min was shown in Hs578T cells (Fig. 4A, right panel). Moreover, the phosphorylation of STAT3α was dramatically reduced by treatment with ALA for 60 min in MDA-MB-231 and Hs578T cells (Fig. 4A). Moreover, all these inhibitors, particularly LY294002, and WP1066, caused dramatic reductions in the Twist1 protein level in MDA-MB-231 and Hs578T cells (Fig. 4B). These results suggest that the reduction in Twist1 protein as the result of ALA treatment may be through inactivation of the MAPKs, PI3K/Akt, and STAT3α signaling pathways.

3.4. ALA accelerates the degradation of Twist1 protein in TNBC cells

Phosphorylation of Ser68 of Twist1 protects Twist1 from

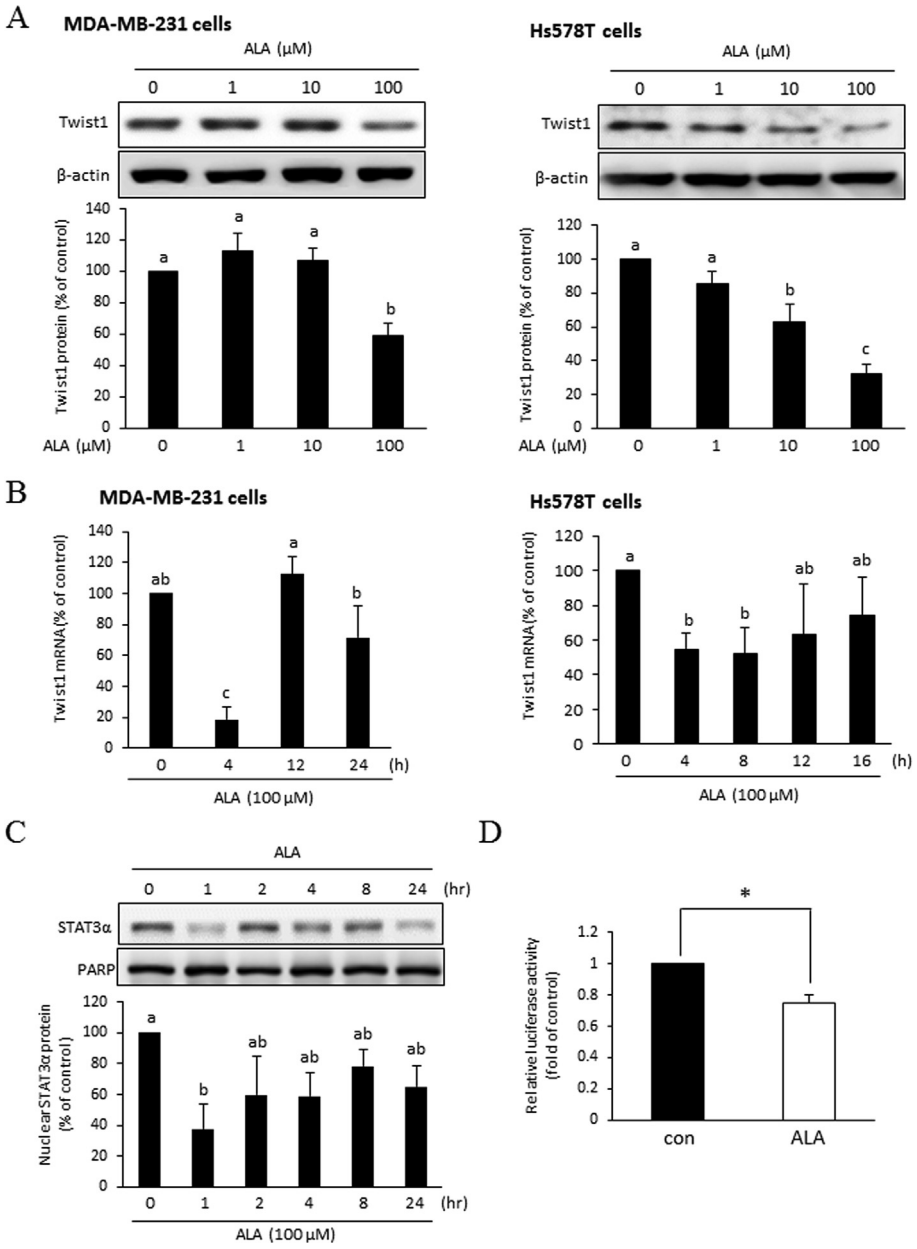

Fig. 3. ALA inhibits Twist1 expression in MDA-MB-231 and Hs578T cells. Cells were treated with various concentrations of ALA for 24 h. (A) Twist1 protein was determined by western blotting. Band intensities were measured by densitometry and are presented in the lower panel. (B) mRNA levels were determined by real-time PCR analysis. (C) The cells were treated with 100 μM ALA for different time periods as indicated. Nuclear extracts (15 μg) were used for western blotting. Analysis of PARP served as a loading control. (D) The pGL4.10 vector containing STAT3 binding site of Twist1 promoter was transfected into MDA-MB-231 cells. After 12 h of transfection, the transfection medium was replaced with normal medium and incubation for an additional 16 h. The cells were then treated with or without ALA for 4 h and cell lysates were collected and luciferase activity was detected and normalized to β-gal activity. Values are mean ± SD (n = 3). [abc]Values not sharing the same letter are significantly different ($P < .05$). The asterisk indicates significant difference between ALA treatment and control at the same time based on Student's *t*-test (*$P < .05$).

