

## Original Article

# GATA1 promotes tumorigenesis and metastasis in breast cancer by cooperating with ZEB2

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**Abstract:** Recent studies have shown that GATA3 plays a significant role in the inhibition of breast cancer cell metastasis, but whether GATA1 plays a role in mediating tumor tumorigenesis and metastasis in breast cancer has not been explored. Here, we find the expression of GATA1 in breast cancer cell lines were higher than the normal breast cell lines MCF-10A. Co-immunoprecipitation assays were utilized to detect that GATA1 was physically associated with the EMT inducer ZEB2. Most importantly, MDA-MB-231 cells transfected with GATA1 shRNA displayed a reversed epithelial morphology and reduced the expression of N-cadherin, while increase the expression of E-cadherin, and the MCF-7 cells transfected with GATA1 displayed the contrary tendency. Transwell invasion assay, growth assays, as well as colony formation and in vivo Living Imaging assay together shown that GATA1 promoted cell epithelial-mesenchymal transition and tumorigenesis in vitro and in vivo. Endothelial tube formation assays indicated GATA1 might regulate the angiogenesis of breast cancer through transcriptional activation of VEGF. qChIP, ChIP as well as Luciferase reporter assays suggested GATA1 binding on the promoter of E-cadherin and VEGF. The changes in the level of protein and RNA implied that suppression of downstream E-cadherin or activation of VEGF was two distinct important mechanisms by which GATA1 triggered EMT and angiogenesis. Further, patient samples collected shown that GATA1 was significantly increased in breast cancer samples, and its higher expression is correlated with poor prognosis, worse over all survivals. Together, our experiments revealed the mechanism for GATA1 in facilitating tumorigenesis, angiogenesis and metastasis of breast cancer cells, suggesting that GATA1 might be a potential therapeutic target for treating breast cancer.

**Keywords:** GATA1, ZEB2, EMT, E-cadherin, VEGF

## Introduction

Metastasis remains a major clinical problem in breast cancer, which is the main cause of disease-related death, it often has a correlation with angiogenesis, and is usually correlated with the low 5-year survival rates [1]. Epithelial-mesenchymal transition (EMT) is believed to be the initial step of breast cancer metastasis, during the process of EMT, epithelial cells with a cobblestone phenotype lost their differentiated characteristics, and acquire mesenchymal cell feature, such as spindle-shaped, motility and invasiveness [2-4]. Epithelial-cadherin (E-cadherin; encoded by CDH1) is a member of the classical cadherins, it usually works as a tumor suppressor in the cancer progression [5, 6]. Generally downregulation of E-cadherin and upregulation of N-cadherin are considered as the mark of EMT [7, 8]. Which play important

roles in the process of embryonic development as well as in tumor metastasis.

Furthermore, angiogenesis, the process of new blood vessels formation, is another important step in tumor metastasis, which involves several signaling between growth factors and endothelial cell receptors. Among them, vascular endothelial growth factor (VEGF), by inducing the formation of new blood vessels, was reported by several groups [9] to get an important role in angiogenesis and tumor metastasis.

GATA1 was a member of the GATA family, the transcription factor GATA family all share 2 highly conserved zinc fingers, the function of which is DNA binding and mediates protein interactions. The function of GATA3 in breast cancer progression was well discussed [10, 11], but the role of GATA1 was still poorly understood.

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To our particular aspects of knowledge, ZEB2/SIP1, which is a potent repressor of E-cadherin expression [12], is considered to be one of the key factors of EMT. Oppositely, vimentin, another mesenchymal marker, is reported to be up-regulated by ZEB2, which is associated with breast tumor cell EMT [13].

### Materials and methods

#### *Cells and cell culture*

Cell lines MCF-7 and MDA-MB-231 cells were acquired from the American Type Culture Collection (ATCC). They were cultured in DMEM or RPMI 1640 (Gibco) respectively, supplemented with 10% fetal bovine serum (FBS) (HyClone) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. MDA-MB-231 medium was in addition supplemented with 1 mM glutamine. Cell lines MCF-10A were from Shanghai institutes for Biological Sciences (CAS), and were cultured in MEGM BulletKit.

#### *Reagents*

Rabbit anti-human GATA1 antibody, ZEB2 antibody, E-cadherin antibody, VEGF antibody,  $\beta$ -actin antibody and secondary antibody were all purchased from Santa Cruz (CA, USA).  $\alpha$ -catenin antibody, N-cadherin antibody, Vimentin antibody, were from abcam (USA). Specific shRNA targeting GATA1, ZEB2 were from Sigma-Aldrich (CA, USA). A negative control shRNA (SCR) was also used as control (Sigma-Aldrich). Matrigel was purchased from BD Biosciences (CA, USA). Lipofectamine2000 (Invitrogen) was used for transfection.

#### *Patients and specimens*

30 breast cancer samples of patients and the adjacent normal tissues were obtained in Tianjin Medical University Cancer Hospital from 2001 to 2008. Samples were chosen with completely clinicopathologic information. Patients who were received radiation therapy or chemotherapy prior to the surgery were excluded. The survival times were calculated based on the operation day to death, via the evaluation of metastasis or recurrence. This study has been approved by the hospital ethical committee.

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA of cell lysates were extracted with Trizol solution (Invitrogen, Carlsbad, CA, USA), and 1  $\mu$ g of total RNA were reverse transcribed

to cDNA using with M-MLV Reverse Transcriptase, according to the manufacturer's instructions (TransGen Beijing, China). Real-time RT-PCR primers were as follows: GATA1, forward 5-CTACCACAAGATGAATGGGC-3, reverse 5-CCTGTAAACATTGTGCAGCT-3; E-cadherin, forward 5-AAATCACATCCTACACTGCC-3, reverse 5-GCAACTGGAGAACCATTGTC-3; VEGF, forward 5-GACATCTCCAGGAGTACCC-3, reverse 5-TCTTCTTTGGTCTGCATTAC-3; ZEB2, forward 5-CCAGTCCAGACCAGTATTCC-3, reverse 5-AGCAATCTCCCTGAAATCC-3; GAPDH, forward 5-CATTCTCTGGTATGACAACGA-3, reverse 5-GTCTACATGGCAACTGTGAG-3. Real-time PCR was performed on an ABI 7500 sequence detection system (Applied Biosystems), using SYBR (Roche). All experiments were performed in three times. GAPDH was used as a normalization control.

