

## ABCB5–ZEB1 Axis Promotes Invasion and Metastasis in Breast Cancer Cells

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ABCB5 belongs to the ATP-binding cassette (ABC) superfamily, which is recognized for playing a role in the failure of chemotherapy. ABCB5 has also been found to be overexpressed at the transcriptional level in a number of cancer subtypes, including breast cancer. However, the exact mechanism ABCB5 uses on cancer cell metastasis is still unclear. In the present study, we demonstrate that ABCB5 expression was increased in metastatic tissues when compared with nonmetastatic tissues. ABCB5 can significantly enhance metastasis and epithelial–mesenchymal transition (EMT), while knockdown of ABCB5 inhibited these processes. Microarray analysis indicated that ZEB1 may function as a downstream factor of ABCB5. Furthermore, the expression of ZEB1 in tissues is positively relevant to ABCB5 in breast cancer. Knocking down ZEB1 inhibits ABCB5 ectopic expression-induced migration and invasion, as well as EMT. Taken together, these results helped to realize the oncogene functions of ABCB5 in breast cancer cells and provided a new direction in treating breast cancer.

**Key words:** ABCB5; Breast cancer; Epithelial–mesenchymal transition (EMT); ZEB1

### INTRODUCTION

In women, breast cancer is a high-incidence malignant tumor<sup>1</sup>. Although there have been significant advances in diagnosis and treatment, breast cancer is still the second leading cause of cancer-related deaths worldwide<sup>2–4</sup>. Therefore, there is a great need to explore new directions in the treatment of breast cancer.

ABCB5 (ATP-binding cassette, subfamily B, member 5) is a human P-glycoprotein family member shown to be highly overexpressed in cancer stem cells (CSCs) in diverse human malignancies<sup>5–7</sup>. ABCB5 is relevant to clinical cancer progression and therapeutic resistance in many tumor types, including breast cancer<sup>8–11</sup>. However, despite the established relationship between ABCB5 and breast, and other, cancers, the intrinsic molecular role of ABCB5 in breast cancer metastasis is currently unclear.

In the present study, we found that ABCB5 expression was increased in metastatic tissues compared with healthy tissues. Knocking down ABCB5 significantly inhibited cancer cell metastasis. Conversely, the ectopic expression of ABCB5 enhanced metastasis and

epithelial–mesenchymal transition (EMT). Furthermore, microarray analysis indicated that ABCB5 acts through the regulation of ZEB1. Knocking down ZEB1 inhibited ABCB5-induced migration and invasion, as well as EMT. Collectively, these results indicate that ABCB5 plays an important part in breast cancer cell metastasis.