ubiquitination and degradation, which maintains the stability of the Twist1 protein and promotes breast cancer cell invasiveness [23]. To determine whether the protein stability of Twist1 is affected by ALA, the cells were treated with 100 μM ALA for the indicated times. As noted, ALA significantly decreased the phosphorylation of Ser68 of Twist1 in the presence of cycloheximide (CHX) in a time-dependent manner in MDA-MB-231 cells (Fig. 5A). ALA dramatically elevated the degradation of Twist1 protein from 16 to 24 h in the presence of CHX in Hs578T cells (Fig. 5B, right panel), whereas a significant degradation of Twist1 in MDA-MB-231 cells was found after treatment with ALA for 24 h (Fig. 5B, left panel). These results suggested that ALA treatment effectively decreased the levels of pSer68-Twist1 and Twist1 total protein in TNBC cells.

MG-132, a proteasome inhibitor, was used to confirm the degradation of Twist1 by ALA via the ubiquitin proteasome pathway. Pretreatment with MG-132 protected against the degradation of Twist1 by ALA in MDA-MB-231 and Hs578T cells (Fig. 5C). We further investigated the effect of ALA on the ubiquitination of Twist1 in the presence of MG-132 in MDA-MB-231 cells. Treatment with 100 μM ALA for 24 h significantly elevated the ubiquitination of Twist1 in the presence of MG-132 in MDA-MB-231 cells (Fig. 5D). To further confirm whether MAPKs, PI3K/Akt, and STAT3α signaling involved in the induction of phosphorylation of Twist1 at Ser68, the SP600125, PD98059, SB203580, LY294002, and WP1066 were used. As noted in Fig. 5E, treatment with 10 μM SP600125, PD98059, SB203580, and LY294002 for 4 h significantly attenuated the level of pSer68-Twist1 in MDA-MB-231 cells. These results suggested that ALA decreased the levels of pSer68-Twist1 may through inactivation of MAPKs, PI3K/Akt, and STAT3α signaling and the degradation of Twist1 protein by ALA is mediated by the ubiquitin–proteasome pathway.

3.5. Twist1 knockdown and ALA reduce the expression of EMT markers in TNBC cells

Loss of E-cadherin expression and induction of expression of N-cadherin, fibronectin, and vimentin contribute to EMT in various cancer cells [6]. To demonstrate whether down-regulation of Twist1 is involved in the inhibition of EMT by ALA, protein levels of markers of