#### *Co-immunoprecipitation*

For immunoprecipitation assays, cells were washed with cold PBS and lysed with cold lysis buffer on a rotator at 4°C for 45 min. Whole cell lysates were incubated with appropriate primary antibodies or normal rabbit/mouse immunoglobulin G (IgG) as negative controls on a rotator overnight at 4°C, then added protein A/G Sepharose CL-4B beads for 2 h at 4°C. Beads were then washed 5 times with lysis buffer (50 mM Tris-Cl, pH 7.4, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate and protease inhibitor cocktail). The immune complexes were subjected to SDS-PAGE (Invitrogen), followed by immunoblotting with secondary antibodies.

#### *Western blot*

Cells were harvested 48 h to 72 h after transfection with the indicated shRNA or recombinant plasmid. Protein concentration was measured using the bicinchoninic acid kit (BCA). Subsequently, 30  $\mu$ g proteins were run by 10% SDS-PAGE gel and then transferred on to NC membranes. 5% skim milk were used to block the NC membranes for 1 h, then incubated with primary antibodies, overnight at 4°C, including GATA1 (1:1000), ZEB2 (1:1000), E-cadherin (1:1000),  $\alpha$ -catenin (1:1000), N-cadherin (1:1000), vimentin (1:500), VEGF (1:1000) and  $\beta$ -Actin (1:500). Goat anti-rabbit secondary antibodies (1:5000), goat anti-mouse secondary antibodies (1:3000) and Western blotting Luminol reagent (Santa Cruz Biotechnology) were used to visualize the protein bands.

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### *Colony formation assay*

After infected the related lentivirus for 3 days, a total of 1000 MCF-7 cells were seeded into 6-well plates, and the medium was changed every 3 days. After 10 days of culturing, the colonies formed were collected, washed with PBS and fixed in 4% paraformaldehyde at 37°C for 15 min, after then, the colonies were stained with Coomassie for 15 min, washed and then air-dried. The colonies were counted using the microscopy (Olympus, Tokyo, Japan). The experiment was performed in triplicate.

### *ChIP and qChIP*

Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's protocol (Upstate). The chromatin was incubated with 4 µg of GATA1 antibody, ZEB2 antibody or normal Rabbit IgG as negative control (Santa Cruz Biotechnology), at 4°C on a rotator overnight. Immunoprecipitated DNA was purified with the Qiagen PCR purification kit. qChIP was analyzed by quantitative PCR using specific primers as follows. For common ChIP assays, the final target DNA sequence was amplified and resolved on standard agarose DNA gels.

Primers used for common ChIP: E-cadherin: 5-AGGGTCACCGCTCTATG-3 (forward) and 5-CTTCCGCAAGCTCACAGG-3 (reverse), VEGF: 5-AAGGAGGAAAGTTAGTGGCTTCCCT-3 (forward) and 5-TGTTGCTGTGGTTTGGTGGA-3 (reverse); qChIP primers: E-cadherin: 5-GCAGGTCCATA-ACCCACCTA-3 (forward) and 5-CATAGACGCG-GTGACCCTCTA-3(reverse), VEGF: 5-AGCCTAGAGCATGGAGCCCAGGTGA-3 (forward) and 5-CC-ATCGGTATGGTGTCTCTAA-3 (reverse).

### *Luciferase reporter assay*

For E-cadherin or VEGF reporter construction, the sequence of the relative promoter and partial exon was obtained by PCR. The pGL3 basic vector (Promega) was utilized to ligate these PCR products to generate pGL3-E-cadherin or pGL3-VEGF luciferase reporter construct. MCF-7 cells were transfected with promoter luciferase reporter, Renilla luciferase plasmid, and the indicated expression constructs, using Lipofectamine LTX-Plus (Invitrogen) in 96-well plates. By addition of empty vector in each transfection, the amount of DNA was retained constant. Renilla plasmid was used as a nor-

malization control of transfection efficiency. 48 hours after transfection, the firefly and Renilla luciferases were assayed according to the manufacturer's protocol (Promega). Each experiment was conducted in triplicate.

### *Transwell invasion assay*

The invasive ability of the MDA-MB-231 cells was investigated using Transwell assay (8-µm pore size; for 24-well plate. Millipore), matrigel used were from BD Biosciences (50 µg/ml). First, 100 µl matrigel was added onto the surface of the chamber, incubated in room temperature for 2 hours for solidification. A total of 100 µl of MDA-MB-231 cell suspension ( $5 \times 10^4$  cells) was in addition to the upper chamber, with serum-free medium. The lower compartment was incubated with 500 µl of RPMI 1640 containing 10% fetal bovine serum. After incubation for 24 hours, the cells invaded into the lower surface were fixed with 2% paraformaldehyde, stained with crystal violet. Photos were taken using an inverted microscope (Olympus Corp. Japan) at 100× magnifications.

