Sphingomyelin synthase 1 regulates the epithelial-to-mesenchymal transition mediated by the TGF-β/Smad pathway in MDA-MB-231 cells

SHUANG LIU[1*], HUAN HOU[1*], PANPAN ZHANG[1*], YIFAN WU[1],
XUANHONG HE[1], HUA LI[2] and NIANLONG YAN[1]

[1]Department of Biochemistry and Molecular Biology, College of Basic Medical Science; [2]Department of Biochemistry
and Molecular Biology, Centre of Experimental Medicine, Nanchang University, Nanchang, Jiangxi 330006, P.R. China

Abstract. Breast cancer is the most common cancer in women and a leading cause of cancer-associated mortalities in the world. Epithelial-to-mesenchymal transition (EMT) serves an important role in the process of metastasis and invasive ability in cancer cells, and transforming growth factor β1 (TGF-β1) have been investigated for promoting EMT. However, in the present study, the role of the sphingomyelin synthase 1 (SMS1) in TGF-β1-induced EMT development was investigated. Firstly, bioinformatics analysis demonstrated that the over-expression of SMS1 negatively regulated the TGFβ receptor I (TβRI) level of expression. Subsequently, the expression of SMS1 was decreased, whereas, SMS2 had no significant difference when MDA-MB-231 cells were treated by TGF-β1 for 72 h. Furthermore, the present study constructed an over-expression cells model of SMS1 and these cells were treated by TGF-β1. These results demonstrated that overexpression of SMS1 inhibited TGF-β1-induced EMT and the migration and invasion of MDA-MB-231 cells, increasing the expression of E-cadherin while decreasing the expression of vimentin. Furthermore, the present study further confirmed that SMS1 overexpression could decrease TβRI expression levels and blocked smad family member 2 phosphorylation. Overall, the present results suggested that SMS1 could inhibit EMT and the migration and invasion of MDA-MB-231 cells via TGF-β/Smad signaling pathway.

Correspondence to: Dr Nianlong Yan, Department of Biochemistry and Molecular Biology, College of Basic Medical Science, Nanchang University, 461 Bayi Road, Nanchang, Jiangxi 330006, P.R. China
E-mail: yannianlong@163.com

*Contributed equally

Introduction

Breast cancer (BC) is the most common cancer in women and a leading cause of cancer-associated mortalities in the world (1). During BC development, the epithelial-to-mesen-chymal transition (EMT) serves a very important role (2). EMT is associated with wound healing, fibrosis and cancer progression; in particular, the metastasis and invasive ability of cancer cells is significantly enhanced (3). Therefore, in the present study, the mechanism of the role of the EMT in one BC cell line was investigated. Previous studies identified that transforming growth factor β1 (TGF-β1) serves a very important role in the EMT process (4,5). TGF-β1 facilitates many responses by binding specifically and activating cell surface receptor serine/threonine kinase complexes, including TGFβ receptor (TβRI and TβRII) (4,6). Activated TGF-β receptors can stimulate the receptor-regulated phosphorylation of Smad family members, an important signal transduction and modulator, by forming complexes (7). Additionally, phosphorylated Smad family members 2 and 3 form a stable complex with Smad family member 4 and move to the nucleus, where the transcription of the target gene is regulated (8,9). Non-Smad signaling pathways, including guanosine triphosphatase, phosphoinositide 3 kinase and mitogen-activated protein kinase signaling pathways, can also be activated by TGF-β (7,10); however, the Smad-dependent signaling pathway is unique but is the most critical to TGF-β-induced EMT (11,12). During this process, cells lose epithelial markers, including E-cadherin, and upregulate mesenchymal markers, including vimentin (13,14).

There are several lines of evidence suggesting that TGF-β receptors are distributed in lipid rafts/caveolae and non-raft membrane microdomains (15). On the cell surface, TGF-β1 receptors are distributed between different microdomains of the cellular membrane and may be internalized via clathrin-and caveolae-mediated endocytic mechanisms (16,17). Lipid rafts, enriched with cholesterol and sphingolipids, are ordered microdomains within plasma membranes. Sphingomyelin (SM) is an essential component of sphingolipids (18). SM is additionally associated with the formation of other

membrane microdomains, including clathrin-coated pits and caveolae, thus serving an important role in the regulation of trans-membrane signaling (19). Sphingomyelin synthase (SMS), with two isoforms, SMS1 and SMS2, is a key enzyme involved in the generation and development of SM. SMS can participate in inflammation, atherosclerosis, proliferation, apoptosis, differentiation and other functions (20).

Furthermore, bioinformatics retrieval from Gene Expression Omnibus (GEO) datasets suggested that SMS1 and TβRI are linked in the mammary gland and breast cancer cells (GSE54491 and GSE89205; https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE89205). The results showed that the expression of SMS1 was decreased, while TβRI was increased, when breast cells were treated with TGF-β1. Therefore, the accumulation of SMS1 inside cells may decrease cellular TβRI expression levels. To test this hypothesis, MDA-MB-231 cells were treated with or without TGF-β1 following transfection with a SMS1 overexpression plasmid. Protein expression levels associated with the development of the EMT were investigated by western blotting and immunofluorescence, and migration and invasion were investigated using a wound healing and a Matrigel invasion assay, respectively.

Materials and methods

Microarray data. All available data on TGF-β1-treated breast cancer cells or normal mammary gland cells from the Gene Expression Omnibus (GEO) database were investigated and an mRNA microarray GSE54491 (https://www. ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54491) was identified. GSE54491 employed an Affymetrix mouse gene 1.0 ST array [transcript (gene) version] to identify mRNAs that were differentially expressed between TGF-β1-treated and TGF-β1-untreated normal murine mammary gland (NMumG) cells. MDA-MB-231 cells were selected for treatment with 10 ng/ml TGF-β1 to assess protein expression, according to previous studies (14,21). After 72 h, the expression of SMS1 and SMS2 was examined by western blot analysis.

Expression of SMS1 in breast cancer cells. MDA-MB-231, MCF-7 and BT549 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Tianjin Haoyang Biological Products Technology Co., Ltd., Tianjin, China) and incubated at 37˚C in a humidified atmosphere (90% relative humidity) containing 5% CO_2. To choose a suitable breast cancer cell line and determine the optimal concentration of TGF-β1, western blotting was performed in order to detect the expression levels of SMS1 in the three breast cancer cells (MDA-MB-231, MCF-7 and BT549). Finally, MDA-MB-231 cells were selected and treated with different concentrations (0, 5, 10, 15 and 20 ng/ml) of TGF-β1 (cat. no. 10804-HNAC; Sino Biological, Inc., Beijing, China). After 72 h, the expression levels of SMS1, E-cadherin and vimentin (EMT markers) were investigated by western blotting.