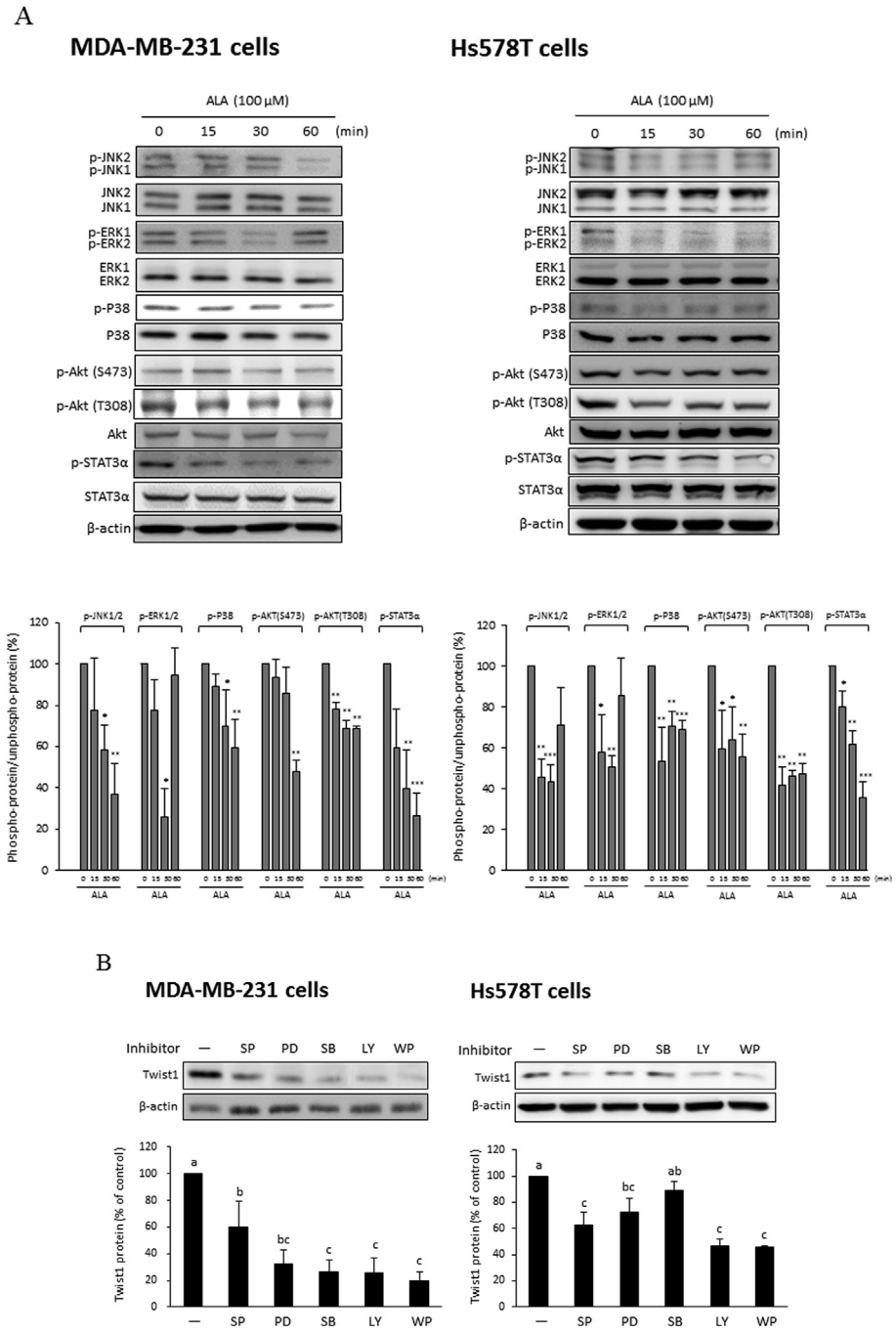

Fig. 4. Effect of specific MAPK, PI3K, and STAT3α inhibitors on Twist1 protein expression and the phosphorylation of MAPKs, Akt, and STAT3α in the presence of ALA. (A) The cells were treated with 100 μM ALA for the indicated time periods. (B) Cells were treated with or without 10 μM of specific MAPKs/JNK inhibitor II (SP600125, SP), ERK inhibitor (PD98059, PD), and p38 inhibitor (SB203580, SB) and PI3K inhibitors (LY294002, LY) or 5 μM of STAT3 inhibitor (WP1066, WP) for 24 h, and aliquots of total protein (10 μg) were used for western blotting. One representative experiment out of three independent experiments is shown. Values are mean ± SD, n = 3. [abc]Values not sharing the same letter differ significantly ($P < .05$). The asterisk indicates significant difference between ALA treatment and control at each indicated time point based on Student's *t*-test (*$P < .05$, **$P < .01$, ***$P < .001$).

EMT, including Twist1, Snail2, N-cadherin, fibronectin, and vimentin, were assayed. The expression of E-cadherin was significantly induced by ALA and Twist1 siRNA, whereas treatment with ALA and Twist1 siRNA down-regulated the expression of Twist1, Snail2, N-cadherin, fibronectin, and vimentin in MDA-MB-231 cells (Fig. 6A) and Hs578T cells (Fig. 6B). Furthermore, knockdown of Twist1 dramatically decreased the total protein level of fibronectin more than did ALA treatment (Fig. 6).

4. Discussion

Twist1 protein is essential not only for embryonic development [36], but also as a crucial component in upregulating EMT, tumor dissemination [37], cancer stemness [38], and multidrug resistance in cancer cells [39]. Although ALA is known to have anticancer potential in various malignant tumors, including tumors of the prostate [40], colon [41], and breast [42], the actual mechanism of this effect is not fully understood. Some research suggests that a number of dietary factors, such as sulforaphane and apigenin, can eliminate the characteristics of cancer stem cells and inhibit cell migration and invasion by down-regulating the expression of Twist1 [43,44]. In this study, our results revealed that Twist1 is a determinative factor of TNBC cell migration. In addition, we have reported for the first time that ALA inhibits Twist1-dependent TNBC cell migration by suppressing the expression of the Twist1 gene and accelerating Twist1 protein degradation.

Compared with the expression in nonmetastatic MCF-7 cells, the basal protein level of Twist1 in metastatic TNBC cells was high, particularly in MDA-MB231 cells (Fig. 1B). These results are consistent with the findings of previous studies showing that the basal level of Twist1