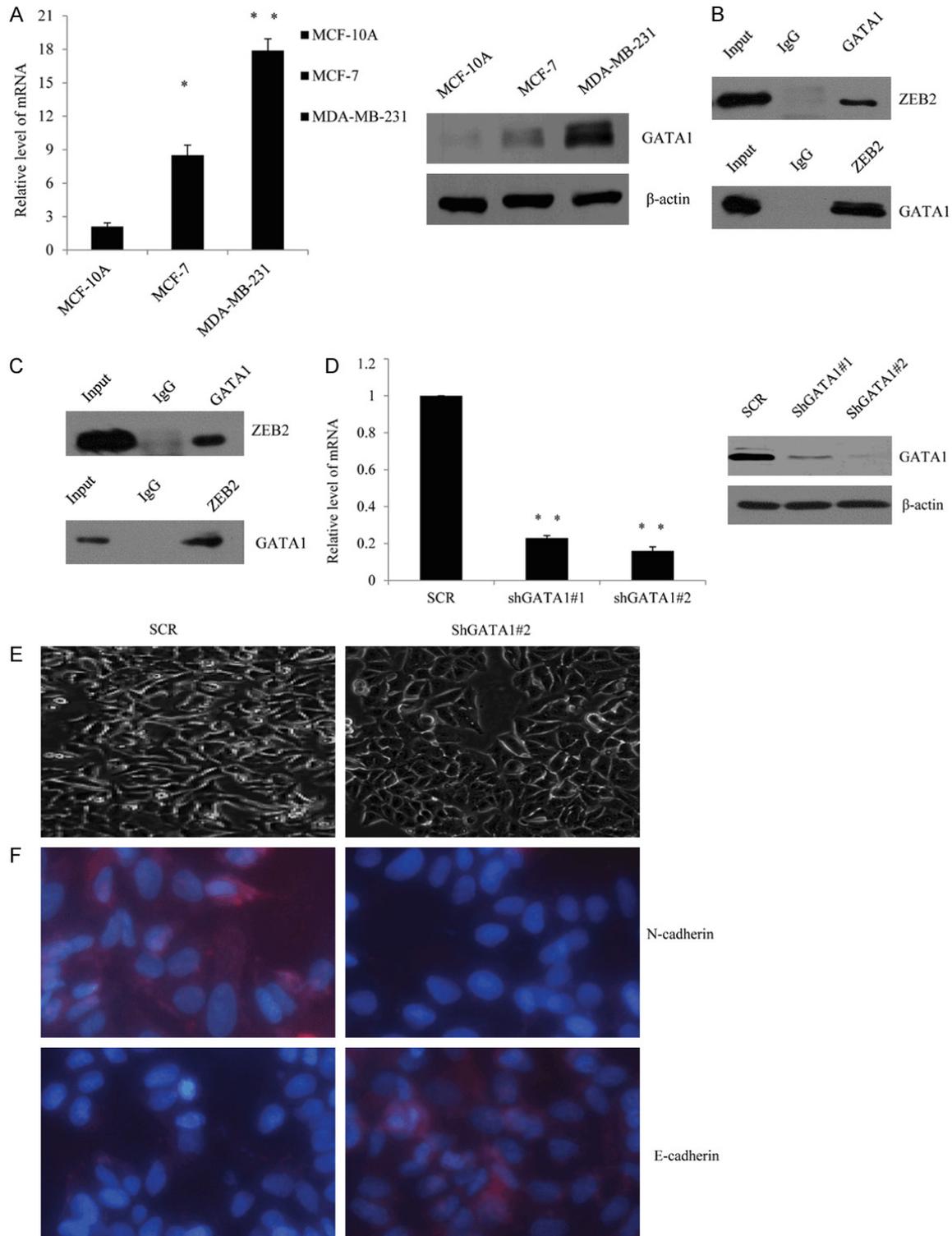
### *In vivo imaging assay*

MDA-MB-231 shGATA1 cells ( $1 \times 10^6$ ) and MDA-MB-231 SCR cells ( $1 \times 10^6$ ) stably expressing firefly luciferase were subcutaneously implanted into nude mice or injected into the caudal vein of SCID mice (Charles River, Beijing, China). Tumor growth was monitored every week. The tumor volumes were measured and statistically analyzed after 30 days. Metastasis bioluminescence imaging was conducted after 5 weeks of initial implantation or 6 weeks of caudal vein injection. Fifteen minutes after the mice were injected with D-luciferin in PBS, and bioluminescence was imaged with a charge-coupled device camera (IVIS; Xenogen).

### *Statistical analysis*

All observations were confirmed by at least three independent experiments. The data was presented as mean ± SD. One-way ANOVA was used to analyze the statistical significance of the mean values. Bivariate correlation was calculated by Spearman's rank correlation coefficients. Cox proportional hazards regression was utilized to test the prognostic significance of factors.  $P < 0.05$  was considered significant.

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**Figure 1.** GATA1 is physically associated with ZEB2 and promotes breast cancer cells EMT in different breast cancer cell lines. A. The expression level of GATA1 was determined in MCF-10A, MCF-7 and MDA-MB-231. Quantitative real-time PCR (left panel) and western blotting (right panel) were repeated three times.  $\beta$ -actin was used as a normalization control. Error bars represent average  $\pm$  SD, \* $P < 0.05$ , \*\* $P < 0.01$ . B. Co-IP analysis of the association between GATA1 and ZEB2. Whole cell lysates of MCF-7 were immunoprecipitated with antibodies against GATA1, with normal IgG as the negative control, while the whole lysates as positive control. The immunocomplexes were then immunoblotted using ZEB2, then inversely. C. Whole cell lysates of MDA-MB-231 were further used to detect the association between GATA1 and ZEB2. D. Knockdown efficiencies of GATA1 in MDA-MB-231 were confirmed by

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Quantitative real-time PCR (left panel) and western blotting (right panel). E. MDA-MB-231 cells were treated with specific shRNA against GATA1. Phase-contrast microscopy was used to examine the morphological alterations of these cells. F. Immunofluorescence staining of epithelial (E-cadherin) and mesenchymal (N-cadherin) markers was visualized by confocal microscopy (Red). DAPI staining was included to visualize the cell nucleus (Blue).

### Results

*GATA1 is physically associated with ZEB2 and promotes breast cancer cells EMT in different breast cancer cell lines*

In order to investigate the role of GATA1 during the progress of breast cancer, we first examine the expression of GATA1 in two breast cancer cell lines (MCF-7 and MDA-MB-231) and the normal breast cell lines MCF-10A by using qRT-PCR and western blot (**Figure 1A**). The expression of GATA1 was significantly increased in MCF-7 and MDA-MB-231, comparing with MCF-10A.

