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Function of GCN5 in the TGF-β1-induced epithelial-to-mesenchymal transition in breast cancer

LIMING ZHAO[1], AIXIA PANG[2] and YUNCHUN LI[3]

[1]Departments of Nuclear Medicine and [2]Urology, Linyi People's Hospital, Linyi, Shandong 276000;
[3]Department of Laboratory Medicine of Jinshan Hospital of Fudan University, Shanghai 201508, P.R. China

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Abstract. Histone acetyltransferase GCN5 is a critical component of the TGF-β/Smad signaling pathway in breast cancer cells; however, it remains unknown whether it is involved in the development and progression of breast cancer. The present study investigated the role of GCN5 in the induction of the EMT by TGF-β1 in breast cancer cells and its underlying molecular mechanism of action. GCN5 activity was elevated and GCN5 mRNA expression and protein expression were increased in MDA-MB231 cells following stimulation with TGF-β1. Furthermore, TGF-β1 stimulation decreased expression of the epithelial cell marker E-cadherin and increased expression of the mesenchymal cell markers, N-cadherin and vimentin, as well as the expression of other EMT markers, including snail and slug. However, these changes were reversed following GCN5 knockdown leading to the downregulation of GCN5 expression. GCN5 knockdown also inhibited the viability, migration and invasion of MDA-MB231 cells, decreased the expression of p-STAT3, p-AKT, MMP9 and E2F1, and increased the expression of p21 in MDA-MB231 cells compared with cells stimulated with TGF-β1 alone. Therefore, GCN5 may work downstream of TGF-β/Smad signaling pathway to regulate the EMT in breast cancer.

Introduction

Breast cancer is the most common malignancy diagnosed in women worldwide and is one of the leading causes of cancer-associated mortality (1). The incidence of breast cancer has been increasing every year and although treatments for breast cancer have improved recently, the clinical outcome of patients remains unsatisfactory (2). The majority of cancer-associated mortalities are due to the metastasis of primary tumors (3). The epithelial-mesenchymal transition (EMT) is a crucial step in cancer invasion and metastasis. Its initiation corresponds with the loss of epithelial properties and the acquisition of migratory mesenchymal characteristics, leading to aggressive cancer progression (4).

Members of the transforming growth factor-β (TGF-β) superfamily are multifunctional proteins that regulate various cellular responses, including cell proliferation, differentiation, migration and apoptosis (5). Exogenous TGF-β1 may serve an important role in determining the migration and invasion capabilities of breast cancer cells, as it induces the EMT (6). Therefore, inhibiting the induction of the EMT by TGF-β1 may be a novel therapeutic strategy to treat patients with breast cancer.

Histone acetyltransferase GCN5 (GCN5; also known as KAT2A) is essential for the development of multiple organs and serves important roles in cell proliferation, differentiation, cell cycle and DNA damage repair (7,8). Previous studies have demonstrated that GCN5 dysfunction is linked to different types of cancer, including breast cancer (9,10). It has been demonstrated that GCN5 is a critical component of the TGF-β/Smad signaling pathway in breast cancer cells and enhances the transcriptional activity of TGF-β1 (6). However, the precise mechanism underlying the TGF-β-induced EMT remains to be elucidated. Epigenetic regulation is recognized as one driving force of the EMT. Upregulation of GCN5, as well as the histone acetylation of certain EMT genes, has been reported in lung cancer cells following treatment with an epidermal growth factor receptor inhibitor (11). Therefore, it is conceivable that GCN5 may work downstream of the TGF-β/Smad signaling pathway to epigenetically regulate the EMT in breast cancer. Thus, the objective of the current study was to investigate the effect of GCN5 on the TGF-β1-induced EMT in breast cancer cells and determine its underlying mechanism of action.

Materials and methods

Cell culture and TGF-β treatment. The human breast cancer cell lines MDA-MB231, MCF-7 and Hs578T were purchased from Shanghai Cancer Institute (Shanghai, China) and cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing sodium bicarbonate, 10% fetal bovine serum (FBS), 2 mmol/l

Correspondence to: Dr Yunchun Li, Department of Laboratory Medicine of Jinshan Hospital of Fudan University, 1508 Longhang Road, Shanghai 201508, P.R. China
E-mail: liyunchunlyc@163.com

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L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified 5% CO_2 atmosphere at 37˚C for 24 h. A total of $5x10^3$ cells/well were plated in 96-well plates. Cells were incubated with or without 5 ng/ml TGF-β1. The protocol and procedure of the experiment were approved by the Ethics Committee of Linyi People's Hospital (Shandong, China). MDA-MB231 cells were treated with TGF-β1 (5 ng/ml) with or without sorafenib (5 μM; Bayer AG, Leverkusen, Germany), and incubated for 0, 1, 2 and 3 days.

Determination of cell viability. MDA-MB231, MCF-7 and Hs578T cells were seeded into a 96-well plate ($1x10^5$ cells/well). When cells reached 90% confluence, they were cultured in serum-free medium for 24 h and then incubated with or without 5 ng/ml TGF-β1 for 24 h. MDA-MB231 cells were divided into three groups depending on different treatments: TGF-β1, sorafenib and TGF-β1+sorafenib. In addition, GCN5 siRNA was used to knockdown GCN5 expression in MDA-MB231 cells. Cell viability was measured using the MTT assay (American Type Culture Collection, Manassas, VA, USA). A total of 10 μl of 12 mM MTT stock solution was added to each well and incubated at 37˚C for 4 h. Subsequently, 500 μl of dimethyl sulfoxide was added to each well and incubated at 37˚C for 10 min. The plate was transferred to a plate reader (Beckman Coulter, Fullerton, CA, USA) and the absorbance was measured at 550 nm. Cell viability was estimated in triplicate.