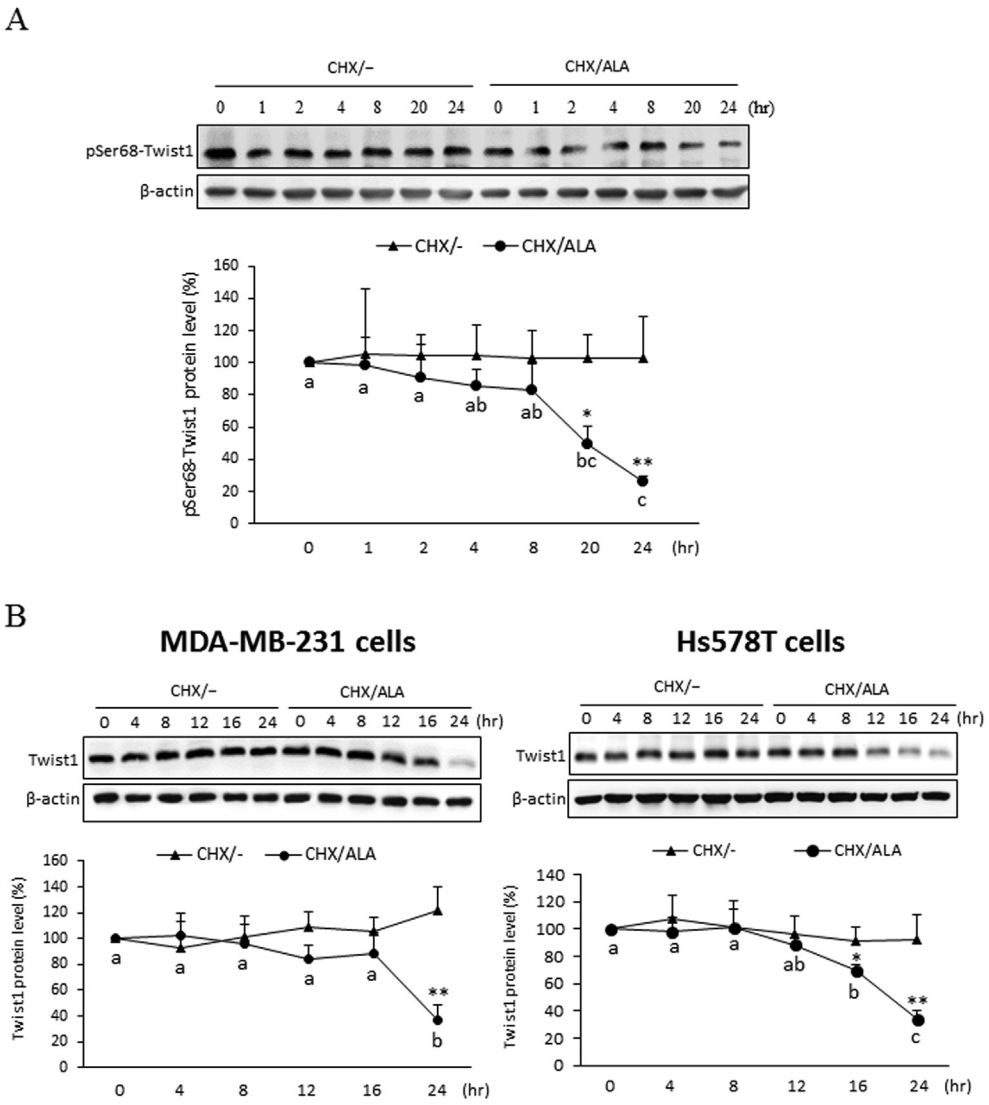

Fig. 5. ALA accelerates Twist1 protein degradation. (A and B) MDA-MB-231 cells were treated with 2.5 μM of CHX to inhibit protein synthesis and with vehicle (ethanol) or 100 μM of ALA for different time periods as indicated. Phospho-Twist1 (Ser68) and Twist1 protein levels were determined by western blotting. Analysis of β-actin served as a loading control. Band intensities were measured by densitometry and are presented in the lower panel. (C) The cells were co-treated with different concentrations of ALA and MG-132 (proteasome inhibitor) for 24 h, and then the Twist1 protein level was determined by western blotting. (D) MDA-MB-231 cells were treated with or without ALA for 24 h in the presence of MG-132, the whole cell protein lysate was prepared, and the protein interaction between Twist1 and ubiquitin was determined by co-immunoprecipitation. (E). Cells treated with 10 μM SP600125, PD98059, SB203580, LY294002 and 5 μM WP1066 for 4 h, and then the protein level of pSer68-Twist1 and Twist1 were determined by western blotting. Values are mean ± SD (n = 3). [ab]Values not sharing the same letter are significantly different ($P < .05$). The asterisk indicates significant difference between the mean of CHX alone and that of CHX with ALA treatment at the same time based on Student's *t*-test (*$P < .05$; **$P < .01$).

protein is low in MCF-7 cells but high in TNBC cells, especially in MDA-MB-231 cells [45]. Elevation of Twist1 expression enhances cell invasion and metastasis [21], whereas depletion of Twist1 suppresses cell migration and invasion [46]. As noted in Fig. 2, knockdown of Twist1 mimics treatment with 100 μM ALA, which significantly suppressed the migration of MDA-MB-231 and Hs578T cells. Moreover, treatment with 100 μM ALA for 24 h significantly decreased Twist1 protein expression (Fig. 3A), which suggests that ALA may exert an anticancer effect against TNBC cell metastasis by down-regulating Twist1-mediated cell migration. Interestingly, the Twist1 mRNA level decreased significantly at 4 h after treatment with ALA and returned to basal levels at 12 to 24 h (Fig. 3B). These results suggest that ALA may inhibit Twist1 either by down-regulating Twist1 transcription or by accelerating Twist1 protein degradation.