### MATERIALS AND METHODS

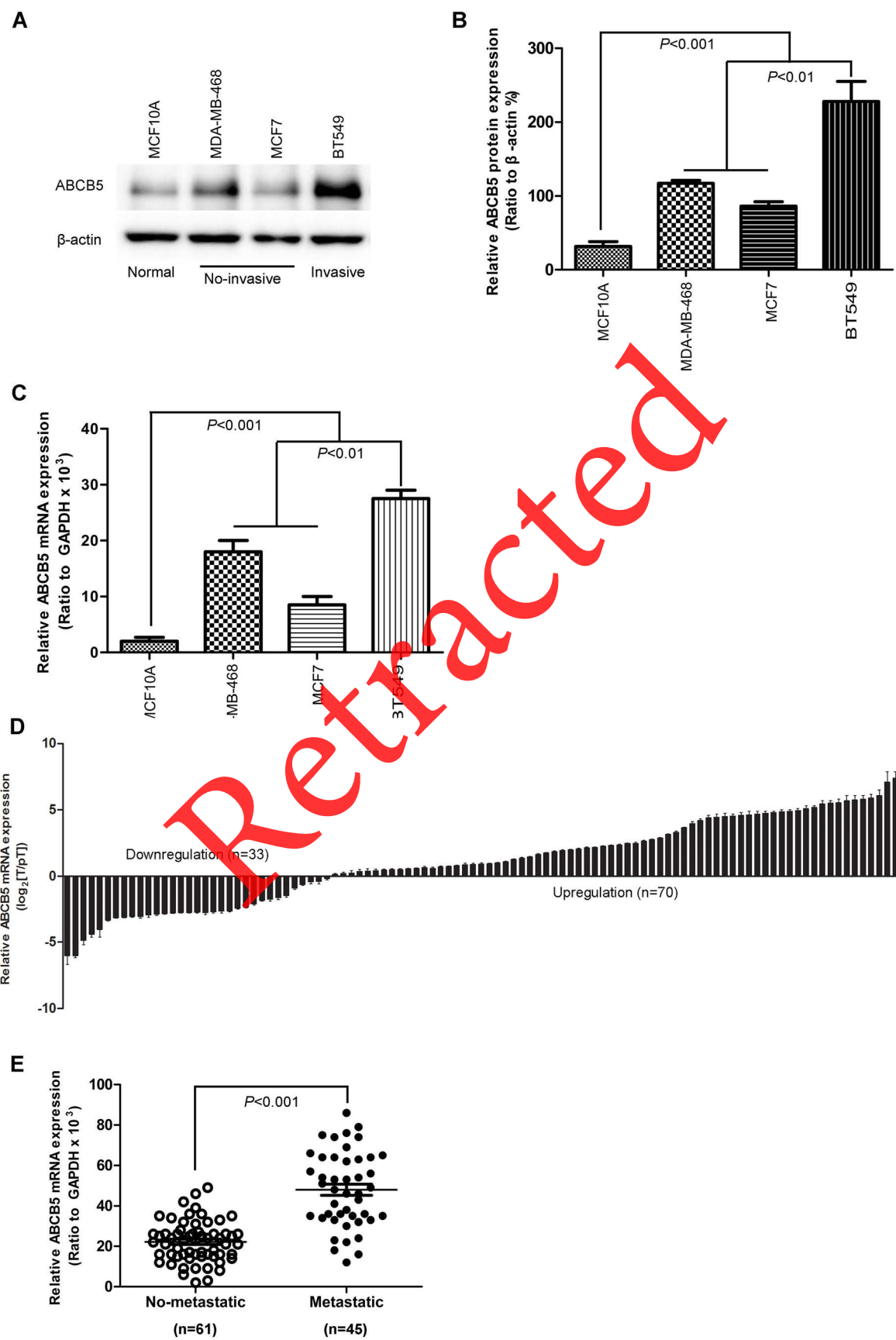
#### *Chemicals and Antibodies*

Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against ZEB1 and ABCB5 were obtained from Abcam (Cambridge, UK). N-cadherin, vimentin, E-cadherin, fibronectin,  $\alpha$ -catenin, and  $\beta$ -actin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

#### *Breast Cancer Tissues*

Breast cancer samples were acquired between 2011 and 2013 at the First Affiliated Hospital of Xi'an Jiaotong University (P.R. China). Control samples were from patients with a curative resection.

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### Real-Time RT-PCR

Total RNA was extracted from tissues and cell using TRIzol reagent. Reverse transcription (RT) was performed using the ThermoScript RT System (Invitrogen). Hot start PCR conditions were set as follows: 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 28–30 cycles (ABC5) or 26 cycles [for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)]. The primers used in the study were as follows: ABC5, 5'-TGTTTTGTTTCGGGACCACCA-3' (sense) and 5'-TTTCTCCGCCAGCATTCAT-3' (antisense); GAPDH, 5'-TGCCTCCTGCACCACCAACT-3' (sense) and 5'-CCCGTTCAGCTCAGGGATGA-3' (antisense).

### Western Blot

Cells were scraped in lysis buffer. Before being transferred onto a nitrocellulose membrane, 20 µg of protein was separated by SDS-PAGE. After blocking with nonfat dry milk, membranes were incubated with a different primary antibody overnight. After three washes, the membrane was incubated for 1 h with the secondary antibody. Proteins were visualized using SuperSignal enhanced chemiluminescence (Pierce, Bonn, Germany).

### Plasmid Constructs

The human cDNA of ABC5 was cloned as previously reported<sup>12,13</sup>. The full-length cDNAs were subcloned into multiple cloning sites of the pBabe plasmid to produce pBabe-ABC5 expression plasmids. Short hairpin RNA (shRNA) targeting ABC5 was initially inserted into the *SalI* and *XbaI* sites of the pSuper plasmid to produce pSuper-shABC5 plasmids.

### Establishment of Stable Cell Lines

The BT549 cell line was transfected using the pSuper and pSuper-shABC5 plasmids according to the manufacturer's instructions. Meanwhile, the MCF7 cell line was transfected with the pBabe and pBabe-ABC5 plasmids using Lipofectamine 2000. Stable transfectants were obtained after selection by puromycin (10 µg/ml; Invitrogen) for 2 weeks. The expression of ABC5 mRNA and protein in stable cell lines was assessed by qRT-PCR and Western blot.

### Invasion and Motility Assay

The invasion and motility assay was performed as described previously<sup>14</sup>. The mean was calculated from three independent experiments done in triplicate.

### Gene Expression Profiling

Affymetrix (Santa Clara, CA, USA) HU U133 plus 2.0 arrays were used according to the manufacturer's protocol. By the robust multiarray average normalization algorithms, all data were normalized using expression console software. Between ABC5 knockdown and control cells, altered genes were considered using scatter plots and included the genes up- and downregulated by ≥10-fold. Clustering analysis was done using a gene list in Gene Cluster v 3.0 software (Stanford University, Stanford, CA, USA), and heat maps were visualized using Java Tree View v1.1.4 r3 software (Stanford University). Gene set enrichment analysis was carried out using ConceptGen (Santa Rosa, CA, USA). Gene sets were obtained either from the ConceptGen or from published gene signatures.