Plasmid production. MDA-MB231 cells were used for the following experiments. To induce GCN5 overexpression, the green fluorescent protein-coding region in the lentiviral vector FUGW was replaced with GCN5 coding sequences. The lentiviral vector pLKO.1 was used for gene knockdown. Plasmids were obtained from Shanghai GenePharma Co, Ltd. (Shanghai, China). The construction of the GCN5 mutant was performed as described previously (12). DNA oligonucleotides for the GCN5-small interfering (si)RNA were synthesized, annealed and cloned into pLKO.1. The sequences of GCN5 siRNA (80 μg, 100 nM) used were as follows: Forward, 5'-GCUCUACACAACCCUCAAATT-3' and reverse, 5'-UUU GAGGGUUGUGUAGAGCTT-3'. MDA-MB231 cells were transfected with GCN5 vector (GCN5-WT) or GCN5 mutant vector (GCN5-MUT), as well as siRNA against GCN5 (GCN5 siRNA) or scramble siRNA (control siRNA) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Following 6 h incubation at 37˚C, the transfection medium was replaced with 2 ml DMEM containing 10% FBS. Cells were subsequently harvested for the assays.

GCN5 activity assay. MDA-MB231 cells were incubated with or without 5 ng/ml TGF-β1 for 24 h. GCN5 activity was determined using the GCN5 chemiluminescent assay kit (cat. no. 50079; BPS Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 40 μl master mixture containing 5 μl cell supernatant, 5 μl 10X HAT assay buffer, 5 μl acetyl-coenzyme A (1 mM) as well as 25 μl water was placed in each well of a 96-well plate. The kit included purified GCN5 as positive control. For the blank control, 5 μl 10X HAT Assay Buffer and 35 μl water were added to the wells. Subsequently, each well was incubated with primary antibody for 1 h at room temperature with slow shaking and then incubated with secondary antibody for 30 min (antibodies were included in the assay kit). A total of 100 μl horseradish peroxidase (HRP) chemiluminescent substrate A and HRP chemiluminescent substrate B with a 1:1 ratio were added to the mixture in each well. Any unused chemiluminescent reagent was discarded following use. Samples were measured using a luminometer or microtiter plate reader capable of measuring chemiluminescence. The Blank value was subtracted from all other values.

Cell migration and invasion assays. In vitro Transwell migration and invasion assays was performed in a modified Boyden chamber assay with a Falcon™ Cell Culture Insert (BD Biosciences, San Jose, CA, USA) in 24-well plates. The membrane was coated with Matrigel to simulate the typical matrices that cancer cells encounter during the invasion process *in vivo*; for the migration assay, only the membrane without coating was used. A total of $1x10^5$ cells/well were suspended in serum-free medium and plated in the top chamber of the Transwell chambers. The lower chamber was filled with medium containing 10% FBS, which acted as a chemoattractant. Following 24 h, cells that migrated through the membrane were subsequently fixed with 100% absolute alcohol at room temperature for 30 min and stained with 0.05% crystal violet at room temperature for 10 min. Following air drying, migrated cells were quantified by counting the optical density of cells per four high-power fields under a light microscope with x40 magnification.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from MDA-MB231 cells using the TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics), according to the manufacturer's instructions. mRNA levels were determined by qPCR using SYBR Green I Master (Roche Diagnostics). Thermal cycling conditions consisted of 2 min at 50˚C, 10 min at 94˚C, and followed by 40 cycles of 94˚C for 15 sec, 60˚C for 15 sec, 72˚C for 15 sec, and 1 sec at 80.5˚C for plate reading. Following the cycling protocol, the final step was applied to all reactions by continuously monitoring fluorescence through the dissociation temperature of the PCR product at a temperature transition rate of 0.1˚C/sec to generate a melting curve. Quantification was conducted according to the $2^{-\Delta Cq}$ method (12). Reactions were performed in triplicate with GAPDH acting as an internal control. The sequences of primers were used: GCN5 forward, 5'-TTCCGAGTGGAGAAGGACA-3' and reverse, 5'-AGCA TGGACAGGAATTTGG-3'; GAPDH forward, 5'-ACAACT TTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACG CCACAGTTTC-3'.

Western blot analysis. Total protein was extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Total cellular protein concentrations were determined using a BCA assay kit

(Beyotime Institute of Biotechnology). A total of 30 μg protein was loaded per lane and separated by 10% SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Following being blocked in 5% milk at room temperature for 2 h, the membrane was immunoblotted with primary antibodies against E-cadherin (cat. no. sc-71008; 1:500), N-cadherin (cat.no.sc-8424; 1:500), vimentin (cat.no.sc-73260; 1:500), snail family transcriptional repressor 1 (Snail; cat. no. sc-271977; 1:1,000), snail family transcriptional repressor 2 (slug; cat. no. sc-166476; 1:1,000), fibronectin (cat. no. sc-18825; 1:1,000), GCN5 (cat. no. sc-6303; 1:500), p-STAT3 (cat. no. sc-293059; 1:1,000), p-21 (cat. no. sc-377515; 1:500), p-AKT (cat. no. sc-7985-R; 1:1,000), MMP9 (cat. no. sc-137213; 1:500), E2F1 (cat. no. sc-251; 1:1,000) and GAPDH (cat. no. sc-69778; 1:5,000) at 4˚C overnight. The membranes were then incubated with anti-rabbit secondary antibody (cat. no. Sc-2357; 1:2,000) or anti-goat secondary antibody (cat. no. Sc-2354; 1:2,000) at 37˚C for 1 h. All antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The visualization reagent was Millipore Western Blot HRP-ECL reagent (cat. no. WBKLS0100; Sigma-Aldrich; Merck KGaA). Western blot quantitative analysis was performed using Scion Image software 4.03 (Scion Corp., Frederick, MD, USA).

Immunohistochemistry. Cells were cultured on cover glass slides and fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min and then permeabilized in 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA). Cells were washed with 2% glycine solution, then blocked with PBS containing 2% FBS and 0.5% saponin for 30 min at 37˚C. Anti-mouse antibodies against E-cadherin (4A2; monoclonal; cat. no. 14472S; Cell Signaling Technology, Inc., Danvers, MA, USA) were diluted at 1:100 in PBS with 1.5% normal goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) and incubated overnight at 4˚C. Cells were then incubated with Alexa Fluor 488-conjugated secondary antibodies (1:1,000 dilution, cat. no. ab201540; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Cells were then stained with DAPI for 2-3 min at room temperature. The slides were mounted with Mowiol solution (SouthernBiotech, Birmingham, AL, USA). Immunofluorescence was viewed using a Zeiss LSM-5Pa confocal microscope (magnification, x40; Zeiss, Oberkochen, Germany).