Transfection and grouping. To construct an SMS1 over-expression cell model, SMS1-overexpressing plasmids [pcDNA3.1(+)], which were constructed by Magus Technology (Shanghai, China), were transfected into MDA-MB-231 cells. Transfection was conducted according to the manufacturer's protocol. First, 12 h prior to transfection, $1x10^6$ cells were seeded into wells of a 6-well plate (Beaver Nano-Technologies Co., Ltd., Suzhou, China) that contained antibiotic-free medium. At the time of transfection, the cell confluency was 60-70% (22). The SMS1-overexpressing plasmid (4 μg; SMS1 group) or a negative plasmid (4 μg; Control group) was diluted with 50 μl DMEM (FBS-free and antibiotic-free medium) or 5 μl Entranster™-D-4000 (Engreen Biosystem New Zealand Ltd., Auckland, New Zealand) and 50 μl DMEM. After 5 min, the dilutions were mixed together and incubated at 37˚C for 20 min and subsequently dispensed into each well. DMEM was replaced with DMEM containing 10% FBS after 6 h (23). The cells were cultured for 24 h, and the Control and the SMS1 groups were treated with 10 ng/ml TGF-β1 at 37˚C, as at that concentration, vimentin and E-cadherin expression had significantly altered. Therefore, the following four groups were created: Control, SMS1, TGF-β1 and SMS1+TGF-β1. After 72 h, all cells were harvested for subsequent experiments.

Wound healing assay. A total of $2x10^5$ MDA-MB-231 cells were seeded in 12-well tissue culture plates. After transfection and 10 ng/ml TGF-β1 treatment (36 h), the cells were maintained in serum-free medium at 37˚C for 8 h. Using a sterile 200-μl pipette tip to gently swipe along the midline of the cell well, the cells were scraped from the well and were washed with PBS three times. Subsequently, the MDA-MB-231 cells were treated with TGF-β1 at the above concentration for 24 h. Finally, the wound closure was measured with a phase contrast inverted microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan; magnification, x4).

Matrigel invasion analysis. The cells were treated as above. Briefly, cells were starved for 8 h. Single-cell suspension ($1x10^5$ cells) was added to serum-free medium with or without 10 ng/ml TGF-β1 on a top well of a 24-well Transwell plate (cat. no. 3413; Corning Incorporated, Corning, NY, USA) precoated with Matrigel (cat. no. 356234). After 36 h, the cells invading the lower chamber containing medium was supplemented with 10% FBS were stained with 0.1% crystal violet solution for 5 min at room temperature (CAS:548-62-9; Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) and counted using a phase contrast inverted light microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan; magnification, x10).

Western blot analysis. Proteins were extracted using radio-immunoprecipitation assay buffer (cat. no. ROO20; Beijing Solarbio Bioscience & Technology Co., Ltd., Beijing, China), and the protein concentration was measured using a bicinchoninic acid assay (cat. no. CW0014; Beijing Kangwei Century Biotechnology Co., Ltd., Beijing, China). Equal amounts of cleared lysates (~50 μg protein) were separated by 10-12% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). Equal transfer was validated by staining with Ponceau red (cat. no. CW0057S; Beijing Kangwei Century
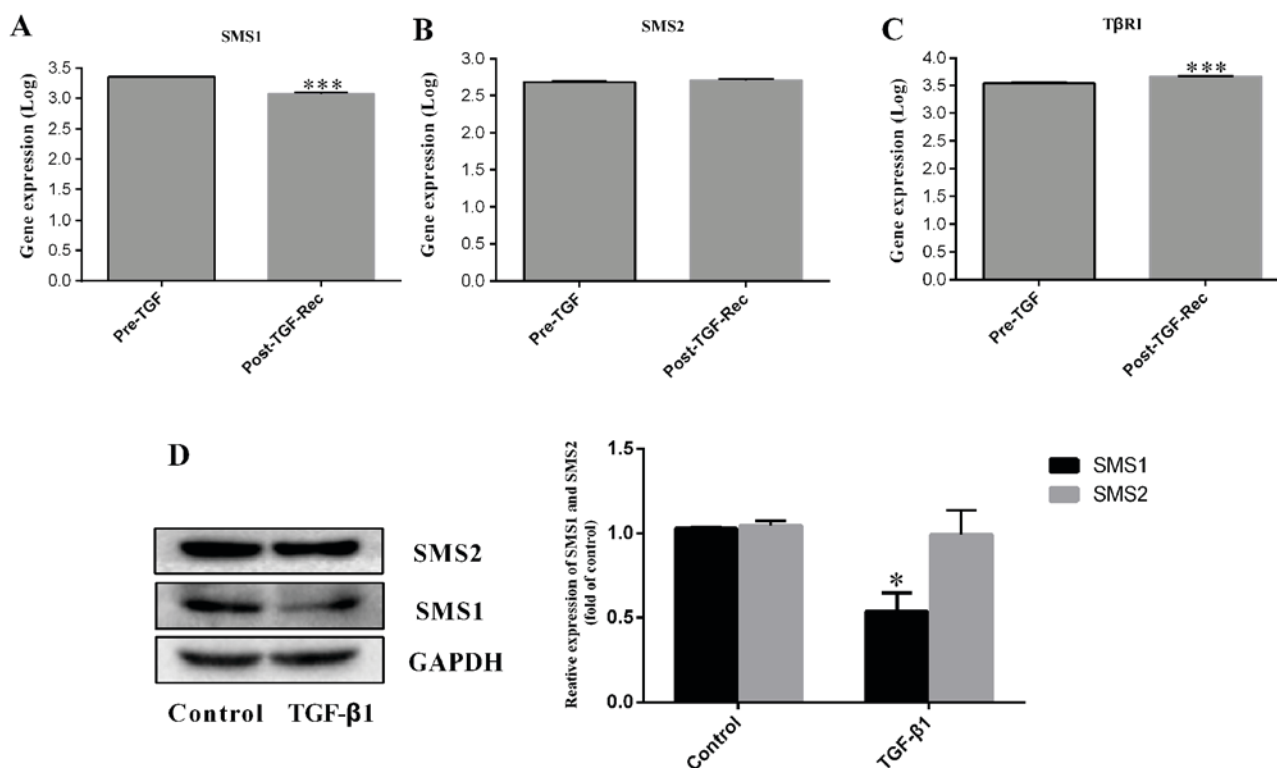
Figure 1. Bioinformatics analysis of the expression of SMS and TGF-β type I receptor. (A) Gene expression of SMS1. (B) Gene expression of SMS2. (C) Gene expression of TβRI. (D) Expression levels of SMS1 and SMS2 in MDA-MB-231 cells as measured by western blotting. Data are presented as the mean ± standard deviation. n=3. *P<0.05, ***P<0.001 vs. the control group. TβRI, transforming growth factor type I receptor; SMS, sphingomyelin synthase; TGF, transforming growth factor.