### Metastasis Assay In Vivo

All the mice were obtained from Vitalriver (Beijing, P.R. China) and maintained in microisolator cages. Different cell lines were suspended in PBS at a density of  $5 \times 10^7$  cells ml<sup>-1</sup>. The cell suspension (0.1 ml) was injected into the tail vein. All of the mice were euthanized by CO<sub>2</sub> 60 days after inoculation.

### Chromatin Immunoprecipitation (ChIP)-qPCR

ChIP kit (#17-371) was purchased from EMD Millipore (Billerica, MA, USA), and ChIP experiments were carried out as previously described<sup>14</sup>. DNA expression was measured by qPCR on the ABI PRISM 7900 HT (Thermo Fisher Scientific, Waltham, MA, USA).

### Statistical Analysis

Data are described as the mean ± SD. Comparisons between different groups were undertaken using the Student's two-tailed *t*-test. The statistical significance of the differences between mean values was determined by *p* < 0.05. Statistical analysis was done with SPSS/Win11.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

### ABC5 Is Overexpressed in Metastatic Cancer Tissues

ABC5 expression in different cells was measured and compared with expression levels in normal cells (Fig. 1A–C). All three cancer cell lines showed a high expression of ABC5 compared to that of the normal cell line (MCF10A). The results also show that the two

### FACING PAGE

**Figure 1.** ABC5 expression is relevant to metastasis in human breast cancer tissues. (A) ABC5 expression in breast cancer cell lines was analyzed by Western blot. (B) Quantitative chart of ABC5 protein level in breast cancer cell lines. (C) ABC5 mRNA expression in breast cancer cell lines was analyzed by qRT-PCR. (D) Semiquantification of ABC5 expression between distant metastasis and no distant metastasis breast cancer tissues. *p* < 0.01 based on Student's *t*-test. Error bars, SD.

noninvasive cell lines showed lower ABCB5 expression (MDA-MB-468 and MCF7) compared with the invasive cell line (BT549). The mRNA levels of ABCB5 in 103 human breast cancer samples were analyzed by RT-PCR (Fig. 1D). The results indicated that ABCB5 is more highly expressed in metastatic cancer tissues than in non-metastatic cancer tissues (Fig. 1E).

#### *Knocking Down ABCB5 Inhibits Migratory, Invasive, and Metastatic Capacity*

We used retroviral vectors to establish breast cancer cell lines with stably silenced ABCB5. The expression levels of ABCB5 in the BT549 cell lines were examined by Western blot (Fig. 2A) and qRT-PCR (Fig. 2B). To determine whether knocking down ABCB5 would influence cell migratory and invasive capacities, Transwell migration and Matrigel invasion assays were performed using established, stable BT549 transfectants. The results show that BT549-shABCB5 cells exhibited a significant decrease in cell migratory activity compared to control cells (Fig. 2C). BT549-shABCB5 cells showed less invasion than control cells (Fig. 2C). To further confirm whether knocking down ABCB5 could inhibit metastasis in vivo, BT549 cells with silenced ABCB5 and control cells were inoculated into the tail vein of mice. Sixty days later, we observed that silencing ABCB5 led to fewer distant metastases (Fig. 2D). Moreover, fewer metastatic foci in the lung (Fig. 2E and F) were counted in each mouse injected with ABCB5-silenced cells.

#### *Overexpression of ABCB5 Promotes Migration, Invasion, and Metastasis of Breast Cancer Cells*

We used retroviral vectors to establish MCF7 cell lines stably overexpressing ABCB5. The expression levels of ABCB5 were examined by Western blot (Fig. 3A) and qRT-PCR (Fig. 3B). MCF7-ABCB5 showed increased migratory activity compared to its control (Fig. 3C). MCF7-ABCB5 exhibited greater invasion ability than control cells (Fig. 3C). To further confirm whether overexpression of ABCB5 promotes metastasis in vivo, MCF7 cells overexpressing ABCB5 and control cells were inoculated into the tail vein of BALB/C athymic mice. Sixty days later, we observed that overexpression of ABCB5 was associated with more distant metastases (Fig. 3D). Moreover, more metastatic foci in the lung (Fig. 3E and F) were counted in each mouse injected with cells overexpressing ABCB5.

#### *Knockdown of ABCB5 Inhibits Epithelial–Mesenchymal Transition*

Western blot and qRT-PCR assays were applied to detect the level of five proteins related to EMT in the BT549-shABCB5 cell line and its control. Knocking

down ABCB5 upregulated E-cadherin and  $\alpha$ -cadherin, and downregulated N-cadherin, fibronectin, and vimentin compared with control cells (Fig. 4A and B).