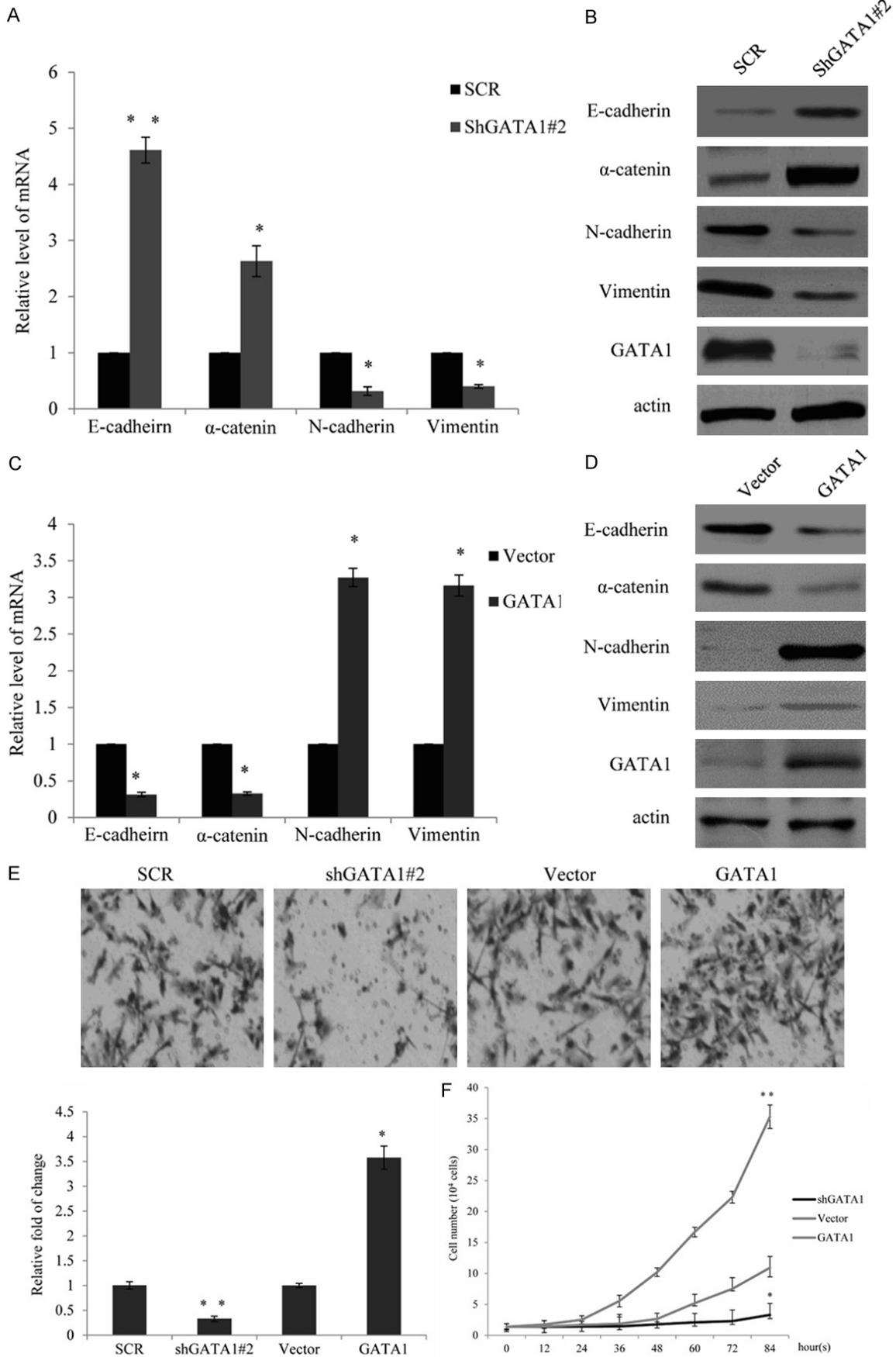
The results of the co-immunoprecipitation experiments revealed that GATA1 copurified with ZEB2 (**Figure 1B** and **1C**), a key factor of epithelial-mesenchymal transition. Total protein extracts from MCF-7 cells were prepared, first, Immunoprecipitation (IP) with anti-GATA1 followed by immunoblotting (IB) with the anti-ZEB2 indicated that GATA1 was co-immunoprecipitated with ZEB2 (**Figure 1B** upper panel), again, IP with anti-ZEB2 followed by IB with anti-GATA1 (**Figure 1B** lower panel). To further support the in vivo interaction between GATA1 and ZEB2, this interaction is also confirmed with endogenous proteins in MDA-MB-231 cells (**Figure 1C**). We also detected another transcription factor, such as snail1 and twist1, both of them had no interaction with GATA1, data were not shown. As showed in **Figure 1D**, MDA-MB-231 cells were infected with control shRNA (SCR), shGATA1#1, or shGATA1#2 groups. Both mRNA level and protein level in different groups were detected. We found that mRNA level of GATA1 was significantly reduced when cells were infected with shGATA1#1, or shGATA1#2 ( $P < 0.05$ , **Figure 1D** left panel). Concomitantly, in western blot analysis, GATA1 was also greatly lowered in shGATA1-infected MDA-MB-231 cells as compared with the control shRNA-infected cells (**Figure 1D** right panel). The results suggested both the two relative shRNA were successfully constructed in MDA-MB-231 cells. Interestingly, when MDA-MB-231 were transfected with the relative shRNA, as shown in **Figure 1E**, while control MDA-MB-231 cells

maintained a spindle-like, fibroblastic morphology, knock down of GATA1 became organized cell-cell adhesion and cobble stone-like epithelial appearance. Further, in MDA-MB-231 cells transfected with shGATA1, the immunofluorescence staining alterations of epithelial marker E-cadherin (**Figure 1F** upper panel) or mesenchymal marker N-cadherin (**Figure 1F** lower panel) were assessed by microscopy. Compared with the SCR cells, shGATA1, the staining of N-cadherin was weaken while the staining of E-cadherin was heighten. Which indicated GATA1 might play an important role in the Epithelial-mesenchymal transition (EMT).