Statistical analysis. All results are presented as the mean ± standard error of the mean. Differences between groups were compared using two-way analysis of variance followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference. All of the statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

GCN5 activity is increased during the EMT induced by TGF-β1 in breast cancer cells. A number of key growth factors are able to induce the EMT, among which TGF-β1 is an important inducer during the metastasis that occurs in breast cancer. Therefore, the effect of TGF-β1 on the viability of breast cancer cells was assessed. Three breast cell lines were treated with 5 ng/ml TGF-β1 for 0, 1, 2, 3 and 4 days to investigate the effect of TGF-β1 on EMT induction. An MTT assay was performed to determine whether TGF-β1 induces toxicity in breast cancer cells. Following incubation with 5 ng/ml TGF-β1, cell viability was not markedly affected in any of the three breast cancer cell lines (Fig. 1A). Thus, 5 ng/ml TGF-β1 was selected to induce the EMT in breast cancer cells for 3 days. It was determined that TGF-β1 significantly increased GCN5 activity by 39.2, 38.9 and 41.3% in MDA-MB231, MCF-7 and Hs578T cells, respectively (all P<0.05; Fig. 1B). Furthermore, levels of GSN5 mRNA increased following treatment with TGF-β1 by 31, 35 and 32% in MDA-MB231, MCF-7 and Hs578T cells, respectively (all P<0.05; Fig. 1C). In addition, MDA-MB231, MCF-7 and Hs578T cells treated with TGF-β1 for 3 days exhibited increased mRNA levels of GSN5 and the mesenchymal cell markers N-cadherin and vimentin (all P<0.05) but decreased levels of the epithelial cell marker E-cadherin (all P<0.05; Fig. 1C). Furthermore, the expression of N-cadherin, vimentin, fibronectin, snail and slug were increased following treatment with TGF-β1, but the expression of E-cadherin was decreased in the MDA-MB231, MCF-7 and Hs578T cell lines, compared with controls (Fig. 1D). All three cell lines exhibited similar responses to stimulation with TGF-β1, any cell can be suitable for the next experiments and represent the other two cells, thus MDA-MB231 were selected for all subsequent experiments.

Treatment with GCN5 inhibitor counteracts the TGF-β1-induced EMT in breast cancer cells. Sorafenib (Nexavar or BAY 43-9006) is approved for the treatment of many tumors and it has been reported that sorafenib is able to attenuate the EMT and cell migration by inhibiting TGF-β1 (11,13). To examine the effect of sorafenib on cell viability following stimulation with TGF-β1, MDA-MB231 cells were treated with TGF-β1 (5 ng/ml) with or without sorafenib (5 μM), and incubated for 0, 1, 2 and 3 days. Cell viability was significantly decreased by 12.2% following treatment with sorafenib compared with cells exposed to TGF-β1 alone (P<0.05; Fig. 2A). Sorafenib was demonstrated to affect viability most significantly following treatment for 72 h, thus, MDA-MB231 cells were exposed to sorafenib for 72 h in subsequent experiments.

It was demonstrated that MDA-MB231 cells treated with TGF-β1 exhibited significantly increased GCN5 activity (P<0.05); however, this was significantly decreased by 25.5% following treatment with sorafenib (P<0.05) (Fig. 2B). The expression of GSN5 mRNA was also reversed to control levels in TGF-β1+sorafenib treated cells (decreased by 14.8%, P<0.05; Fig. 2C). TGF-β1 stimulation significantly increased N-cadherin and vimentin levels and decreased E-cadherin levels (all P<0.05). However, following exposure to sorafenib under TGF-β1 induction, E-cadherin expression recovered by 27.7%, whereas N-cadherin and vimentin expression decreased by 31.9 and 70.7%, respectively (all P<0.05).

Subsequently, the effect of sorafenib on the expression of proteins associated with the TGF-β1-induced EMT in breast cancer cells was evaluated. TGF-β1 treatment decreased the expression of E-cadherin and increased the expression of N-cadherin, vimentin, fibronectin, snail and slug in