There are several potential mechanisms by which ALA may decrease breast cancer cell growth and induce apoptotic cell death [42,47]. Treatment with 50–100 μM ALA for 72 h dose-dependently reduces the growth of TNBC and luminal breast cancer cells [42]. In the present study, treatment with 100 μM ALA for 24 h did not cause a reduction in the growth of MDA-MB-231 and Hs578T cells, indicating that the cytotoxicity of ALA was dependent on its exposure time. Our previous study showed that docosahexaenoic acid (DHA) can inhibit TPA-induced phosphorylation of STAT3α in MCF-7 cells [48]. However, 100 μM of ALA, but not other n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid or DHA, significantly suppressed the phosphorylation of STAT3α and the protein level of Twist1 in Hs578T cells (data not shown). These results suggest that attenuation of the phosphorylation of STAT3α is involved in the down-regulation of Twist1 by ALA in TNBC cells.

Transcription factors such as STAT3 are involved in the upregulation of transcriptional activity of Twist1 [20]. STAT3 is constitutively expressed and activated in numerous cancer types, including more than

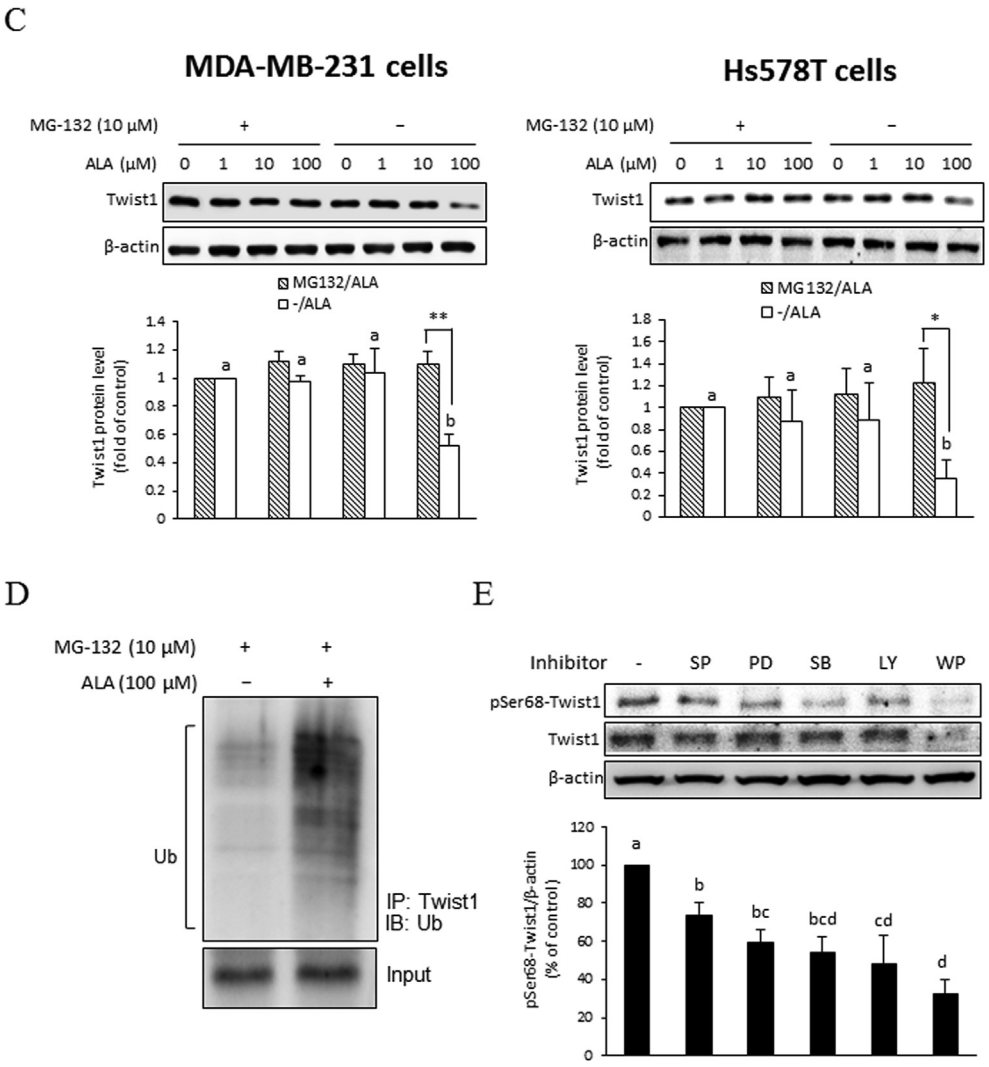

Fig. 5. (*continued*)

40% of breast cancers [49]. Induction of STAT3 expression or STAT3 phosphorylation (Tyr705) has been found to promote cancer cell proliferation, angiogenesis, and metastasis and to enhance resistance to conventional chemo- and radio-therapies by inducing the expression of STAT3 target proteins, including Bcl-2, c-myc, cyclin-D1, HIF-1α, survivin, and Mcl-1 [50,51]. Constitutive activity of STAT3 has also been shown to directly up-regulate the gene expression of HIF-1α [52], a potent tumorigenic factor, and ROS/STAT3/HIF-1alpha signaling is involved in EGF-induced Twist1 expression in prostate cancer cell invasion [53]. In gastric cancer cells, activation of Notch1 induces the phosphorylation of STAT3 and subsequently enhances the interaction between nuclear STAT3 and Twist promoter [14].