*GATA1 promotes the tumorigenesis of breast cancer cells in vitro*

Considering the expression of GATA1 in MCF-7 was lower than that of MDA-MB-231, we choose GATA1 “loss of function” experiment in MDA-MB-231 cells, the expression of the epithelial markers such as E-cadherin, a-catenin were up regulated; while the mesenchymal markers N-cadherin, Vimentin were raised as measured by real-time RT PCR (**Figure 2A**) or western blotting (**Figure 2B**). When GATA1 was overexpressed in MCF-7 cells, the opposite result was shown. E-cadherin, a-catenin were decreased while N-cadherin, Vimentin were up regulated (**Figure 2C** and **2D**). Considering EMT as the initiation step of metastasis, we further focus our attention on whether GATA1 could also take part in the metastasis, transwell assay was performed to assess the effect of GATA1 on cell invasion. Migrated MDA-MB-231 cells infected with shGATA1 were counted for one third than those infected with control, while GATA1 overexpression was related with 3.5 times increase of the invasion cells, which indicated GATA1 might take a role in the metastasis of breast cancer (**Figure 2E**). In order to further understand the role of GATA1 in the tumorigenicity, we performed growth assays in MCF-7 cells transfected with vector, GATA1 gain-of-function, GATA1 loss-of-function. As data shown, cells with GATA1 overexpression showed an obvious growth promotion, while GATA1 was knocked down, there was an evident growth inhibition (**Figure 2F**).

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**Figure 2.** GATA1 promotes the tumorigenesis of breast cancer cells in vitro. A. MDA-MB-231 cells were transfected with shGATA1, the expressions of the epithelial markers such as E-cadherin,  $\alpha$ -catenin; mesenchymal markers N-cadherin, Vimentin were measured by real-time RT PCR. Error bars represent the mean  $\pm$  S.D. \* $P < 0.05$ ; \*\* $P < 0.01$ . B. MDA-MB-231 cells were transfected with shGATA1, western blotting was used to detect the alteration of the epithelial or mesenchymal markers. C. MCF-7 cells were transfected with GATA1 constructs. The expressions of the epithelial and mesenchymal markers were measured using real-time RT PCR. D. MCF-7 cells were transfected with GATA1 constructs, western blotting was used to detect the alteration of the epithelial or mesenchymal markers. E. MDA-MB-231 cells were transfected with control shRNA, shGATA1, empty vector, or GATA1 over expression construct. After 48 h of transfection, cell invasion assays were performed, the invaded cells were stained and counted. Representative photos were shown in each group. Statistically analyzed were presented as fold of change over vector. Error bars represent the mean  $\pm$  S.D. Experiments were repeated three times. \* $P < 0.05$ ; \*\* $P < 0.01$ . F. MCF-7 cells transfected with vector, GATA1, SCR, shGATA1 were subjected to growth curve analysis. Equal numbers of cells were mixed, and seeded into replicate plates, the cells were counted every 12 hours.

### *The molecular mechanism of GATA1 in promoting breast cancer cell invasion and angiogenesis*

To further understand the molecular mechanism of GATA1 in regulating transcription, quantitative ChIP (qChIP) assays were performed in MCF-7 cells, several key genes in different pathways which were involved in metastasis were chosen to detect, such as E-cadherin,  $\alpha$ -catenin, Ep-CAM, N-cadherin, vimentin, HIF-1A, caspase3, caspase7, VEGF, CCL14 and ATM. On the promoter of E-cadherin and VEGF, the bindings of GATA1 were obviously higher than that of the normal IgG (**Figure 3A**). Since ZEB2 was reported as a repressor of E-cadherin [14] and therefore worked an inducer of EMT, furthermore, the physical interaction between ZEB2 and GATA1 was detected in our study. It was reasonable to suppose there was functional consistency between ZEB2 and GATA1. Under this assumption, ChIP-PCR assays were performed in MCF-7 cells, on the promoter of E-cadherin or VEGF, not only GATA1, but also ZEB2, there was an obvious band compared with normal IgG, total ChIP DNA (Input) was as positive control (**Figure 3B**). To further support the argument, luciferase reporter activity assays were carried out in MCF-7 cells with E-cadherin or VEGF promoter-driven luciferase reporter under GATA1 over expression or depletion. These experiments indicated that GATA1 over expression or knockdown resulted in repressed or enhanced E-cadherin reporter activity, contrary to E-cadherin, on the promoter of VEGF, GATA1 over expression or silencing led to a significant effect on the activation or repression of the VEGF reporter activity (**Figure 3C** left panel). The up regulation or down regulation of ZEB2 got the similar results on the two promoters (**Figure 3C** right panel).