Biotechnology Co., Ltd.) for 30 min at room temperature. The membranes were blocked with 10% skimmed milk or 10% bovine serum albumin (BSA; cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) in TBS for 1 h at room temperature and subsequently incubated with primary antibodies in TBS containing 0.05% Tween-20, 2% BSA and 0.05% sodium azide overnight at 4˚C (24). The following antibodies were used at the indicated dilutions: SMS1 (cat. no. sc-133135; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), vimentin (cat. no. 10366-1-AP; 1:2,000; ProteinTech Group, Inc., Chicago, IL, USA), E-cadherin (cat. no. 20874-1-AP; 1:2,000; ProteinTech Group, Inc.), phospho (p)-Smad2 (cat. no. AF8314; 1:2,000; Affinity Biosciences, Shanghai, China), Smad2 (cat. no. WL03369; 1:1,500; Wanleibio Co., Ltd., Shanghai, China) and TβR1 (cat. no. AF5347; 1:2,000; Affinity Biosciences), and a GAPDH antibody (cat. no. HRP-60004; 1:12,000; ProteinTech Group, Inc.). Subsequently, the membranes were incubated at room temperature for 1.5 h with secondary horseradish peroxidase-conjugated anti-rabbit antibodies (cat. no. SA0000I-2; 1:8,000; ProteinTech Group, Inc.) or anti-mouse antibodies (cat. no. SA0000I-I; 1:8,000; ProteinTech Group, Inc.) in TBS containing 0.05% Tween 20. Signals were determined using an enhanced chemiluminescence reagent (cat. no. CW0049M; Beijing Kangwei Century Biotechnology Co., Ltd.) and an autoradiography system (Image Lab; version 5.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA) (14). Each assay was repeated at least three times.

Immunofluorescence and confocal microscopy imaging. A total of $2x10^4$ cells were seeded on coverslips in a 24-well plate. Followign the treatment, the cells were gently washed with PBS. Subsequently, they were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization using 0.5% Triton X-100 (cat. no. T8200; Beijing Solarbio Science & Technology Co., Ltd.) in PBS for 20 min at room temperature. The coverslips were subsequently blocked in 5% BSA in PBS for 60 min at room temperature. Subsequently, the cells were incubated with anti-TGF-βRI (cat. no. AF5347; 1:100; Affinity Biosciences), anti-vimentin (cat. no. 10366-1-AP; 1:200; ProteinTech Group, Inc.) and anti E-cadherin (cat. no. 20874-1-AP; 1:50; ProteinTech Group, Inc.) antibodies overnight at 4˚C and subsequently washed in PBS. The cells were exposed to secondary antibody conjugated with fluorescein isothiocyanate (cat. no. BA1105; 1:50; Wuhan Boster Biological Technology, Ltd., Wuhan, China) or TRITC (cat. no. E032420-01; 1:250; Earthox Life Sciences Millbrae, CA, USA) for 60 min at room temperature. DAPI was used to stain the nuclei (cat. no. AR1177; Wuhan Boster Biological Technology, Ltd.) for 5 min at room temperature, and the cells were washed with PBS. Finally, an inverted fluorescence (Olympus IX71; Olympus Corporation; magnification, x20) was used to acquire the data of vimentin and E-cadherin and a confocal microscope (Leica SP8; Leica Microsystems, Ltd., Milton Keynes, UK; magnification, x40) was used to acquire the data of TGF-βRI. Images were analyzed by Image J software (Ver. 2.1; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean ± standard deviation. Unpaired t-test was used for single comparisons. For
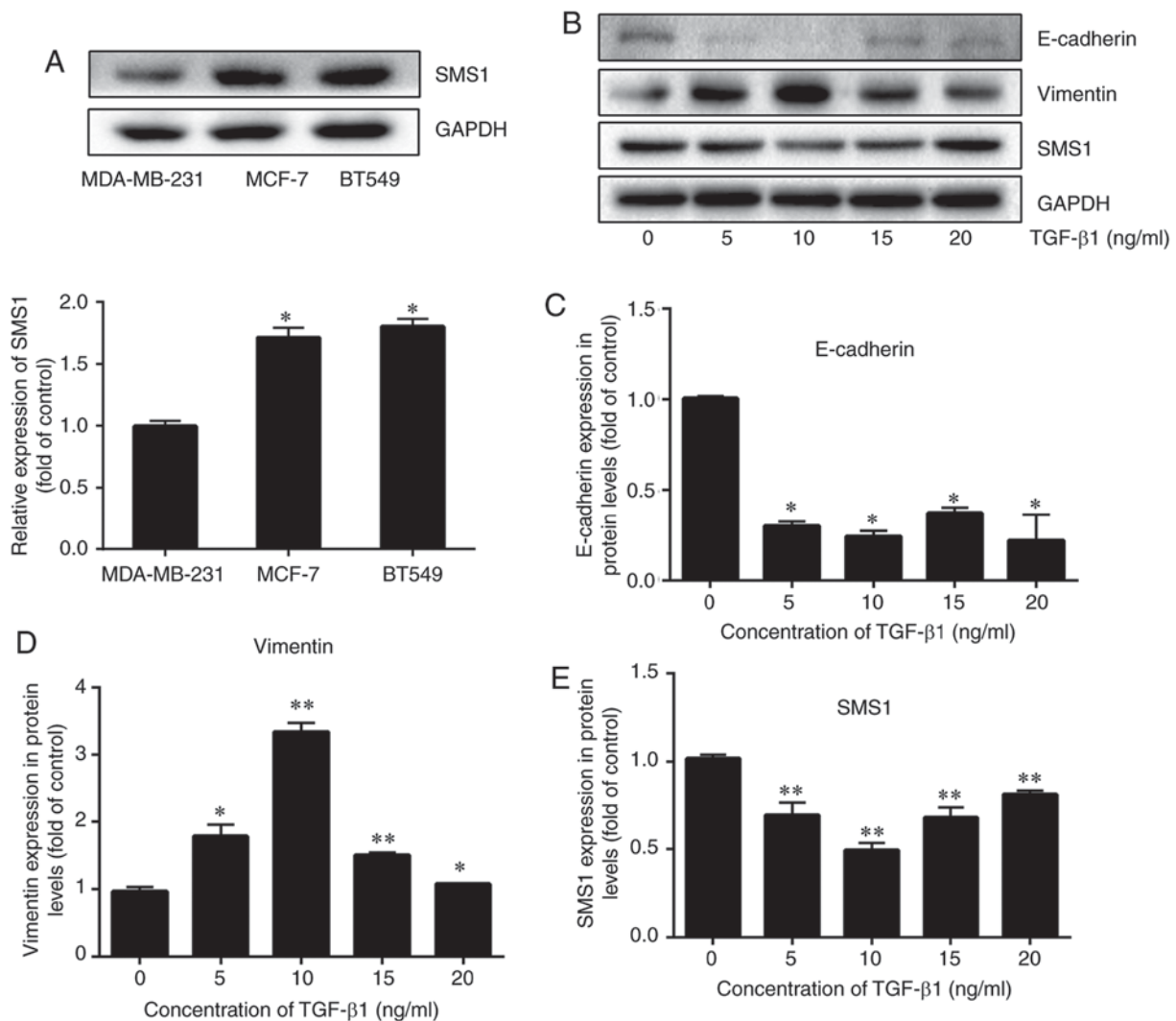
Figure 2. Expression of SMS1 in breast cancer cell lines. (A) Expression of SMS1 was measured by western blot in three cell lines. Data are presented as the mean ± standard deviation. n=3. **P<0.01 vs. MDA-MB-231 group. (B) Expression of E-cadherin, vimentin and SMS1 as measured by western blotting in MDA-MB-231 cells and relative quantification of (C) E-cadherin, of (D) vimentin and of (E) SMS1 as measured by western blotting in MDA-MB-231 cells. Data are presented as the mean ± standard deviation. n=3. *P<0.05, **P<0.01 vs. the control group. SMS, sphingomyelin synthase 1; TGF, transforming growth factor.