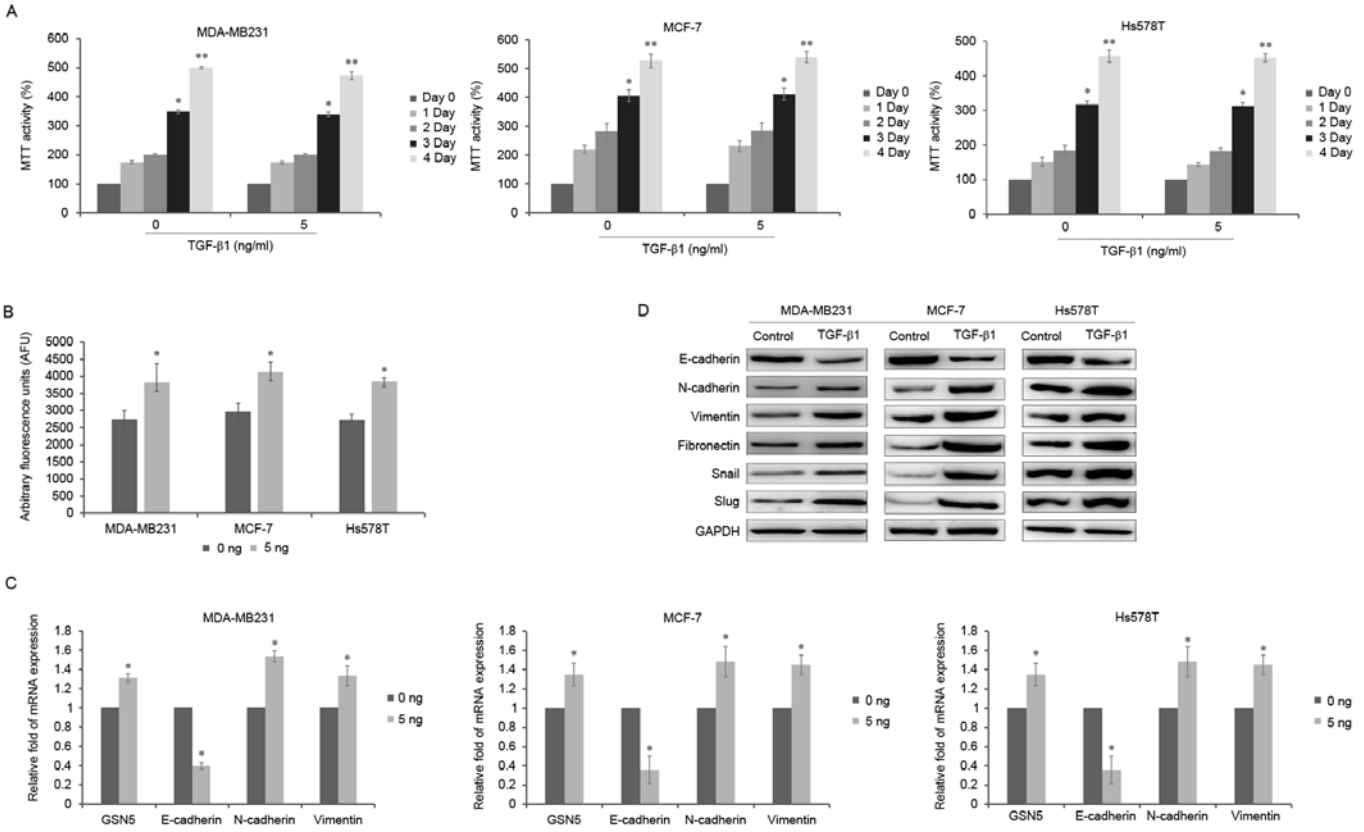

Figure 1. Effects of TGF-β1 treatment on cell viability, GCN5 activity and the expression of GSN5 and EMT markers in MDA-MB231, MCF-7 and Hs578T cells. (A) MTT assay of cell viability following incubation of MDA-MB231, MCF-7 and Hs578T cells with 5 ng/ml TGF-β1 for 0, 1, 2, 3 and 4 days. (B) GCN5 activity following stimulation with 5 ng/ml TGF-β1 was measured using a GCN5 chemiluminescent assay kit. (C) Reverse transcription-quantitative polymerase chain reaction measured the levels of GSN5, E-cadherin, N-cadherin and vimentin mRNA in MDA-MB231, MCF-7 and Hs578T cells treated with or without 5 ng/ml TGF-β1 for 3 days. (D) Western blot analysis of E-cadherin, N-cadherin, vimentin, fibronectin, snail and slug expression in MDA-MB231 cells treated with 5 ng/ml TGF-β1 for 3 days compared with untreated cells. Values are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. day 0 and **P<0.01 vs. day 0 or 0 ng. EMT, epithelial-mesenchymal transition; TGF-β1, transforming growth factor-β1.

MDA-MB231 cells (Fig. 2D). However, sorafenib-treated MDA-MB231 cells cultured with TGF-β1 exhibited increased expression of E-cadherin and decreased expression of vimentin, fibronectin, snail and slug. The same results were identified by immunohistochemistry; E-cadherin expression was decreased in cells treated with TGF-β1 but recovered to control levels in TGF-β1 treated cells following treatment with sorafenib (Fig. 2E).

It has been demonstrated that TGF-β1 induces the invasion and migration of cancer cells (14). Therefore, to determine whether sorafenib prevents the TGF-β1-induced migration and invasion of breast cancer cells, cell migration and invasion assays were performed. Compared with untreated MDA-MB231 cells, TGF-β1 significantly increased the number of migrating cells (P<0.05; Fig. 2F). However, migration in MDA-MB231 cells treated with sorafenib was significantly decreased compared with cells treated with TGF-β1 alone (P<0.05). TGF-β1 also significantly increased the invasive capacity of MDA-MB231 cells (P<0.05), however, sorafenib significantly inhibited this invasive capacity (P<0.05; Fig. 2G).

Knockdown of GCN5 by siRNA inhibits the EMT induced by TGF-β1 in breast cancer cells. To further determine the biological functions of GCN5 in the TGF-β1-induced EMT in breast cancer, GCN5 siRNA was used to knockdown GCN5 expression in MDA-MB231 cells. Cell viability was significantly decreased following GCN5 knockdown following stimulation with TGF-β1 compared with the control (P<0.05; Fig. 3A). By contrast, the viability of cells treated with TGF-β1 and transfected with control siRNA was similar to that of the control group. The increases in GCN5 activity and GCN5 mRNA expression following stimulation with TGF-β1 were significantly decreased to levels similar to the control group following transfection with GCN5-siRNA (all P<0.05 vs. transfection with control siRNA; Fig. 3B and C). Knockdown of GCN5 also normalized the expression of EMT markers; following stimulation with TGF-β1, E-cadherin mRNA levels were significantly increased whereas N-cadherin and vimentin mRNA levels were significantly decreased compared with cells transfected with control siRNA (P<0.05; Fig. 3C).