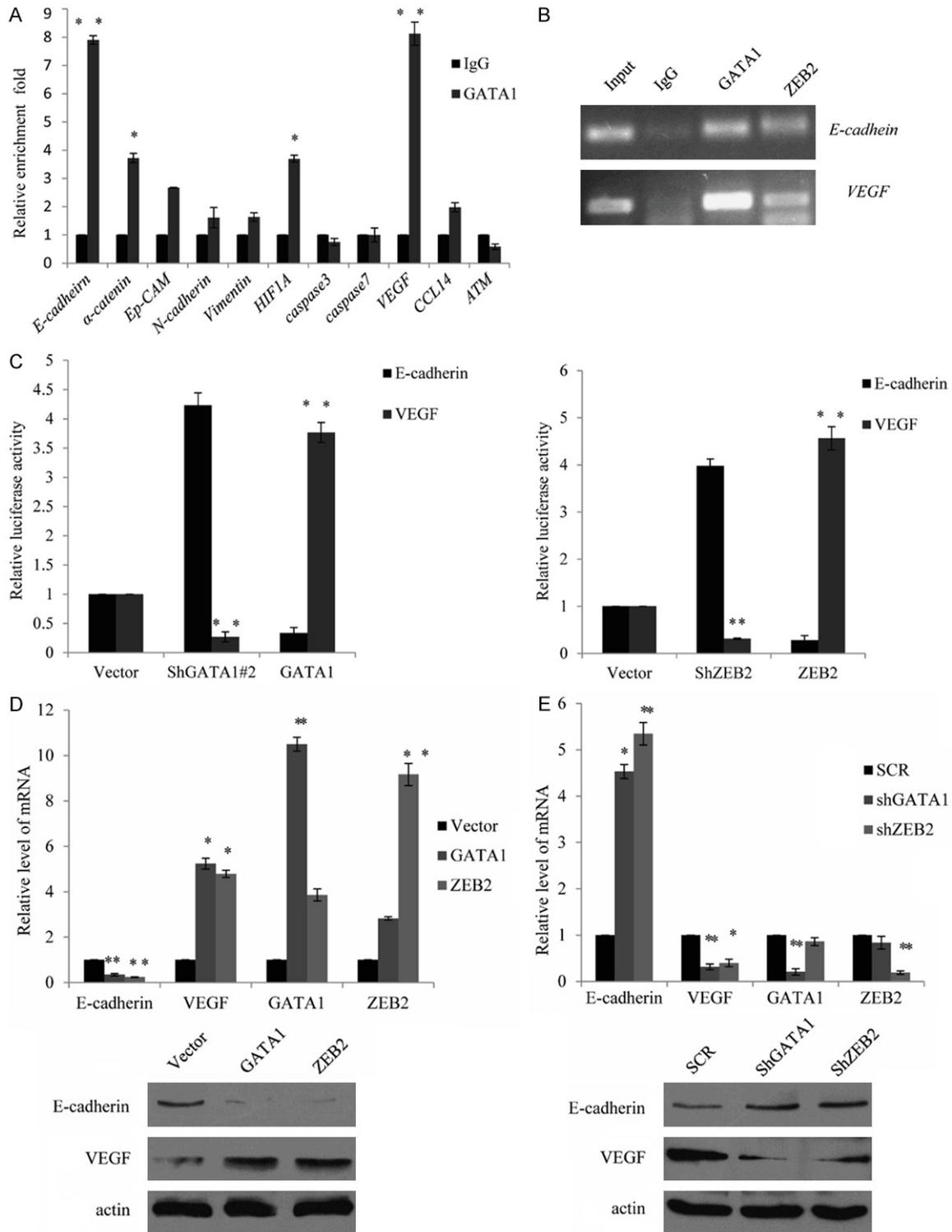
Consistent with the promoter occupancy, in GATA1, ZEB2 overexpressed MCF-7 cells, the

mRNA (**Figure 3D** left panel) and protein (**Figure 3D** right panel) expression of E-cadherin decreased, to the contrary. VEGF increased. Respectively, while GATA1 or ZEB2 was knock-down in MDA-MB-231 cells, in both mRNA and protein level, E-cadherin increased, VEGF decreased, which further supporting the notion that both GATA1 and ZEB2 have dual transcriptional activities (**Figure 3E**).

### *GATA1 enhances breast cancer cells invasion and angiogenesis in vivo*

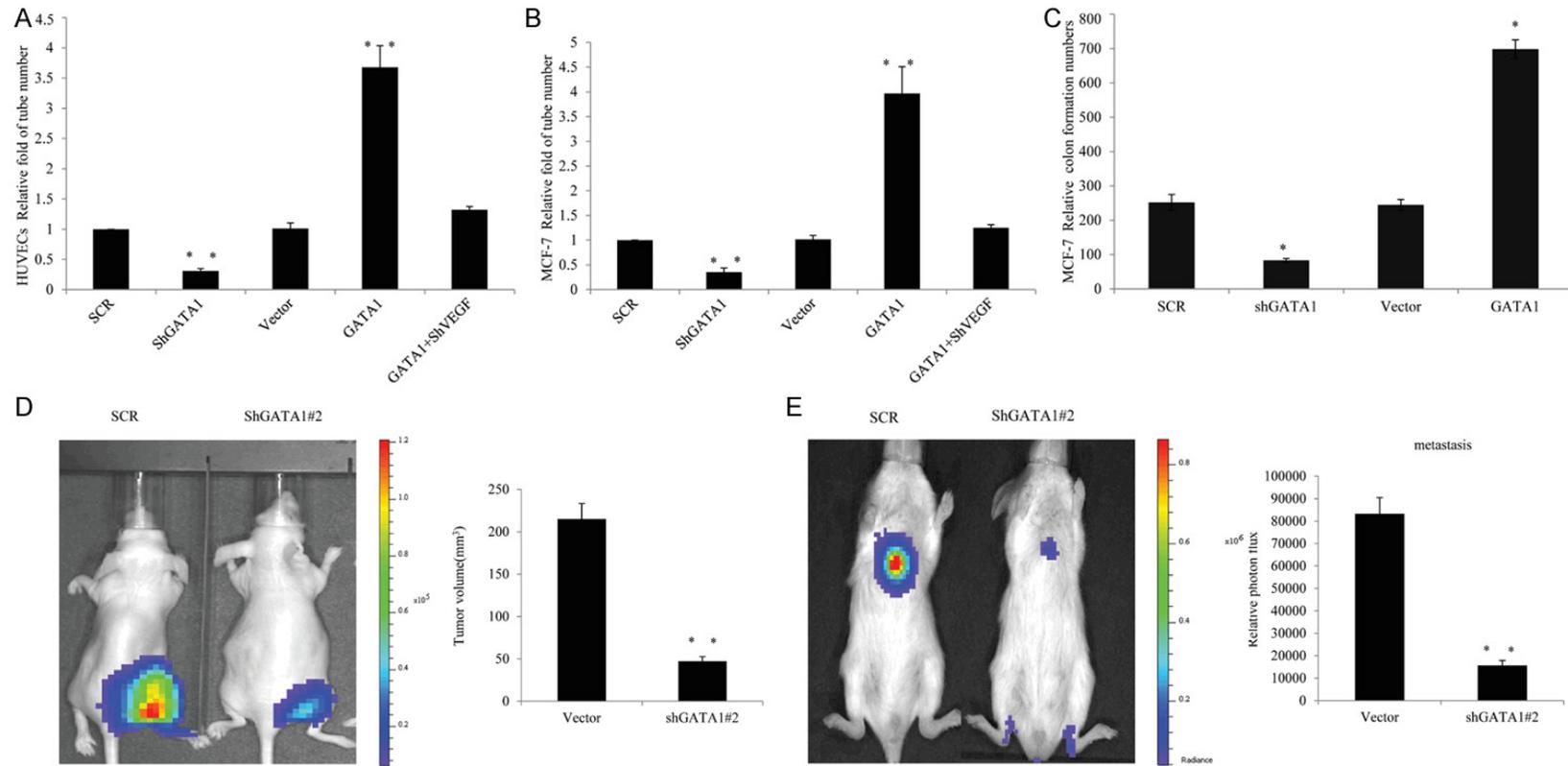
Considering VEGF as a target gene of GATA1, it means that GATA1 might regulate the angiogenesis of breast cancer through transcriptional activation of VEGF. For this purpose, endothelial tube formation assays were carried out. First, Human umbilical vein endothelial cells (HUVECs) infected with lentiviruses carrying SCR, ShGATA1, vector, GATA1, or GATA1 plus ShVEGF (**Figure 4A**), or cultured with different conditioned media from MCF-7 that were infected with the above lentiviruses (**Figure 4B**). As shown, HUVECs with shGATA1 or cultured in the CM with knockdown GATA1, formed significantly fewer tubes than those in the SCR or vector group, whereas HUVECs with GATA1 overexpression or that which were cultured in the CM with GATA1 overexpression generated more tubes than the control. Moreover, the effect of GATA1 over expression on the potential of HUVECs angiogenesis could be rescued by the addition of shVEGF (**Figure 4A** and **4B**). To further explore the tumorigenic ability of GATA1, in vitro, colony formation was performed, the average number of colonies in the SCR group or vector group was similar to each other, whereas the number of colonies in GATA1 knockdown was only 30%, while GATA1 overexpression was about 2.7 fold ( $P < 0.05$ ). The data indicated there was an obviously increase in colony formation because of the high expression of GATA1 (**Figure 4C**). In vivo, tumor formation in nude mice or tumor