#### *Overexpression of ABCB5 Promotes Epithelial–Mesenchymal Transition*

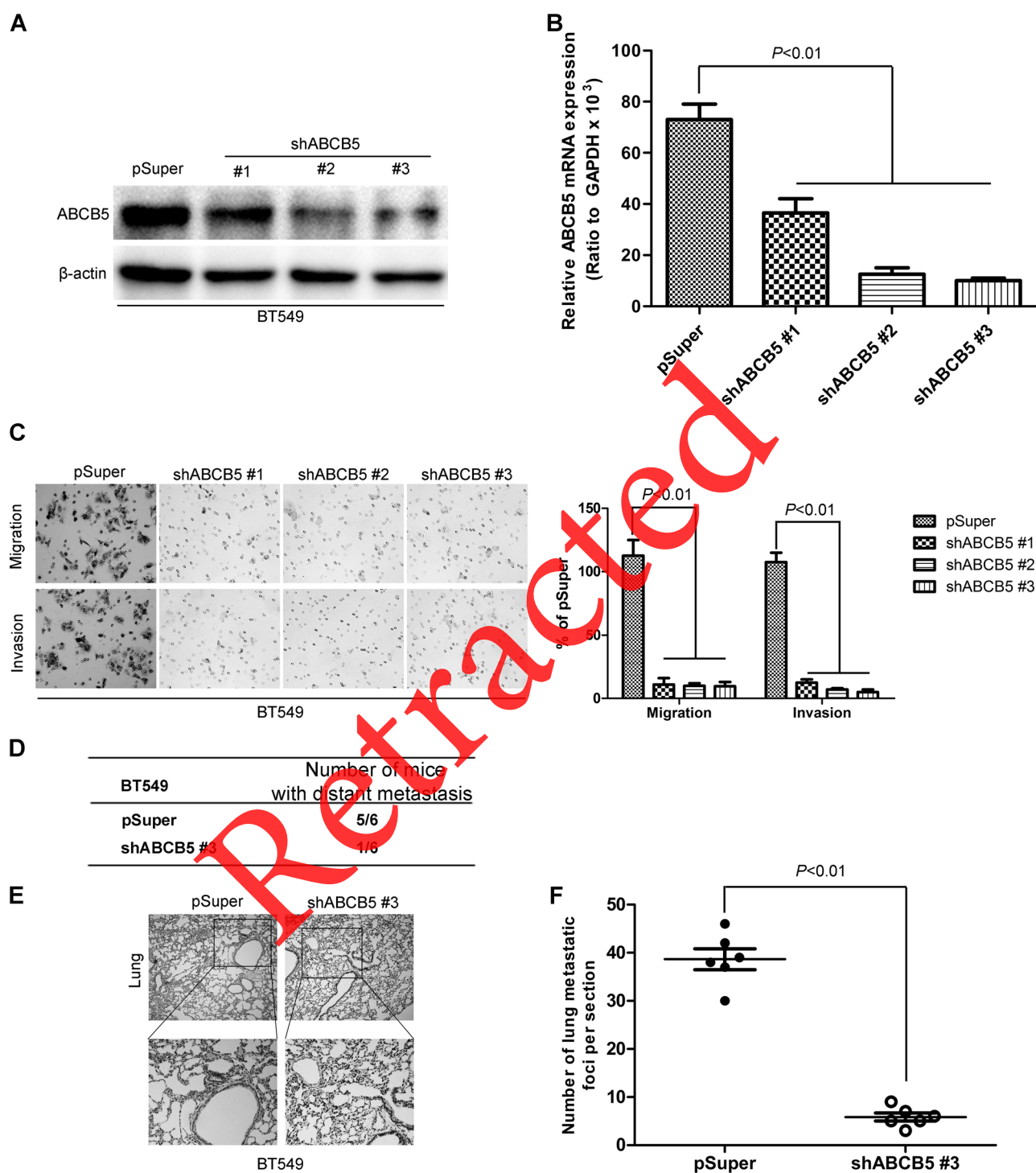
Western blot and qRT-PCR assays were applied to assess the expression of five proteins (E-cadherin,  $\alpha$ -cadherin, N-cadherin, fibronectin, and vimentin), which are related to EMT, in the MCF7-ABCB5 cell line and its control. Overexpression of ABCB5 downregulated E-cadherin and  $\alpha$ -cadherin, and upregulated N-cadherin, fibronectin, and vimentin compared with control cells (Fig. 4C and D).

#### *ABCB5 Regulates the Expression of ZEB1 at the Transcriptional Level*

To better characterize the mechanisms by which ABCB5 is engaged in the development and progression of breast cancer cell metastasis, a microarray was performed using the cell line BT549-shABCB5 and its control transfected with the empty pSuper plasmid. The results indicate that many genes were significantly differentially expressed after ABCB5 knockdown (Fig. 5A). In addition, enrichment assays found that ZEB1 was enriched in ABCB5 knockdown cells (Fig. 5B). These results suggest that ABCB5 enhances breast cancer cell metastasis mediated by ZEB1.