multiple comparisons, one-way analysis of variance followed by Tukey's or Games-Howell post-hoc test was used. All experiments were repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Bioinformatics analysis of the possible trends of SMS1 and TGF-β type I receptors in the development of EMT. The GSE54491 dataset was downloaded from National Center for Biotechnology Information GEO DataSets. For the GSE54491 dataset, normal murine mammary gland (NMumG) cells transformed by overexpression of endothelial growth factor receptor (NME) cells were cultured in the presence of TGF-β1 (5 ng/ml) for 4 weeks, at which point TGF-β1 supplementation was discontinued and the cells were allowed to recover for an additional 4 weeks (Post-TGF-Rec). Total RNA was prepared from unstimulated cells (Pre-TGF) at similar passage numbers and compared by microarray analysis. In

the present study, the two groups were analyzed in triplicate, including three Pre-TGF and three Post-TGF-Rec samples. The results showed that the expression of SMS1 decreased by 0.48-fold (P<0.001; n=3; Fig. 1A), whereas, the expression of SMS2 was not significantly different (P=0.731; n=3; Fig. 1B), in the Post-TGF-Rec group compared with the Pre-TGF group. However, TβRI (Fig. 1C) was increased by 0.32-fold (P<0.001; n=3). To validate the bioinformatics results, the expression of SMS1 and SMS2 in MDA-MB-231 cells following treatment with 10 ng/ml TGF-β1 was measured. The results confirmed that the expression of SMS1 was significantly decreased (Fig. 1D; P<0.05; n=3), whereas SMS2 was not significantly different between groups (Fig. 1D; P>0.05; n=3); these results were consistent with the bioinformatics data.

Expression of SMS1 is significantly decreased in the TGF-β1-induced EMT process in MDA-MB-231 cells. To examine whether the expression of SMS1 is different in breast cancer cells, the expression of SMS1 in three breast cancer
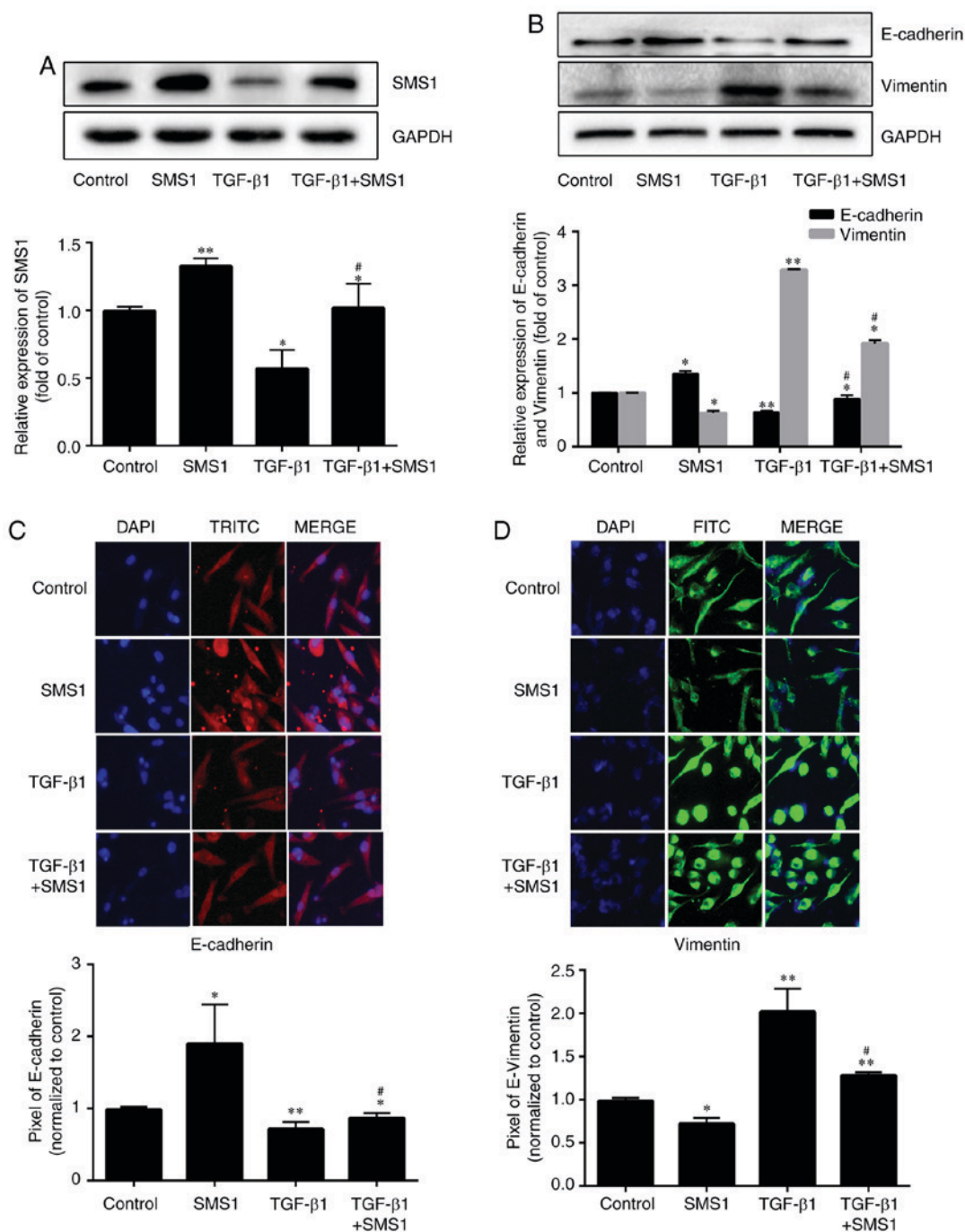
Figure 3. Overexpression of SMS1 inhibits TGF-β1-induced EMT. (A) Expression of SMS1 and of (B) E-cadherin and Vimentin were measured by western blotting in MDA-MB-231 cells. (C) Immunofluorescence staining of E-cadherin and of (D) Vimentin by a fluorescence microscope in MDA-MB-231 cells. Magnification, x20. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group; #P<0.05 vs. the TGF-β1 group. SMS, sphingomyelin synthase 1; TGF, transforming growth factor.

cells, including MDA-MB-231, MCF-7 and BT549 was investigated. The results demonstrated that the expression of SMS1 was the lowest in MDA-MB-231, followed by MCF-7 and BT549 (Fig. 2A; P<0.05, n=3). Therefore, in the present study, MDA-MB-231 was selected.