Furthermore, the effect of GCN5 knockdown on the TGF-β1-induced EMT in breast cancer cells was assessed. GCN5 knockdown reversed the decrease in E-cadherin expression induced by TGF-β1 and reversed the increase in N-cadherin, vimentin, fibronectin, snail and slug expression induced by TGF-β1 (Fig. 3D). As expected, GCN5 knockdown significantly reversed the effects of exogenous TGF-β1 stimulation on the migration and invasion capabilities of MDA-MB231

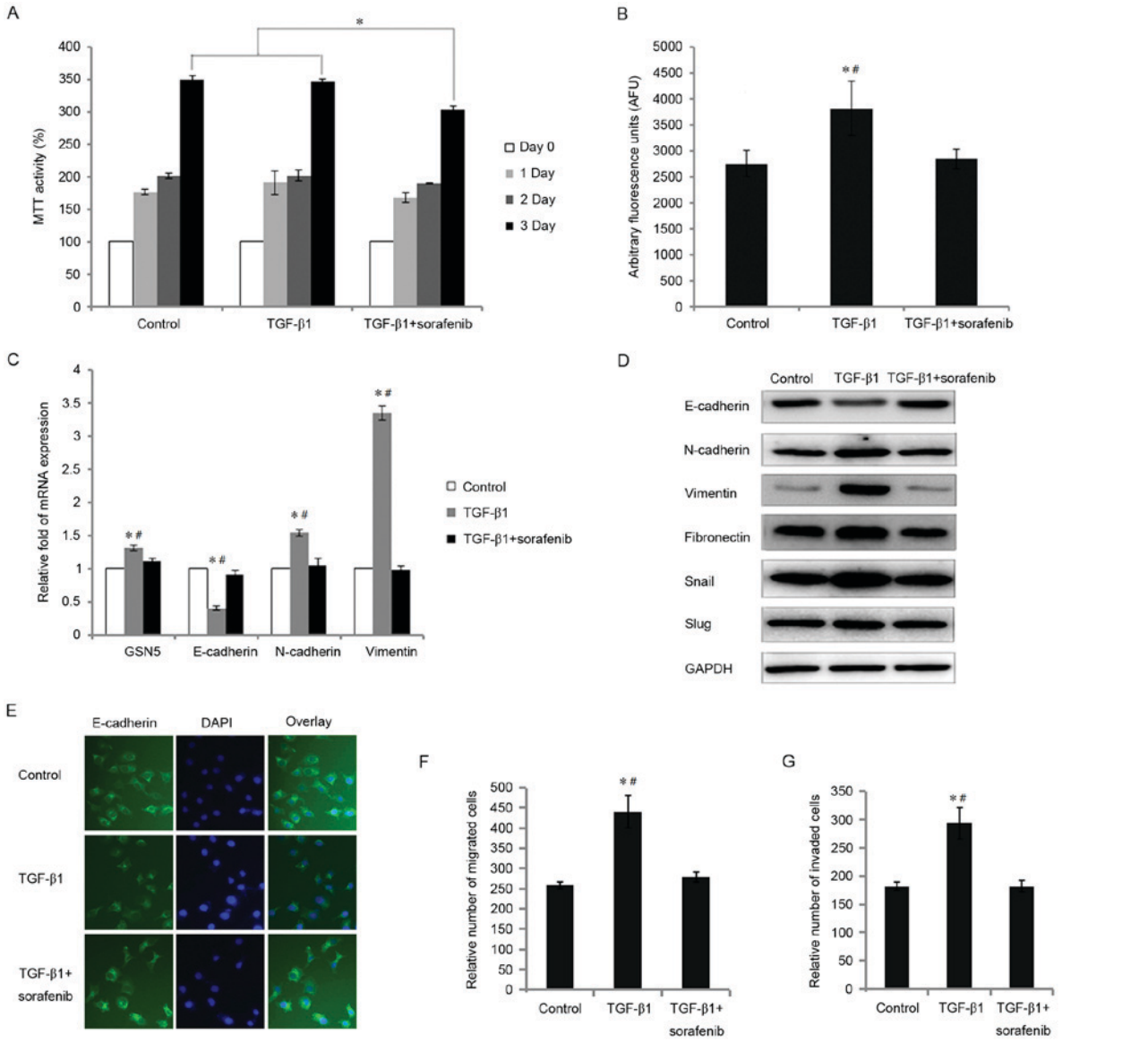

Figure 2. Effects of treatment with sorafenib in cells incubated with TGF-β1 on cell viability, GCN5 activity, the expression of GSN5 and markers of the epithelial-mesenchymal transition, and the migration and invasion of MDA-MB231 cells. (A) MTT assay measuring cell viability following incubation with sorafenib (5 μM) and stimulation with TGF-β1 for 0, 1, 2 and 3 days. (B) GCN5 activity in cells treated with sorafenib (5 μM) and TGF-β1 was detected using a GCN5 chemiluminescent assay kit. (C) Reverse transcription-quantitative polymerase chain reaction measured levels of GSN5, E-cadherin, N-cadherin, and vimentin mRNA in MDA-MB231 cells stimulated with TGF-β1 and treated with or without sorafenib (5 μM) for 3 days. (D) Western blot analysis measuring E-cadherin, N-cadherin, vimentin, fibronectin, snail and slug expression in MDA-MB231 cells treated with sorafenib (5 μM) and stimulated with TGF-β1 for 3 days. (E) Immunohistochemistry results (magnification, x40) indicating the expression of E-cadherin in MDA-MB231 cells treated with sorafenib (5 μM) and stimulated with TGF-β1 for 3 days. (F) The results of an *in vitro* Transwell migration assay indicating the relative number of migrated cells treated with TGF-β1 or TGF-β1+sorafenib compared with the control group. (G) *In vitro* Transwell invasion assay identifying the relative number of invaded cells treated with TGF-β1 and TGF-β1+sorafenib treatment, compared with the control group. Values are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control group and #P<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; GCN5, histone acetyltransferase GCN5; snail, snail family transcriptional repressor 1; slug, snail family transcriptional repressor 2.

cells; the migration and invasion of MDA-MB231 cells were decreased by 39.0 and 33.8%, respectively (P<0.05 vs. TGF-β1+contol siRNA group; Fig. 3E and F).