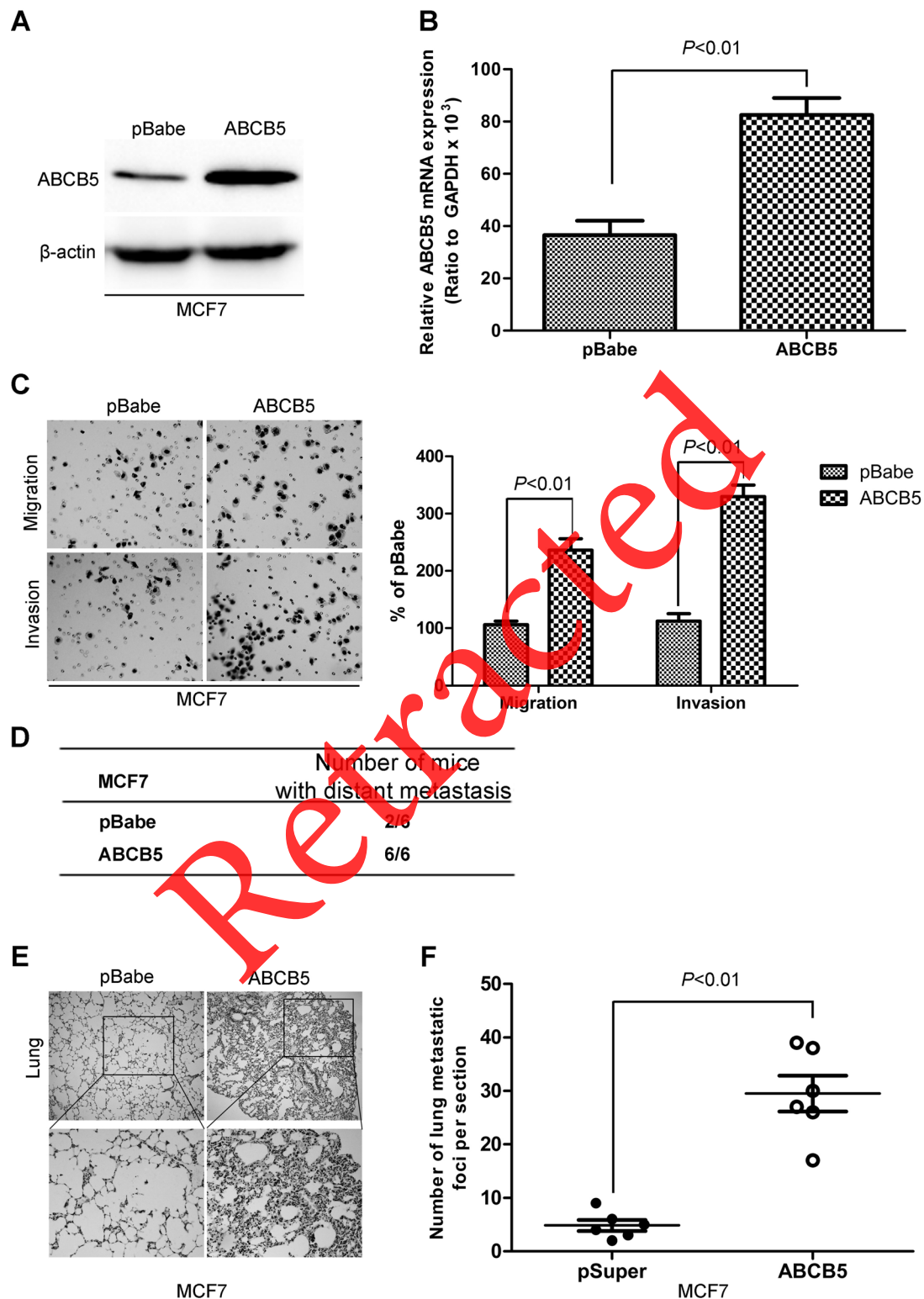
To further investigate the relationship between ABCB5 and ZEB1, we assessed ZEB1 levels in the breast cancer cell lines described above using Western blot and RT-PCR. Compared with control cells (Fig. 6A and B), the expression level of ZEB1 was significantly decreased in ABCB5 knockdown cells, while overexpression of ABCB5 increased ZEB1 expression on the protein and mRNA levels (Fig. 6C and D). In human breast cancer tissues, the expression of ZEB1 was positive relevant to ABCB5 (Fig. 6E).