MDA-MB-231 cells were cultured with different concentrations of TGF-β1 (0, 5, 10, 15 and 20 ng/ml) for 72 h and the expression of SMS1, E-cadherin and vimentin was examined. An increase in vimentin and a decrease in E-cadherin expression were evident when MDA-MB-231 cells were treated with 10 ng/ml TGF-β1 (P<0.05 and P<0.01, respectively; n=3;

Fig. 2B-D, respectively). Similarly, the decrease in SMS1 expression was evident when MDA-MB-231 cells were treated with 10 ng/ml TGF-β1 (P<0.05 and P<0.01, respectively; n=3; Fig. 2E).

Overexpression of SMS1 inhibits TGF-β1-induced EMT. Protein expression levels of the EMT markers E-cadherin and vimentin were measured to evaluate the influence of the overexpression of SMS1 on the TGF-β1-induced EMT process (Fig. 3A; P<0.05 and P<0.01, respectively, n=3). Following treatment with TGF-β1, the expression of E-cadherin was decreased by 0.39-fold, and vimentin was increased by 2.3-fold
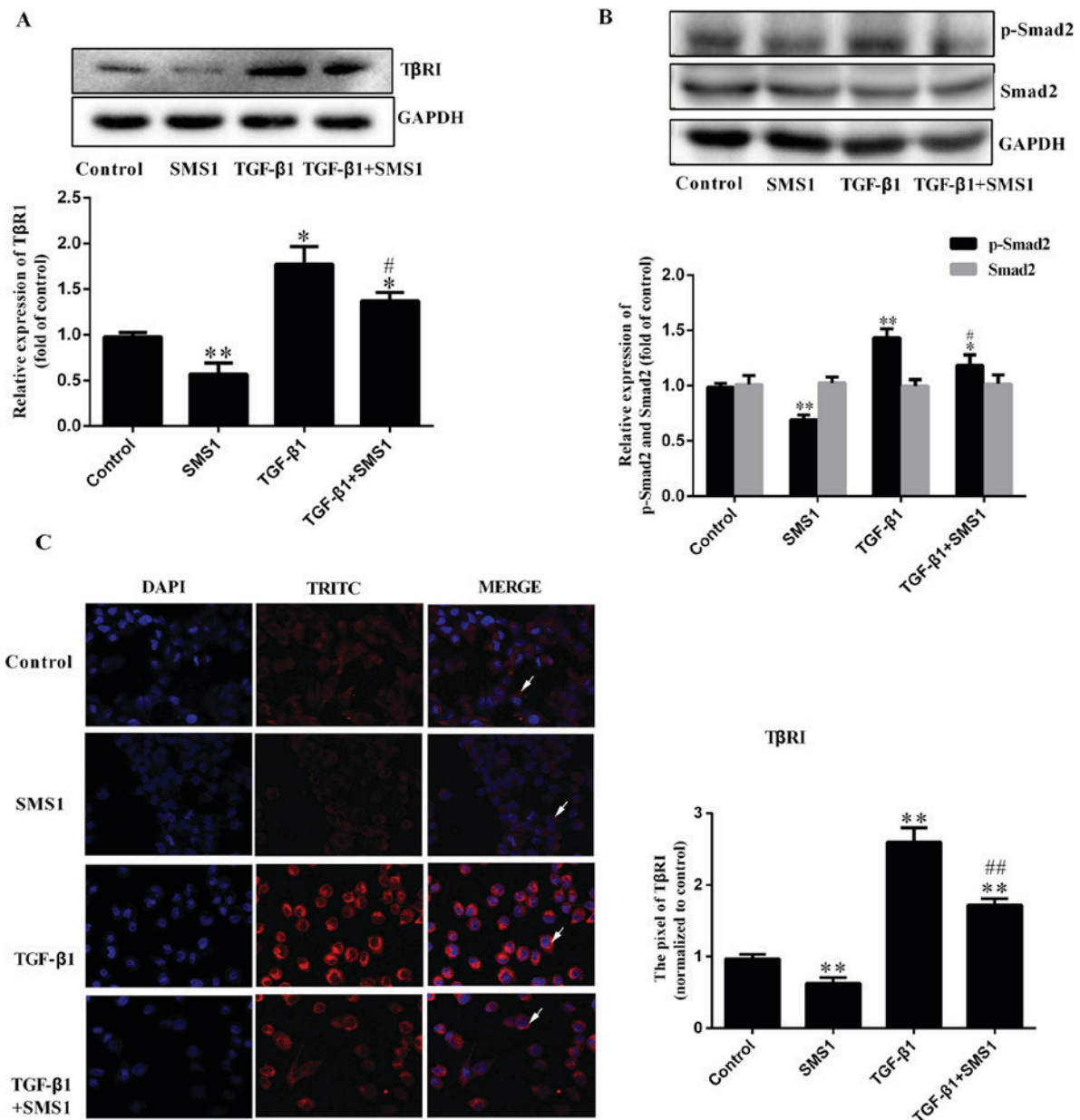
Figure 4. Overexpression of SMS1 regulates TGF-β1-induced EMT via TGF-β type I receptors. (A) Expression of TβRI and (B) phosphorylation of Smad2 and Smad2 were measured by western blotting in MDA-MB-231 cells. (C) Immunofluorescence staining of TβRI was shown by a confocal microscope in MDA-MB-231 cells. Magnification, x40. Data are presented as the mean ± standard deviation. n=3. *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the TGF-β1 group. P, phosphorylated; SMS, sphingomyelin synthase 1; TGF, transforming growth factor; TβRI, TGF β type I receptor.

compared with the control group (Fig. 3B; P<0.05 or P<0.01; n=3). In contrast, overexpression of SMS1 together with TGF-β1 treatment blocked TGF-β1-induced EMT, in which the expression of E-cadherin was decreased by 0.23-fold and vimentin was increased by 0.92-fold. The immunofluorescence and confocal microscopy imaging results verified these results to some extent (Fig. 3C and D).