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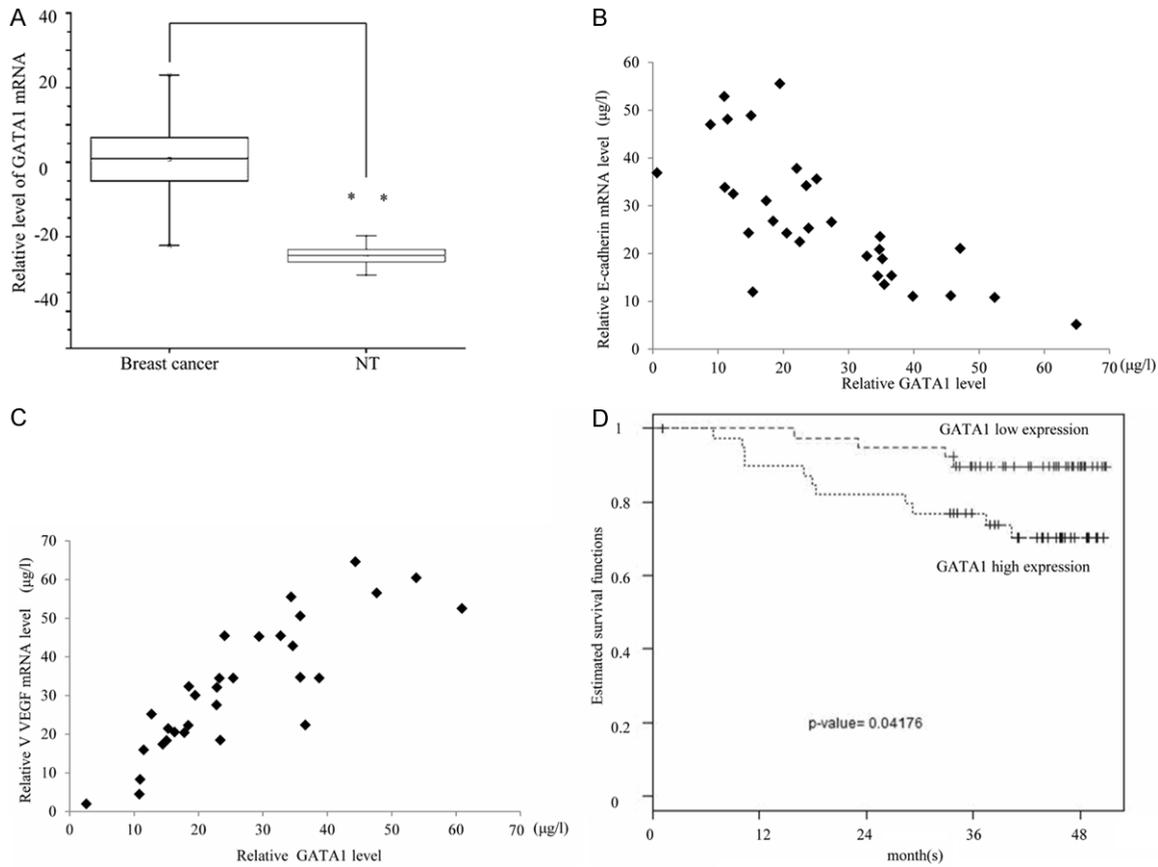
**Figure 3.** The molecular mechanism of GATA1 in promoting breast cancer cell invasion and angiogenesis. A. Recruitment of GATA1 on E-cadherin and VEGF promoters. qChIP experiments were performed in MCF-7 cells with indicated antibodies. Each bar indicates mean  $\pm$  S.D. of three independent experiments. B. Recruitment of GATA1 and ZEB2 the promoters of E-cadherin and VEGF, ChIP-PCR experiments were carried out. Further supported the idea. C. The regulation of E-cadherin or VEGF promoter driven luciferase activity. MCF-7 cells were transfected with promoter luciferase constructs together with GATA1/ZEB2 over expression constructs or relative shRNAs. Luciferase activities were measured and normalized to those of Renilla, of three independent experiments. D. MCF-7 cells were transfected with GATA1 constructs or shGATA1. The mRNA and protein levels of E-cadherin and VEGF were detected. E. The mRNA and protein levels of E-cadherin or VEGF were detected 48 h after MCF-7 cells were transfected with ZEB2 constructs or shZEB2.