Reduced STAT3 signaling, either through downregulation of the STAT3/mcl-1 pathway or reduced focal adhesion kinase (FAK)-STAT3 signaling, causes mitochondrial dysfunction and apoptosis [54,55]. In a study in adipose-derived stem cells from obese adipose tissue, ALA suppressed the activation of Th17 inflammatory immune cells and IL-17A secretion by inhibiting STAT3 activity [56], indicating that ALA can reduce STAT3 phosphorylation and subsequently down-regulate STAT3 target genes relevant to human cancer. Inhibiting JAK/STAT3 blocks the self-renewal of breast cancer stem cells and the expression of diverse lipid metabolic genes, such as carnitine palmitoyltransferase 1B (CPT1B), as well as glucose metabolic genes, including glucose transporter-1 (LUT-1), phospho-fruktokinase-liver type (PFK-L), and enolase-1 (ENO-1), contributing to down-regulating lipid metabolism and glycolysis prior to tumour growth arrest and cell death [57,58]. These results indicate that blocking STAT3 signaling may also contribute to down-regulating lipid and glucose metabolism and mitochondrial activity, which protects against cancer development and chemoresistance. In the present study, a higher level of nuclear STAT3α accumulated in untreated cells, whereas treatment with 100 μM ALA for 1 h transiently decreased the nuclear accumulation of STAT3α (Fig. 3C) and STAT3α transactivation (Fig. 3D) in MDA-MB-231 cells. These results suggest that a reduction in STAT3α transactivation may be involved in the down-regulation of Twist1 expression by ALA in TNBC cells.

Twist1 has been recognized as a ubiquitin substrate of the Skp1-Cul1-F-box protein ($SCF^{\beta\text{-}TRCP}$) E3 ligase complex [59]. Twist1 is degraded by the ubiquitin–proteasome system, which can suppress tumor cell motility and cancer metastasis [59,60]. The phosphorylation of Twist1 at Ser68 (pSer68-Twist1) by Ras-activated MAPKs maintains the protein stability of Twist1 and promotes EMT in breast cancer cells [23]. Compared with the level of phosphorylated Twist1 in non-tumorigenic epithelial human breast cells, a high level of pSer68-Twist1 was shown in untreated metastatic 4 T1 breast cancer cells. Furthermore, the same study indicated that the high level of pSer68-Twist1 in invasive human breast ductal carcinomas was positively correlated with the levels of Twist1 protein [23]. As noted in Fig. 5A and B, pSer68-Twist1 and total Twist1 were constitutively expressed in untreated cells, whereas treatment with ALA for 4 h significantly decreased the pSer68-Twist1 level in TNBC cells. The MAPKs family, including JNK,