Overexpression of SMS1 regulates TGF-β1-induced EMT via TGF-β type I receptor. Previous studies have demonstrated that the Smad-dependent signaling pathway is induced by TGF-β1, including the activation of TβRI and phosphorylated Smad2 and Smad3 (10,13). To further investigate the mechanism of SMS1 in regulating the TGF-β1 induced EMT process, the effect on Smad-dependent signaling pathway

proteins was investigated. Western blot analysis revealed that TGF-β1 treatment induced the expression of TβRI and the phosphorylation of Smad2, which were increased by 0.77 and 0.43-fold, respectively. The expression of Smad2 demonstrated no significant difference (Fig. 4B; P>0.05, respectively; n=3). However, SMS1 overexpression downregulated TβRI expression and blocked Smad2 phosphorylation (Fig. 4A and B; P<0.05 and P<0.01, respectively, n=3). The expression of TβRI was investigated by immunofluorescence. The results demonstrated that the overexpression of SMS1 altered the expression of TβRI on the cell membrane, which may be associated with the endocytosis of TβRI (Fig. 4C) (16,17).

Overexpression of SMS1 inhibits MDA-MB-231 cell migration and invasion induced by TGF-β1. To further clarify the role
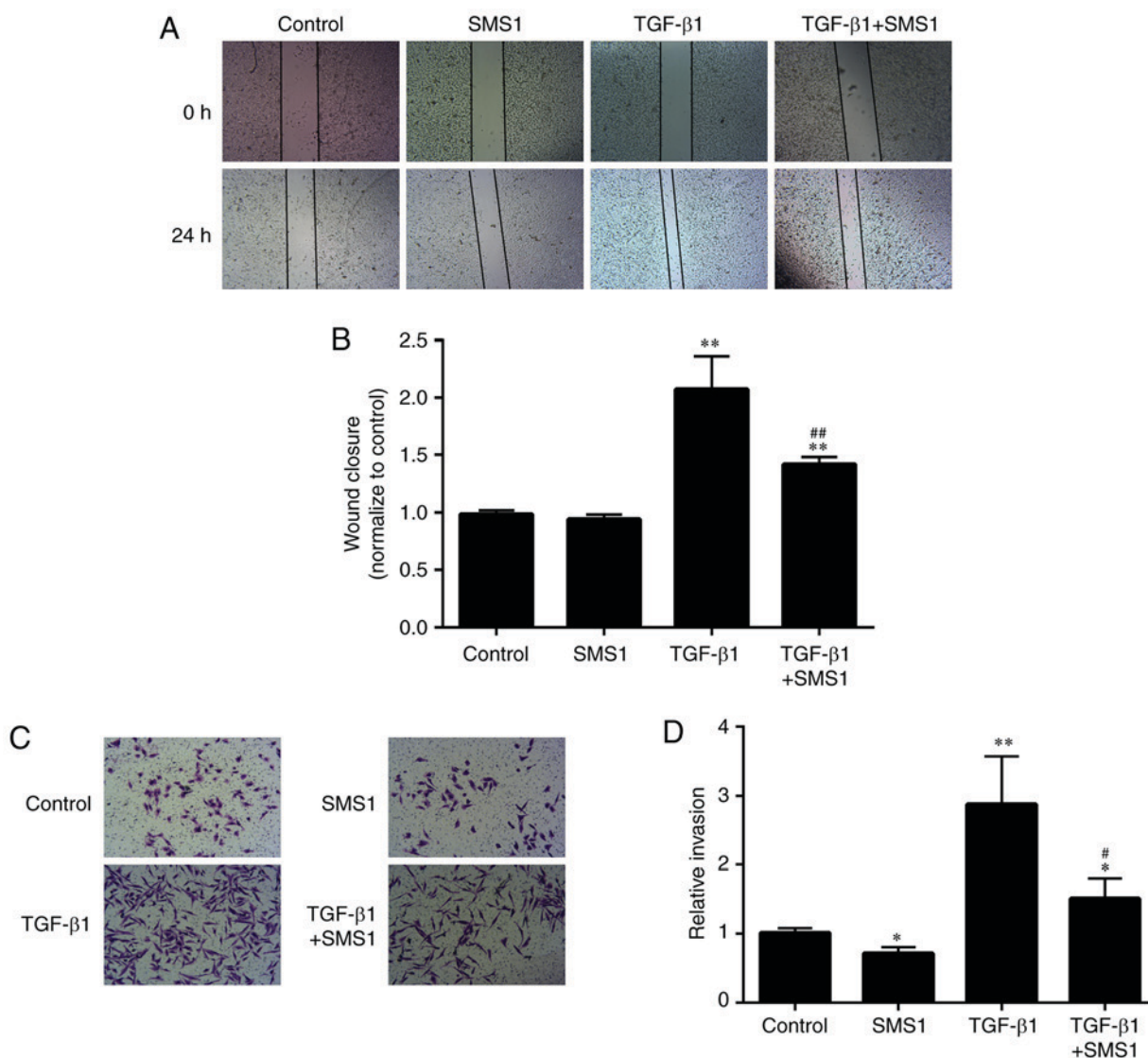
Figure 5. Overexpression of SMS1 inhibits MDA-MB-231 cells migration and invasion induced by TGF-β1. (A and B) Migration abilities were shown by wound healing analysis in MDA-MB-231 cells. Magnification, x4. (C and D) Invasion abilities were shown by Matrigel invasion assay in MDA-MB-231 cells. Magnification, x10. Data are presented as the mean ± standard deviation. n=3. *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the TGF-β1 group. SMS, sphingomyelin synthase 1; TGF, transforming growth factor.

of SMS1 in the TGF-β1-induced EMT in MDA-MB-231 cells, changes in the migration and invasion abilities of cells treated with or without TGF-β1 following transfection with an SMS1 plasmid, were investigated. Treatment with TGF-β1 significantly increased the cell migration abilities in MDA-MB-231 cells by 1.1-fold, as demonstrated by wound healing analysis. In addition, the increased migration abilities of MDA-MB-231 cells were significantly inhibited (by 0.32-fold) by transfection with an SMS1 plasmid (Fig. 5A and B; P<0.01; n=3). Furthermore, treatment with TGF-β1 for 72 h significantly increased the invasion abilities of MDA-MB-231 cells by 1.9-fold in a Matrigel invasion assay. Overexpression of SMS1 significantly suppressed the TGF-β1-induced invasion abilities of MDA-MB-231 cells by 0.47-fold (Fig. 5C and D; P<0.05 and P<0.01, respectively; n=3).

Discussion

The aim of the present study was to assess the importance of SMS1 in TGF-β1-induced EMT development. The results showed that SMS1 overexpression can downregulate TβRI expression and interfere with TGF-β1-induced Smad2 phosphorylation, it can increase the expression of E-cadherin and decrease the expression of vimentin. Finally, the migration and invasion of MDA-MB-231 cells were suppressed following SMS1 overexpression together with treatment with TGF-β1. These results demonstrated that SMS1 overexpression can inhibit the EMT in MDA-MB-231 cells.