GCN5 rescue confirms the roles of GCN5 in the TGF-β1-induced EMT in breast cancer cells. To ensure that the induction of the EMT by TGF-β1 requires GCN5 activity, a GCN5 vector (GCN5-WT) was used to induce the overexpression of GCN5 to counteract the knockdown of GCN5 expression by transfection with GCN5 siRNA, leading to a recovery of GCN5 activity and increase in the expression of GCN5 mRNA (Fig. 4A and B). Furthermore, in the GCN5 rescue experiments, it was determined that the expression of GCN5, E-cadherin, N-cadherin and vimentin mRNA, and the expression of GCN5, E-cadherin, N-cadherin, vimentin, fibronectin, snail and slug protein were also significantly altered (P<0.05), compared with the cells treated with GCN5 siRNA. The mRNA and protein expression were as similar in the TGF-β1+GCN5 siRNA+GCN5-WT- and the TGF-β1-treated groups, therefore it is suggested that the function of GCN5 siRNA was counteracted by GCN5-WT-induced GCN5 overexpression (Fig. 4B and C).

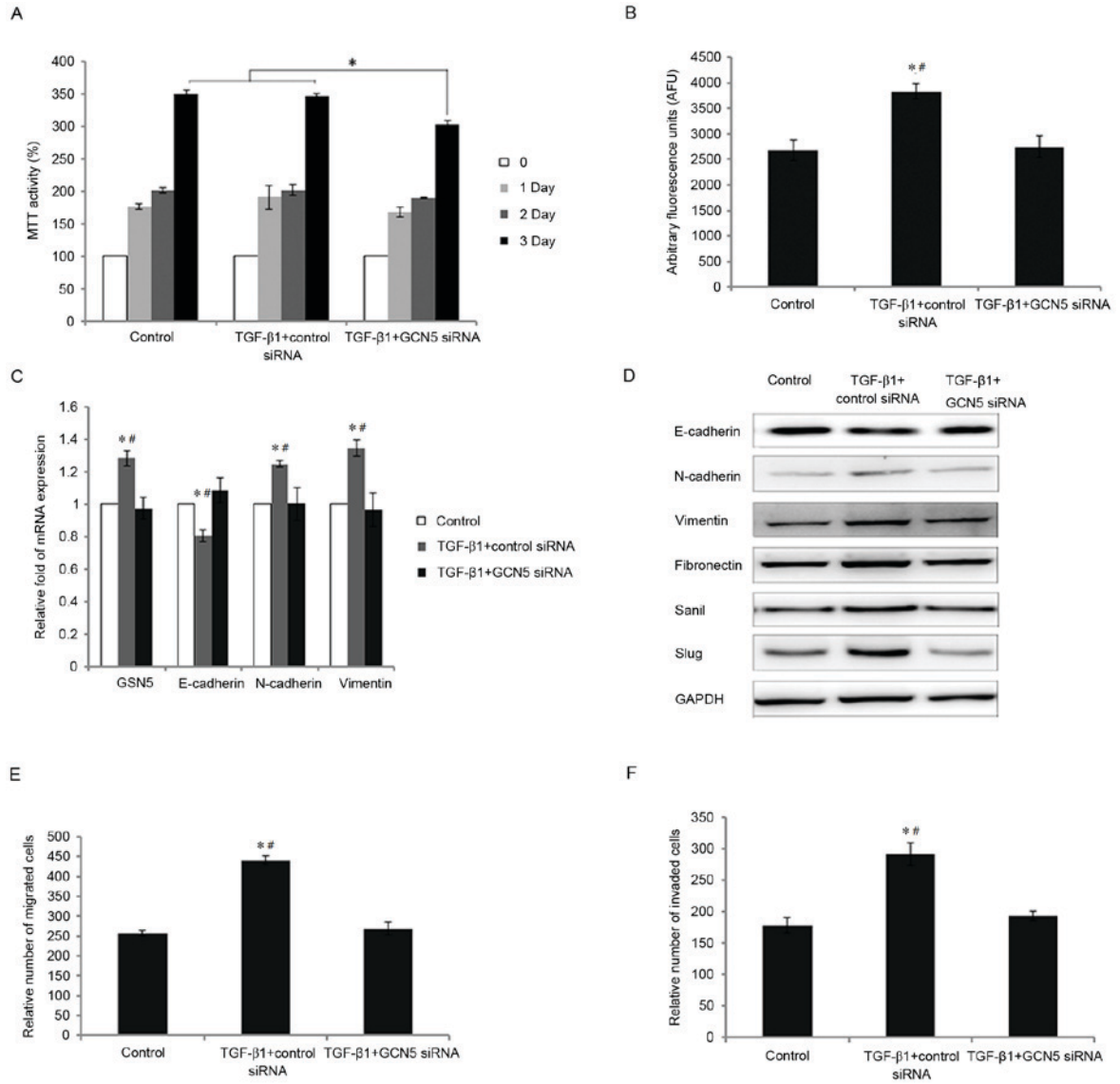

Figure 3. Effects of GCN5 knockdown following TGF-β1 treatment on cell viability, GCN5 activity, the expression of GSN5 and markers of the epithelial-mesenchymal transition, and the migration and invasion of MDA-MB231 cells. (A) MTT assay measuring cell viability following transfection with GCN5 siRNA (40 nM) or control siRNA in cells treated with TGF-β1 for 0, 1, 2 and 3 days. (B) GCN5 activity following transfection with GCN5 siRNA (40 nM) or control siRNA in cells stimulated with TGF-β1 was detected using a GCN5 chemiluminescent assay kit. (C) Reverse transcription-quantitative polymerase chain reaction measured the expression of GSN5, E-cadherin, N-cadherin and vimentin mRNA in MDA-MB231 cells transfected with GCN5 siRNA or control siRNA following stimulation with TGF-β1. (D) Western blot analysis of E-cadherin, N-cadherin, vimentin, fibronectin, snail and slug expression in MDA-MB231 cells transfected with GCN5 siRNA or control siRNA following stimulation with TGF-β1. (E) An *in vitro* Transwell migration assay identified the relative number of migrated cells in cells transfected with TGF-β1+control siRNA or TGF-β1+GCN5 siRNA, compared with the control group. (F) An *in vitro* Transwell invasion assay identifying the relative number of invading cells in cells transfected with TGF-β1+control siRNA or TGF-β1+GCN5 siRNA, compared with the control group. All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control group and $^{\#}$P<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; GCN5, histone acetyltransferase GCN5; snail, snail family transcriptional repressor 1; slug, snail family transcriptional repressor 2; siRNA, small interfering RNA.