To assess the regulation of ZEB1 expression by ABCB5, we performed a luciferase assay. The results show that knocking down ABCB5 significantly decreased ZEB1 promoter activity (Fig. 7A), while overexpression of ABCB5 increased ZEB1 promoter activity (Fig. 7B). To better determine whether ABCB5 regulates ZEB1 on the transcriptional level, qChIP assays were performed in BT549-shABCB5 and MCF7-ABCB5 cells. Antibodies against ABCB5 and IgG were used to pull down the chromatin complex, and three primer pairs against the ZEB1 gene promoter region (#1, #2, and #3) were used to assess the occupancy of the ZEB1 gene promoter (Fig. 7C). Silencing ABCB5 was associated with decreased activity levels in regions #1 and #3 of the ZEB1 gene promoter region in BT549-shABCB5 cells (Fig. 7D and E). Greater occupancy of those regions by ABCB5 was found

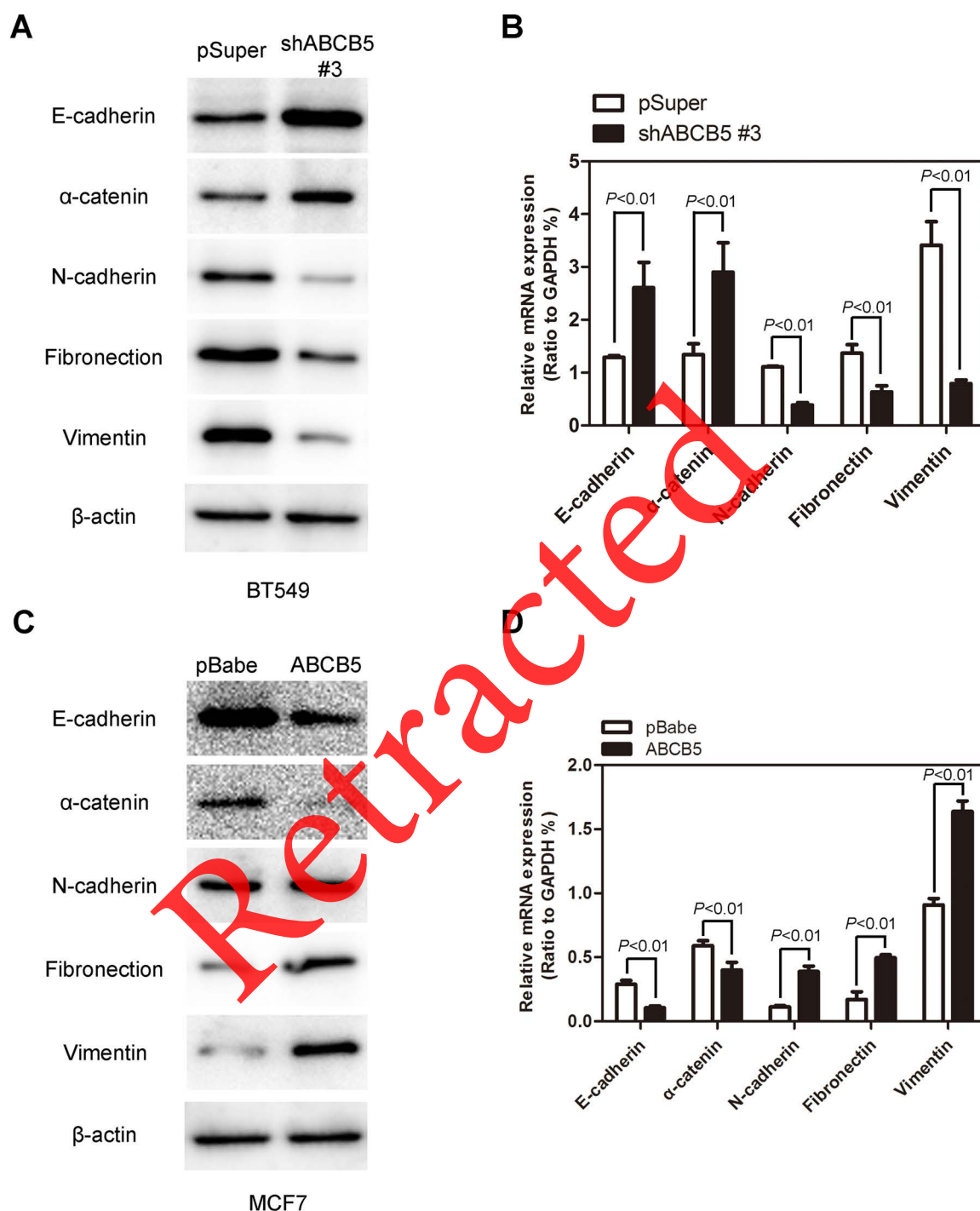


**Figure 2.** Silencing ABCB5 in breast cancer cells decreases the migration and invasion capacities of breast cancer cells. (A) Western blot analysis of ABCB5 levels in established cell lines. (B) qRT-PCR analysis of ABCB5 mRNA levels in established cell lines. (C) Breast cancer cells with silent expression of ABCB5 showed reduced invasion capacity in the Transwell and Matrigel assays. (D) Silencing ABCB5 significantly decreased the number of mice with distant metastasis. (E, F) Fewer metastatic foci in the lung were counted in each mouse injected with breast cancer cells with silenced ABCB5 or control cells.  $p < 0.01$  based on Student's  $t$ -test. Error bars, SD.