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**Figure 4.** GATA1 enhances breast cancer cells invasion and angiogenesis in vivo. A. HUVECs were infected with lentiviruses carrying SCR, ShGATA1, vector, GATA1, or GATA1 plus ShVEGF, was added onto solidified extracellular matrix. After incubation, endothelial cell tube formation was assessed and the tubes were counted under light microscopy. B. HUVECs cultured with different CM from MCF-7 infected with the above mentioned lentiviruses, endothelial cell tube formation was assessed and the tubes were counted, statistically analyzed were represented. C. MCF-7 cells transfected with vector, GATA1 or SCR, shGATA1 were maintained in the culture media for 10 days, 1 mg/ml G418 were used to prior, the colonies were stained with crystal violet, statistically analyzed were represented. D. The effect of shGATA1 on tumorigenesis of orthotopic breast cancer cells. Representative in vivo bioluminescent images are shown; Representative tumors formed in nude mice by control MDA-MB-231 cells and GATA1 loss-of-function cells. E. The effect of shGATA1 on lung metastasis. MDA-MB-231-vector, or MDA-MB-231-shGATA1 cells were injected intravenously through the tail vein of the SCID mice. Lung metastasis was quantified using bioluminescence. Error bars indicate mean  $\pm$  S.D. \*P<0.05.

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**Figure 5.** The abnormal expression of GATA1 in breast cancer indicated a poorer prognosis. A. The expression of GATA1 was determined by real-time PCR in 30 pairs of breast cancer samples and adjacent noncancerous tissue (NT). Error bars represent standard error of the mean, Student's t-test, \* $P < 0.05$ ; \*\* $P < 0.01$ . B. Correlation between GATA1 and E-cadherin in the resected breast cancer samples from 30 patients. \* $P < 0.05$ . C. Correlation between GATA1 and VEGF in the resected breast cancer samples from 30 patients. \* $P < 0.05$ . D. Clinical data were plotted using Kaplan-Meier curves, and the 5-year survival rate was compared using the Cox log-rank test (\*\* $P < 0.001$ ). The y-axis represents the survival probability, and the x-axis represents the survival in months.

metastasis in SCID mice was conducted, we injected Luci-MDA-MB-231 cells with shGATA1 lentivirus or SCR lentivirus to the subcutaneous of the nude mice, or to the caudal vein of the SCID mice. The results shown that cells with GATA1 knockdown, the volume of the tumor, was much smaller (**Figure 4D**), and the lung metastasis was inhibited (**Figure 4E**).

### *Abnormal expression of GATA1 in breast cancer indicated a poorer prognosis*

In order to understand the clinical significance of GATA1 during the progress of breast cancer, we collected 30 breast cancer samples, and paired with adjacent noncancerous samples. The expression levels of GATA1 were examined, using qRT-PCR. The results shown that the expression level of GATA1 in breast cancer samples was significantly higher than the non-

cancerous tissues ( $P < 0.01$ , **Figure 5A**). Further, we performed correlation tests in the resected patient samples. An inverse correlation was observed between GATA1 and E-cadherin ( $R = -0.5438$ ;  $P < 0.001$ ; **Figure 5B**), as to GATA1 and VEGF, there was a strong positive correlation ( $R = 0.6996$ ;  $P < 0.0001$ ; **Figure 5C**). Further, depending on the median GATA1 expression level, patients were divided in to two groups, the group of GATA1 over expression was found to have a poorer prognosis for an overall 5-year survival ( $P < 0.05$ ) (**Figure 5D**).

### Discussion

In conclusion, we found that the expression of GATA1 in breast cancer was obviously higher than that of the noncancerous samples. Furthermore, the expression of E-cadherin was negatively associated with GATA1 in the tumor

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tissues, while the expression of VEGF was positively correlated with GATA1. The concept of ZEB2 as a powerful repressor of E-cadherin has been widely accepted [15-17], here we reported not only the physical interaction between GATA1 and ZEB2, but also the functional consistency between the two factors. We declare that GATA1 exerts its dual transcriptional regulatory function via activate the expression of genes implicated in angiogenesis, including VEGF and suppress the expression of E-cadherin. Further, the over expression of GATA1 meaning for a worse overall survival than that of patients with GATA1 low expression, it indicates GATA1 may act as an independent prognostic factor. Especially, Tube formation assay and in vivo MRI assays the reveal overexpression of GATA1 may influence the depth of tumor invasion and angiogenesis which are associated with the worst prognosis.

We demonstrate that GATA1 promotes breast cancer cell angiogenesis and invasion in vitro and in vivo, implies that GATA1 promotes EMT and enhances the invasive capacity of breast cancer. Together, our experiments reveal the mechanism for GATA1 in facilitating EMT and tumorigenesis of breast cancer, uncover the distinct role of GATA1 resulting in altered expression of the downstream target genes, suggesting that GATA1 might be a potential therapeutic target of breast cancer for treating the angiogenesis and metastasis. It is well known that the changes in the development of breast cancer should not be regarded as the alternations of a small a small part of genes. Thus, we predict other targets of GATA1 that are related to breast cancer angiogenesis and invasion will be discovered in the future. Although GATA1 and GATA3 share high similarity sequences, the function of the two genes tends to be opposite in breast cancer. So it is necessary for further exploration of the potential role of other GATA family numbers which might contribute to the breast cancer.

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### Disclosure of conflict of interest

None.

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