MS1 and SMS2 are the key enzymes in SM biosynthesis, but SMS1 is found on the Golgi apparatus, and SMS2 exists in the Golgi apparatus and plasma membranes (20). The expression levels of SMS1 and TβRI were negatively associated with each other when breast cancer cells were treated with TGF-β1; however, the expression of SMS2 was not altered and was not correlated with that of TβRI. This suggests that SMS1 and SMS2 may have different functions in the EMT. Additionally, three breast cancer cell lines expressed SMS1; however, the expression of

SMS1 was lower in MDA-MB-231 cells. Furthermore, MDA-MB-231 is a triple-negative breast cancer cell line, which has high metastatic and invasive ability (25). Therefore, in the present study, MDA-MB-231 cells was used to demonstrate that SMS1 can regulate the EMT in MDA-MB-231 cells.

Previous studies have demonstrated that TGF-β1 induces EMT to promote tumor invasion and metastasis (11-14). During EMT development, the morphological alterations are characterized by upregulated expression of the mesenchymal marker vimentin and downregulated expression of the epithelial marker E-cadherin. Our findings were consistent with their findings, however, SMS1 can inhibit these changes, which shown that overexpression SMS1 can inhibit EMT (Figs. 3 and 5).

Similar to other cell surface receptors (26), TGF-β receptors are mainly internalized via clathrin-dependent endocytosis, which is an essential regulatory event in signal transduction (27,28). In addition, lipid rafts/caveolae negatively regulate TGF-β1 signaling pathway through lipid raft-induced internalization of TGF-β receptors, promoting receptor degradation (29). Therefore, altering the contents of the main components of lipid raft would affect the distribution of TGF-β receptors in lipid raft/caveolae and no lipid raft, and the signal transduction of TGF-β receptors. In the present study, overexpression of SMS1 negatively regulated TβRI expression, moreover, SMS1 overexpression may block the TGF-β1-induced phosphorylation of Smad 2 and Smad2-mediated transcriptional activity without affecting total Smad 2 expression levels and additionally suppressed the TGFβ-1-induced EMT and cell migration. As demonstrated in previous studies (27-29), caveolin-1 and TβRI have distributed colocalization in the cell membrane. A possible explanation for this might be that SMS1 promotes lipid raft/caveolae-mediated internalization and the interaction between TβRI and caveolin-1, resulting in decreased surface expression of TβRI and Smad activation. Unlike clathrin-dependent endocytosis, lipid raft/caveolae-mediated internalization facilitates the degradation of TβRI receptors and therefore inhibits TGF-β1 signaling pathway (15,29).

In fact, a number of previous studies identified that altering the components of lipid rafts/caveolae may affect the TGF-β/Smad signaling pathway (30,31). In addition to SM, cholesterol is additionally a primary component of lipid rafts (30). Cholesterol precursors and cholesterol biosimilars can regulate signal transduction and the TGF-β/Smad pathway. For example, 7-DHC (precursor) and Euphol (biosimilar) can suppress TGF-β-stimulated luciferase activity by promoting lipid raft/caveolae formation and subsequently recruiting cell-surface TGF-β receptors from non-lipid raft microdomains to lipid rafts/caveolae, where TGF-β receptors become inactive in transducing canonical signaling and undergo rapid degradation upon TGF-β binding (29-31). In contrast, methyl-β-cyclodextrin, a sterol-chelating agent, reverses 7-DHC-induced suppression of TGF-β-stimulated luciferase activity by extrusion of 7-DHC from resident lipid rafts/caveolae (31). Furthermore, other factors that can affect the distribution of TGF-β receptors between lipid rafts/caveolae and non-lipid raft microdomains can also regulate the TGF-β/Smad pathway (27-29). Previous studies identified that cholest-4-en-3-one and dimethyl sulfoxide may increase lipid raft and/or caveolae accumulation of TGF-β receptors and facilitate the rapid degradation of TGF-β, thus suppressing TGF-β-induced signaling (32,33). In addition, ethanol also disrupts the location of other membrane proteins in lipid rafts/caveolae that utilize lipid rafts/caveolae as signaling platforms and enhances canonical TGF-β signaling by increasing the non-lipid raft microdomain localization of TGF-β receptors (34). Altogether, canonical TGF-β signaling pathway is tightly associated with lipid rafts and their primary components.

Although the studies presented thus far have indicated that receptor endocytosis is not essential for TGF-β1 signaling, lipid raft-mediated endocytosis of TGF-β receptors facilitates receptor degradation and thus turns off the signaling pathway (32,35). However, this is the first study, to the best of the authors' knowledge to investigate that the attenuation of the TGF-β1-induced EMT by SMS1, which could influence the formation of lipid rafts via TβRI expression and could be an important mechanism for the controlled progression of developmental events. The present study provides novel insight into the impact of SMS1 in signal transduction, and it has a number of important implications for future targeted therapy for breast cancer.