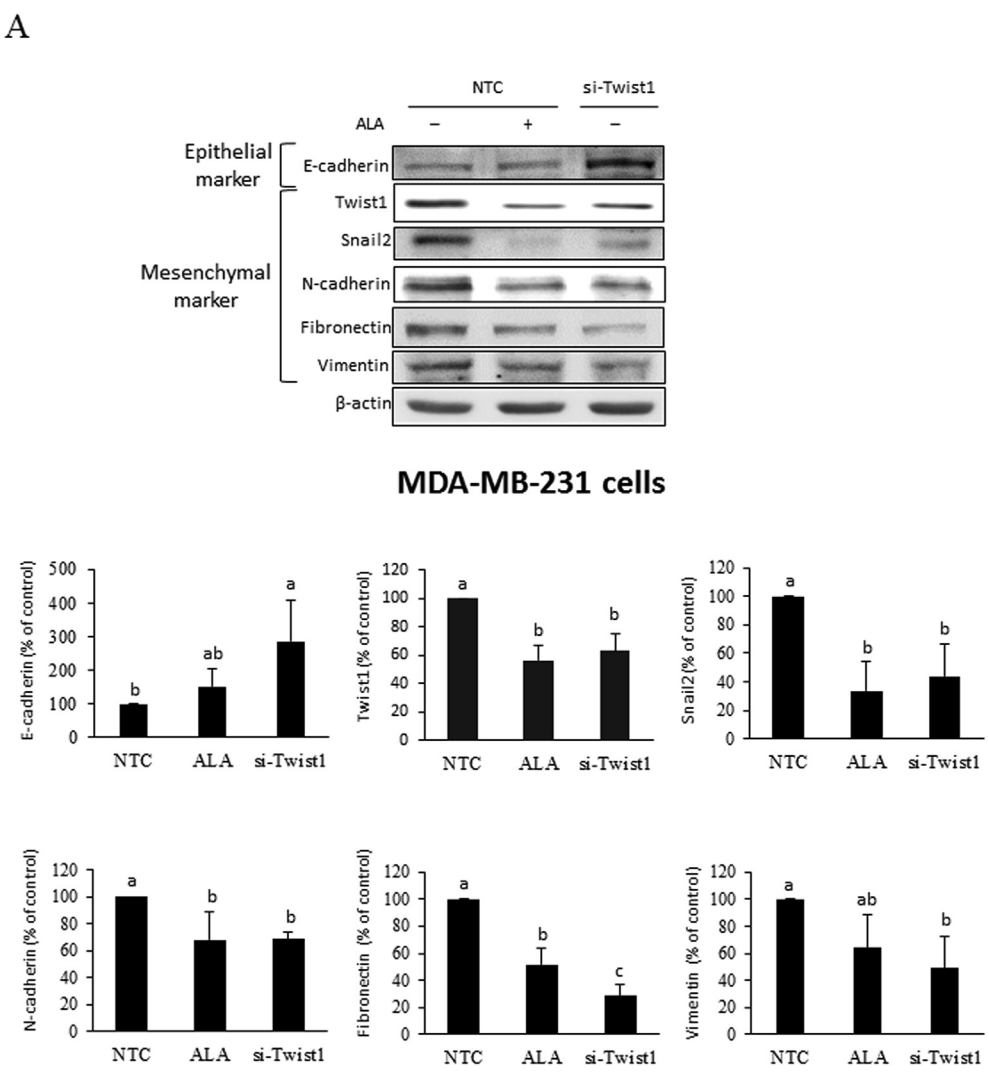

Fig. 6. Effect of ALA and Twist1 siRNA on EMT-associated protein expression in TNBC cells. (A) MDA-MB-231 and (B) Hs578T cells were treated with 100 μM of ALA for 24 h or transfected with Twist1 siRNA or nontargeting control (NTC) for 8 h and were then incubated with normal medium for an additional 24 h. E-cadherin, Twist1, Snail2, N-cadherin, fibronectin, and vimentin proteins were determined by western blotting. Analysis of β-actin served as a loading control. Band intensities were measured by densitometry and are presented in the lower panel. Values are mean ± SD, n = 3. [abc]Values not sharing the same letter differ significantly ($P < .05$).

ERK1/2, and p38, phosphorylates Ser68 in the N-terminal 112 amino acids of Twist1 [23]. In this study, ALA rapidly inhibited the phosphorylation of Akt as well as of JNK, ERK1/2, and p38-MAPK, within 60 min (Fig. 4A). Compared with untreated cells, treatment with inhibitors of MAPKs, PI3K, and STAT3 for 4 h significantly attenuated the level of pSer68-Twist1 in MDA-MB-231 cells (Fig. 5E), indicating the possibility of degradation of Twist1 by ALA through inhibition of JNK, ERK1/2, p38, PI3K/Akt, and STAT3-mediated phosphorylation of Ser68-Twist1 expression.

A previous study revealed that the PI3K/Akt pathway is constitutively activated in MDA-MB-231 cells [61]. Activation of PI3K/Akt is positively correlated with Snail expression and negatively correlated with E-cadherin expression in cancer cells [62,63]. In this study, the basal activation of Akt was high in untreated cells (Fig. 4A), as was the protein expression of Snail2 and Twist1 (Fig. 6). Treatment with ALA significantly inhibited the phosphorylation of Akt (Fig. 4A) as well as the protein expression of Snail2 and Twist1 (Fig. 6). These results suggest that phosphorylation of Akt is also positively correlated with Twist1 expression, which contributes to elevate N-cadherin expression in TNBC cells.

EMT-related genes such as E-cadherin, Snail2, N-cadherin, fibronectin, and vimentin have been shown to be regulated by Twist1 at the transcriptional level [64–66]. In this study (Fig. 6), the expression of EMT-related genes was shown to correlate positively with the level of Twist1, which is consistent with the findings of a previous study [63]. Upon knockdown of Twist1 by RNA interference, the expression of these mesenchymal markers was significantly reversed, which is likely the result of ALA treatment (Fig. 6). Twist1 was able to bind to an evolutionarily conserved E-box on the proximate Snail2 promoter to induce its transcription, indicating that induction of Snail2 is essential for Twist1-induced cell invasion and distant metastasis in mice [67]. A recent study revealed that sterol regulatory element-binding protein 1 (SREBP1) interacts with c-MYC to enhance the binding of c-MYC to the promoter of Snail, thereby increasing Snail expression and accelerating EMT [68]. Overexpression of SREBP1 is accompanied by induction of EMT-related genes, including Snail and Twist1, in colorectal cancer [68]. We cannot rule out that SREBP1 may contribute to enhanced upregulation of Snail by interaction of Twist1 or c-MYC in TNBC cells. The issue of the effect of ALA on the interaction between SREBP1 and Twist1 or other coactivators will be considered in a future study. These results indicate that ALA suppresses TNBC cell migration through suppression of Twist1-mediated EMT-related gene expression. In