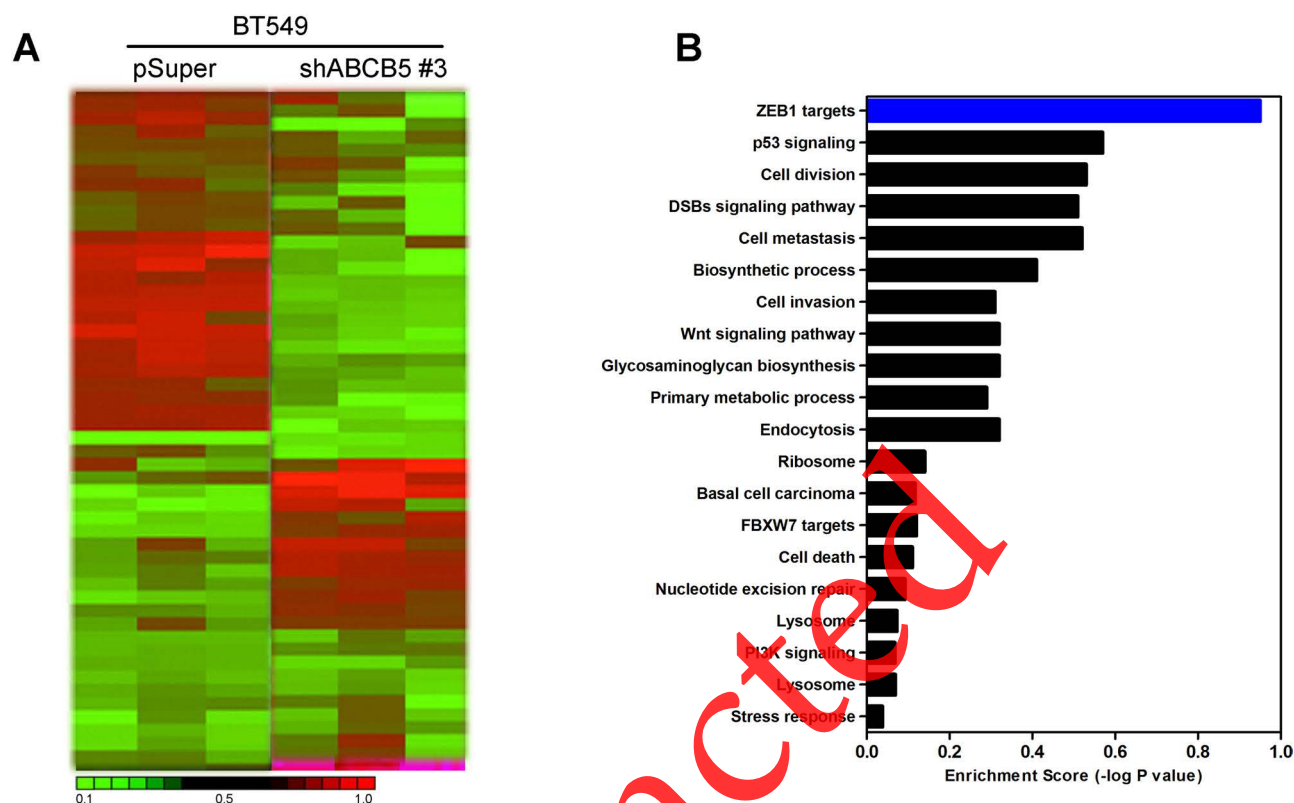




**Figure 3.** Overexpression of ABCB5 in breast cancer cells increases the migration and invasion capacities of breast cancer cells. (A) Western blot analysis of ABCB5 levels in established cell lines. (B) qRT-PCR analysis of ABCB5 mRNA levels in established cell lines. (C) Breast cancer cells with high expression of ABCB5 possessed stronger invasion capacity in the Transwell and Matrigel assays. (D) Overexpression of ABCB5 significantly increased the number of mice with distant metastasis. (E, F) More metastatic foci in the lung were counted in each mouse injected with breast cancer cells overexpressing ABCB5 or control cells.  $p < 0.01$  based on Student's  $t$ -test. Error bars, SD.



**Figure 4.** ABCB5 regulates the transition between epithelial and mesenchymal phenotypes in breast cancer cells. Western blot analysis (A) and qRT-PCR analysis (B) show that silencing ABCB5 causes the upregulation of epithelial cell markers and downregulation of mesenchymal cell markers. Western blot analysis (C) and qRT-PCR analysis (D) show that overexpression of ABCB5 causes the downregulation of epithelial cell markers and upregulation of mesenchymal cell markers.  $p < 0.01$  based on Student's  $t$ -test. Error bars, SD.