Availability of data and materials

The datasets used and/or generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL, HH and PZ performed the experiments and bioinformatics analysis, and were major contributors in writing the manuscript. YW analyzed and interpreted the data of the manuscript, and was responsible for the design and drafting of the manuscript. XH and HL were responsible for the statistical analysis. NY designed the study and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, Allen C, Alsharif U, Alvis-Guzman N, Amini E, Anderson BO, *et al*: Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: A systematic analysis for the global burden of disease study. JAMA Oncol 4: 1553-1568, 2018.
2. Lee JM, Dedhar S, Kalluri R and Thompson EW: The epithelial-mesenchymal transition: New insights in signaling, development, and disease. J Cell Biol 172: 973-81, 2006.
3. Singh A and Settleman J: EMT, cancer stem cells and drug resistance: An emerging axis of evil in the war on cancer. Oncogene 29: 4741-4751, 2010.
4. Chen Q, Yang W, Wang X, Li X, Qi S, Zhang Y and Gao MQ: TGF-beta1 induces EMT in bovine mammary epithelial cells through the TGFbeta1/Smad signaling pathway. Cell Physiol Biochem 43: 82-93, 2017.
5. Yeh YC, Wei WC, Wang MG, Lin SC, Sung JM and Tang MJ: Transforming growth factor-β1 induces smad3-dependent β 1, integrin gene expression in epithelial-to-mesenchymal transition during chronic tubulointerstitial fibrosis. Am J Pathol 177: 743-1754, 2010.
6. Attisano L and Wrana JL: Signal transduction by the TGF-beta superfamily. Science 296: 1646-1647, 2002.
7. Hwangbo C, Tae N, Lee S, Kim O, Park OK, Kim J, Kwon SH and Lee JH: Syntenin regulates TGF-β1-induced Smad activation and the epithelial-to-mesenchymal transition by inhibiting caveolin-mediated TGF-β type I receptor internalization. Oncogene 35: 389-401, 2016.
8. Thiery JP and Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7: 131-42, 2006.
9. Miettinen PJ, Ebner R, Lopez AR and Derynck R: TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: Involvement of type I receptors. J Cell Biol 127: 2021-2036, 1994.
10. Derynck R and Zhang YE: Smad-dependent and smad-independent pathways in TGF-beta family signalling. Nature 425: 577-584, 2003.
11. Lin XL, Liu M, Liu Y, Hu H, Pan Y, Zou W, Fan X and Hu X: Transforming growth factor beta1 promotes migration and invasion in HepG2 cells: Epithelialtomesenchymal transition via JAK/STAT3 signaling. Int J Mol Med 41: 129-136, 2017.
12. Kim JY, An HJ, Kim WH, Gwon MG, Gu H, Park YY and Park KK: Anti-fibrotic effects of synthetic oligodeoxynucleotide for TGF-beta1 and smad in an animal model of liver cirrhosis. Mol Ther Nucleic Acids 8: 250-263, 2017.
13. Boldbaatar A, Lee S, Han S, Jeong AL, Ka HI, Buyanravjikh S, Lee JH, Lim JS, Lee MS and Yang Y: Eupatolide inhibits the TGF-beta1-induced migration of breast cancer cells via downregulation of SMAD3 phosphorylation and transcriptional repression of ALK5. Oncol Lett 14: 6031-6039, 2017.
14. Wang Y, Liu X, Zheng H, Wang Q, An L and Wei G: Suppression of CUL4A attenuates TGF-beta1-induced epithelial-to-mesenchymal transition in breast cancer cells. Int J Mol Med 40: 1114-1124, 2017.
15. Le Roy C and Wrana JL: Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat Rev Mol Cell Biol 6: 112-26, 2005.
16. Midgley AC, Rogers M, Hallett MB, Clayton A, Bowen T, Phillips AO and Steadman R: Transforming growth factor-beta1 (TGF-beta1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (HA)-facilitated epidermal growth factor receptor (EGFR) and CD44 co-localization in lipid rafts. J Biol Chem 288: 14824-14838, 2013.
17. Chen YG: Endocytic regulation of TGF-beta signaling. Cell Res 19: 58-70, 2009.
18. Li J, Xia K, Xiong M, Wang X and Yan N: Effects of sepsis on the metabolism of sphingomyelin and cholesterol in mice with liver dysfunction. Exp Ther Med 14: 5635-5640, 2017.
19. Taniguchi M and Okazaki T: The role of sphingomyelin and sphingomyelin synthases in cell death, proliferation and migration-from cell and animal models to human disorders. Biochim Biophys Acta 1841: 692-703, 2014.
20. Yeang C, Ding T, Chirico WJ and Jiang XC: Subcellular targeting domains of sphingomyelin synthase 1 and 2. Nutr Metab (Lond) 8: 89, 2011.
21. Torki S, Soltani A, Shirzad H, Esmaeil N and Ghatrehsamani M: Synergistic antitumor effect of NVP-BEZ235 and CAPE on MDA-MB-231 breast cancer cells. Biomed Pharmacother 92: 39-45, 2017.
22. Luo S, Pan Z, Liu S, Yuan S and Yan N: Sphingomyelin synthase 2 overexpression promotes cisplatin-induced apoptosis of HepG2 cells. Oncol Lett 15: 483-488, 2018.
23. Yan N, Ding T, Dong J, Li Y and Wu M: Sphingomyelin synthase overexpression increases cholesterol accumulation and decreases cholesterol secretion in liver cells. Lipids Health Dis 10: 46, 2011.
24. Hu S, Ding Y, Gong J and Yan N: Sphingomyelin synthase 2 affects CD14 associated induction of NFkappaB by lipopolysaccharides in acute lung injury in mice. Mol Med Rep 14: 3301-3306, 2016.
25. Kwiatkowska E, Wojtala M, Gajewska A, Soszynski M, Bartosz G and Sadowska-Bartosz I: Effect of 3-bromopyruvate acid on the redox equilibrium in non-invasive MCF-7 and invasive MDA-MB-231 breast cancer cells. J Bioenerg Biomembr 48: 23-32, 2016.
26. Xu Y, Xia J, Liu S, Stein S, Ramon C, Xi H, Wang L, Xiong X, Zhang L, He D, *et al*: Endocytosis and membrane receptor internalization: Implication of F-BAR protein carom. Front Biosci (Landmark Ed) 22: 1439-1457, 2017.
27. Conner SD and Schmid SL: Regulated portals of entry into the cell. Nature 422: 37-44, 2003.
28. Mitchell H, Choudhury A, Pagano RE and Leof EB: Ligand-dependent and -independent transforming growth factor-beta receptor recycling regulated by clathrin-mediated endocytosis and Rab11. Mol Biol Cell 15: 4166-4178, 2004.
29. Razani B, Zhang XL, Bitzer M, von Gersdorff G, Bottinger EP and Lisanti MP: Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. J Biol Chem 276: 6727-6738, 2001.
30. Zhang B, Paffett ML, Naik JS, Jernigan NL, Walker BR and Resta TC: Cholesterol regulation of pulmonary endothelial calcium homeostasis. Curr Top Membr 82: 53-91, 2018.
31. Huang SS, Liu IH, Chen CL, Chang JM, Johnson FE and Huang JS: 7-Dehydrocholesterol (7-DHC), but not cholesterol, causes suppression of canonical TGF-beta signaling and is likely involved in the development of Atherosclerotic Cardiovascular Disease (ASCVD). J Cell Biochem 118: 1387-1400, 2017.
32. Chen CL, Wu DC, Liu MY, Lin MW, Huang HT, Huang YB, Chen LC, Chen YY, Chen JJ, Yang PH, *et al*: Cholest-4-en-3-one attenuates TGF-beta responsiveness by inducing TGF-beta receptors degradation in Mv1Lu cells and colorectal adenocarcinoma cells. J Recept Signal Transduct Res 37: 189-199, 2017.
33. Huang SS, Chen CL, Huang FW, Hou WH and Huang JS: DMSO enhances TGF-beta activity by recruiting the type II TGF-beta receptor from intracellular vesicles to the plasma membrane. J Cell Biochem 117: 1568-1579, 2016.
34. Huang SS, Chen CL, Huang FW, Johnson FE and Huang JS: Ethanol enhances TGF-beta activity by recruiting TGF-beta receptors from intracellular vesicles/lipid rafts/caveolae to non-lipid raft microdomains. J Cell Biochem 117: 860-871, 2016.
35. Chen CL, Huang SS and Huang JS: Cellular heparan sulfate negatively modulates transforming growth factor-beta1 (TGF-beta1) responsiveness in epithelial cells. J Biol Chem 281: 11506-11514, 2006.