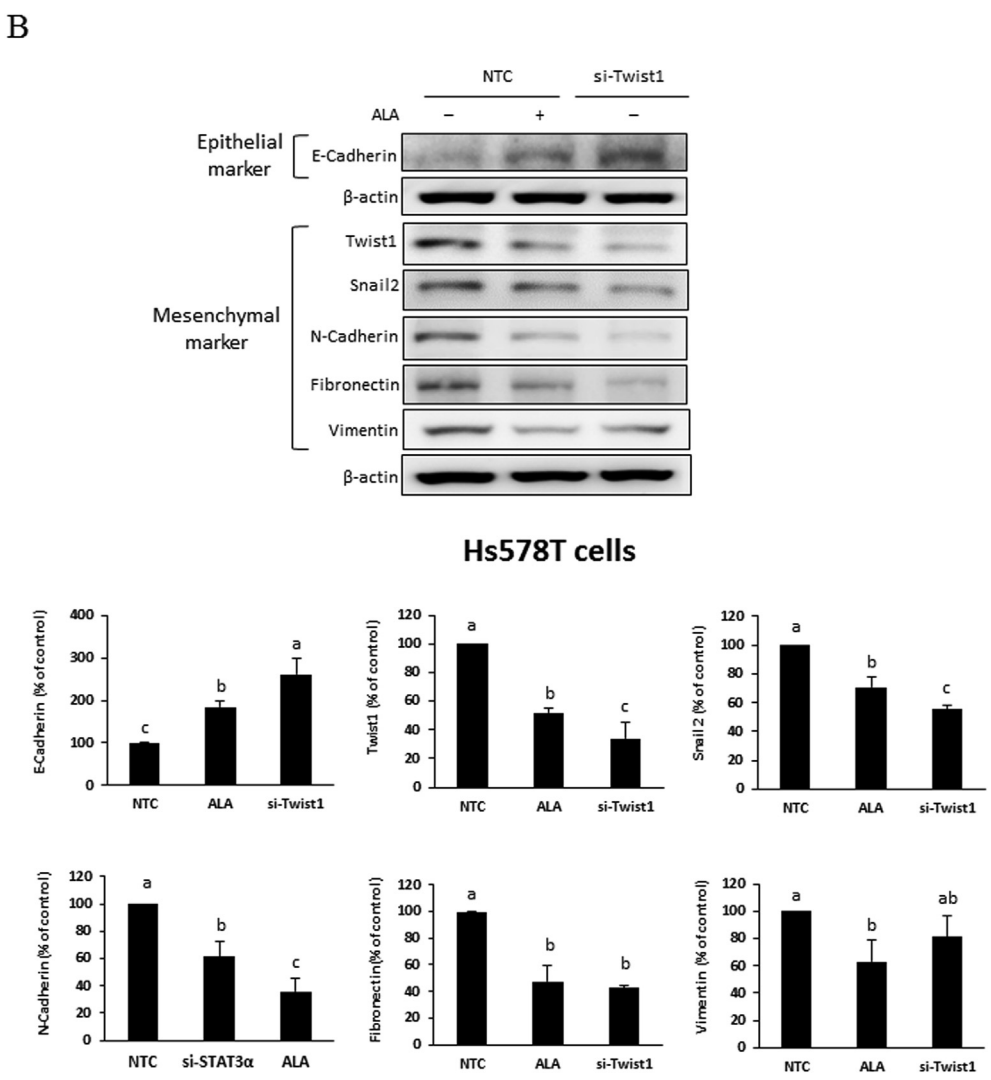

Fig. 6. (*continued*)

addition to EMT-related genes, oncogenic genes including OCT4, MAGEA4, and discoidin domain receptor 2 (DDR2) have been shown to be induced by Twist1, which leads to tumor cell self-renewal, aggressiveness, and invasiveness [69–71]. Our study showing that Twist1 was dramatically down-regulated by ALA indicates that ALA may play a role in the down-regulation of the expression of other oncogenic genes mediated by Twist1.

In conclusion, we have shown that ALA effectively suppresses EMT-related gene expression and cell migration at least in part through attenuation of Twist1 transcription by decreasing the nuclear accumulation and transactivation of STAT3α as well as by accelerating Twist1 protein degradation by the ubiquitin-proteasome pathway. These results provide evidence of a mechanism whereby ALA may prove to be a potent anti-metastasis agent in TNBC cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

The authors' responsibilities were as follows: Li C-C (Principal Investigator) and Wang S-C designed the study; Sun H-L and Lii C-K analyzed data and performed statistical analysis; Hsu Y-H, Liu S-H, Huang C-S, and Tsai C-H contributed to sample measurements and data collection; Li C-C wrote the paper; Li C-C had primary responsibility for final content. All authors contributed to the manuscript review and approved the final version.

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