Immunochemistry also demonstrated that E-cadherin expression was decreased following treatment with TGF-β1, but its expression increased to normal levels following GCN5 knockdown even under treatment with TGF-β1. However, when GCN5-WT was transfected with GCN5 siRNA, the expression of E-cadherin decreased (Fig. 4D).

GCN5 regulates the EMT by enhancing the signal transducer and activator of transcription 3 (STAT3), protein kinase B (AKT) and E2F1 signaling pathways. It has been reported that GCN5 can promote cell proliferation by enhancing the expression of E2F1 in non-small cell lung cancer (9) and that GCN5 can regulate cell proliferation and invasion by stimulating the STAT3 and AKT signaling pathways (15). To further explore the mechanism by which the downregulation of GCN5 inhibits TGF-β1-induced cell proliferation and invasion, the expression of various genes that regulate cell molecular signaling was measured. Compared with the group transfected with siRNA control, GCN5 knockdown by GCN5 siRNA significantly decreased the expression of phosphorylated (p)-STAT3, p-AKT, matrix metalloproteinase 9 (MMP9) and E2F1, and significantly increased the expression of p21

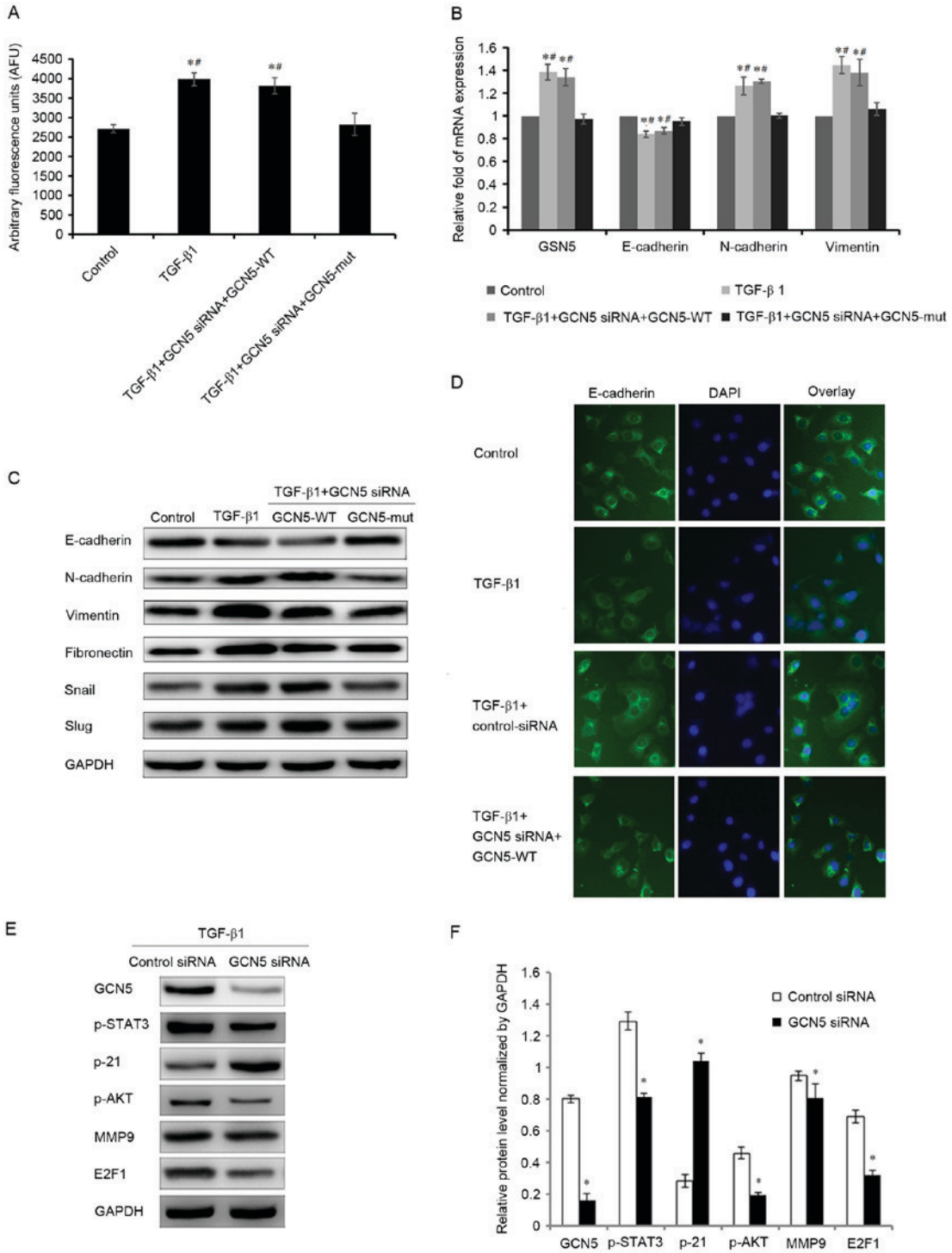

Figure 4. Effects of GCN5 rescue in cells stimulated with TGF-β1 on GCN5 activity and the expression of GSN5 and markers of the epithelial-mesenchymal transition. (A) GCN5 activity in cells transfected with GCN5 siRNA+GCN5-WT following TGF-β1 stimulation was measured using a GCN5 chemiluminescent assay kit. (B) Reverse transcription-quantitative polymerase chain reaction analysis measured the expression of GSN5, E-cadherin, N-cadherin, and vimentin mRNA in MDA-MB231 cells treated with GCN5 siRNA and GCN5-WT or GCN5-mut under TGF-β1 stimulation for 3 days. (C) Western blotting measuring E-cadherin, N-cadherin, vimentin, fibronectin, snail and slug levels in MDA-MB231 cells treated with GCN5 siRNA and GCN5-WT or GCN5-MUT following stimulation with TGF-β1 for 3 days. (D) Immunohistochemical staining (magnification, x40) indicating the expression of E-cadherin in MDA-MB231 cells treated with GCN5 siRNA and GCN5-WT or GCN5-MUT following stimulation with TGF-β1 for 3 days. (E) Western blots with (F) quantitative analyses of GCN5, p-STAT3, p-21, p-AKT, MMP9 and E2F1 levels in MDA-MB231 cells treated with GCN5 siRNA (40 nM) following stimulation with TGF-β1 (5 ng/ml) for 3 days. GAPDH was used as an internal control. *P<0.05 vs. control group and $^{\#}$P<0.05 vs. TGF-B1+GCN5 siRNA+GCN5-mut group. TGF-β1, transforming growth factor-β1; GCN5, histone acetyltransferase GCN5; snail, snail family transcriptional repressor 1; slug, snail family transcriptional repressor 2; MMP9, matrix metalloproteinase 9; p, phosphorylated; AKT, protein kinase B; STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; GCN5-WT, GCN5 vector; HCN5-MUT, GCN5 mutant vector.

(Fig. 4E and F). These results indicate that GCN5 promotes the proliferation, migration and invasion of breast cancer cells, at least in part, by enhancing the STAT3, AKT and E2F1 signaling pathways.

Discussion

Histone acetylation serves a vital role in establishing an active chromatin environment for transcriptional regulation (16). Histone lysine acetyltransferases (KATs) help to maintain the balance between histone acetylation and deacetylation (17). GCN5 is a Smad-binding transcriptional co-activator for TGF-β-specific R-Smads and stimulates TGF-β and bone morphogenetic protein signaling; thus, GCN5 is considered to be an essential component in the transcriptional regulation induced by TGF-β/Smad in certain cells (6). Although TGF-β may serve different roles during the different stages of breast cancer progression, it is a strong inducer of the EMT (18) and it has been demonstrated that the TGF-β1 signaling pathway promotes cancer cell proliferation, invasion and metastasis, thus stimulating the EMT in breast cancer (5,8). GCN5 regulates breast cancer development (7,9) and it has been reported that following treatment with TGF-β1 for 3 days, the expression of GSN5 increases in MDA-MB231 cells, increasing the expression of EMT markers, thus enhancing cell migration and invasion (19). It has been suggested that modifying GSN5 expression may affect the TGF-β1 signaling events required to induce the EMT in cancer cells, leading to the increased migration and invasion of MDA-MB231 cells (20).

The results of the present study demonstrate that GCN5 promotes the induction of the EMT by TGF-β1, as well as the migration and invasion of breast cancer MDA-MB231 cells. GCN5 activity was elevated following treatment with TGF-β1 and the expression of GCN5 mRNA and protein were increased. TGF-β1 treatment decreased the expression of the epithelial cell marker E-cadherin in MDA-MB231 cells and increased the expression of the mesenchymal cell markers N-cadherin and vimentin. The expression of other EMT markers, including snail and slug, were also increased following TGF-β1 treatment. However, following the inhibition of GCN5 activity, the expression of these EMT transition markers were reversed to levels observed in cells that did not undergo TGF-β1 treatment. In MDA-MB231 cells, knockdown of GCN5 expression with specific siRNA demonstrated that GCN5 downregulation effectively suppressed cell growth, cell migration and cell invasion. Furthermore, in a rescue experiment that utilized a GCN5-expressing vector to overexpress GCN5 and neutralize the knockdown effects of GCN5 siRNA, levels of EMT markers were increased to levels similar to the TGF-β1 induced group. These results indicate that GCN5 may be able to reverse the effects of TGF-β1 stimulation that serve crucial roles during EMT, providing a possible epigenetic mechanism for its clinical benefits in breast cancer metastasis.

To the best of our knowledge, the function of GCN5 in TGF-β signaling was previously unknown. It has previously been demonstrated in glioma cells that STAT3 and AKT may be involved in the GCN5-regulated migration and survival (15). Furthermore, it has been demonstrated that STAT3 may help regulate cell proliferation, oncogenesis and angiogenesis in tumors (21) and the AKT pathway may regulate various cellular functions, including migration and survival in cancer cells (22). Therefore, the present study examined whether downregulation of GCN5 expression following TGF-β1 stimulation regulates the STAT3 and AKT signaling pathways. It was demonstrated that GCN5 knockdown is able to significantly repress the TGF-β1-induced elevation of p-STAT3 and p-AKT expression. This indicates that GCN5 can promote the TGF-β1-induced EMT transition, at least partly via the STAT3 and AKT signaling pathways (16). GCN5 and E2F1 may have synergistic effect (23), and the results of the current study also demonstrated that E2F1 is the downstream targeted protein of the STAT3 and AKT pathways; there is an interaction between GCN5 and E2F1 following GCN5 knockdown. Furthermore, GCN5 potentiates lung cancer cell growth in conjunction with the transcription factor E2F1 to regulate the cell cycle (9) and it has been reported that the expression of GCN5 promotes cell growth and the G1/S-phase transition in multiple lung cancer cell lines. The results of the current study indicated that GCN5 knockdown inhibits cell growth, decreases the expression of E2F1, but increases the expression of the cell cycle inhibitor p21 in MDA-MB231 cells, suggesting that GCN5 also serves an important role in the E2F1 pathway. Furthermore, it has been determined in glioma tissues that GCN5 expression is correlated with MMP9 (15), which has been implicated in cancer progression, invasion and metastasis. The results of the present study demonstrated that GCN5 knockdown significantly represses MMP9 levels and suggested that GCN5 may enhance the TGFβ1-induced EMT process by increasing MMP9 levels.

In conclusion, the results of the current study suggest that GCN5 may be involved in regulating the induction of the EMT by TGF-β1 via mediation of the STAT3, AKT and E2F1 signaling pathways. These results indicate that GCN5 may be an important inducer of EMT transition and may therefore be a potential target of novel therapeutic strategies to treat breast cancer.

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