**Figure 5.** ABCB5 regulates the expression level of ZEB1. (A) Clustering of the genes differentially expressed after silencing ABCB5. (B) The enrichment scores of differentially expressed genes in an ABCB5-silenced cell line.

in MCF7-ABCB5 cells (Fig. 7F and G). ZEB1 silencing was verified by Western blot and qRT-PCR (Fig. 8A and B). Knocking down ZEB1 decreased the migratory and invasion capacities of MCF7-ABCB5 cells (Fig. 8C). Epithelial markers were increased and mesenchymal markers were decreased (Fig. 8D). These results indicate that these cells were transformed from a mesenchymal to an epithelial state.

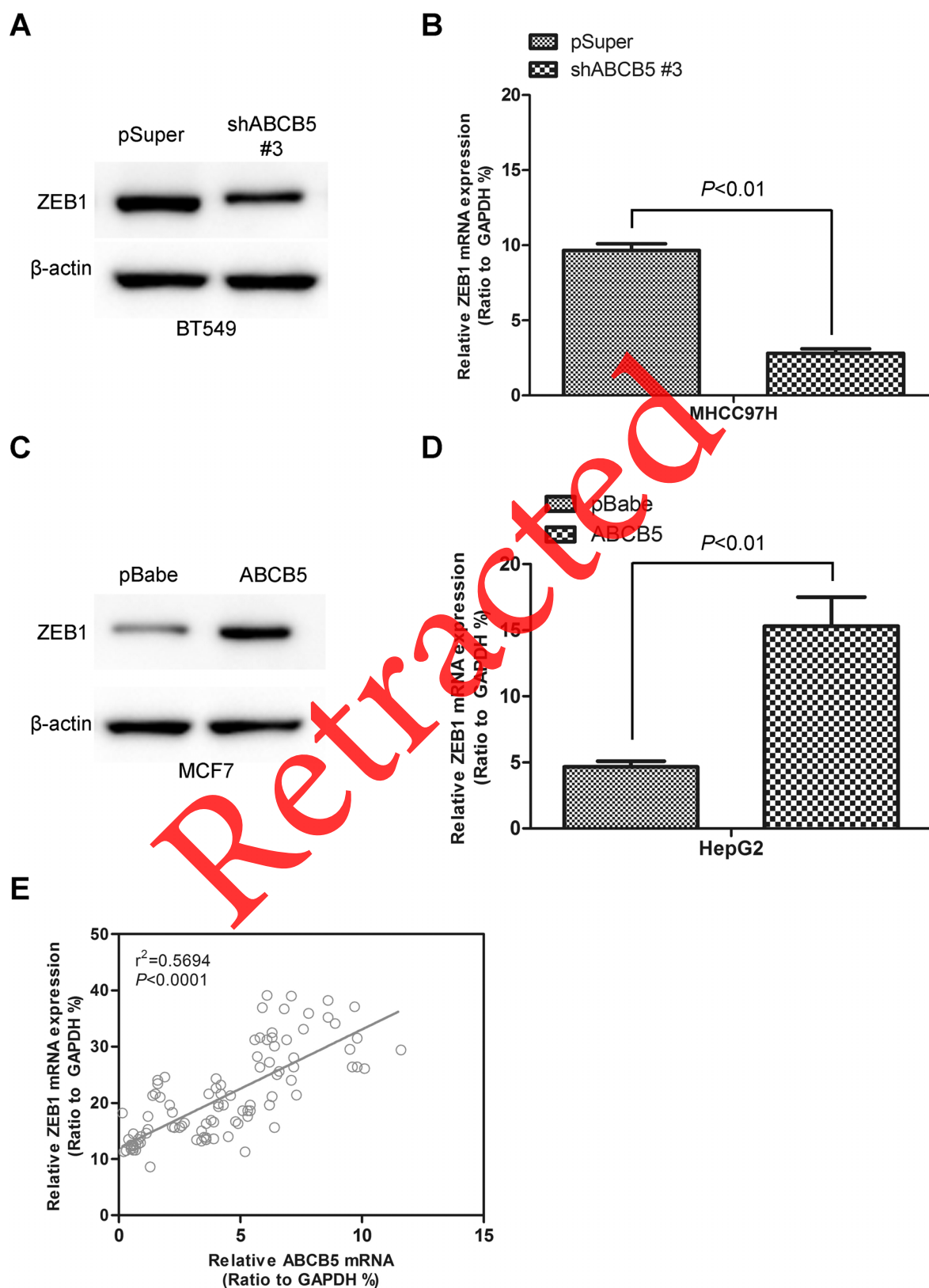
## DISCUSSION

In this study, the role of ABCB5 in breast cancer metastasis was characterized. Increased levels of ABCB5 were detected in the metastatic breast cancer tissues compared to healthy breast tissues. Knocking down ABCB5 was found to inhibit cell migration and invasion as well as EMT progression. We also found that overexpression of ABCB5 in breast cancer cells significantly promoted tumor cell migration, invasion, and EMT progression. ABCB5 affected the expression of ZEB1 in breast cancer cells. Silencing ZEB1 in ABCB5-overexpressing cells resulted in a phenomenon similar to that caused by ABCB5 knockdown.

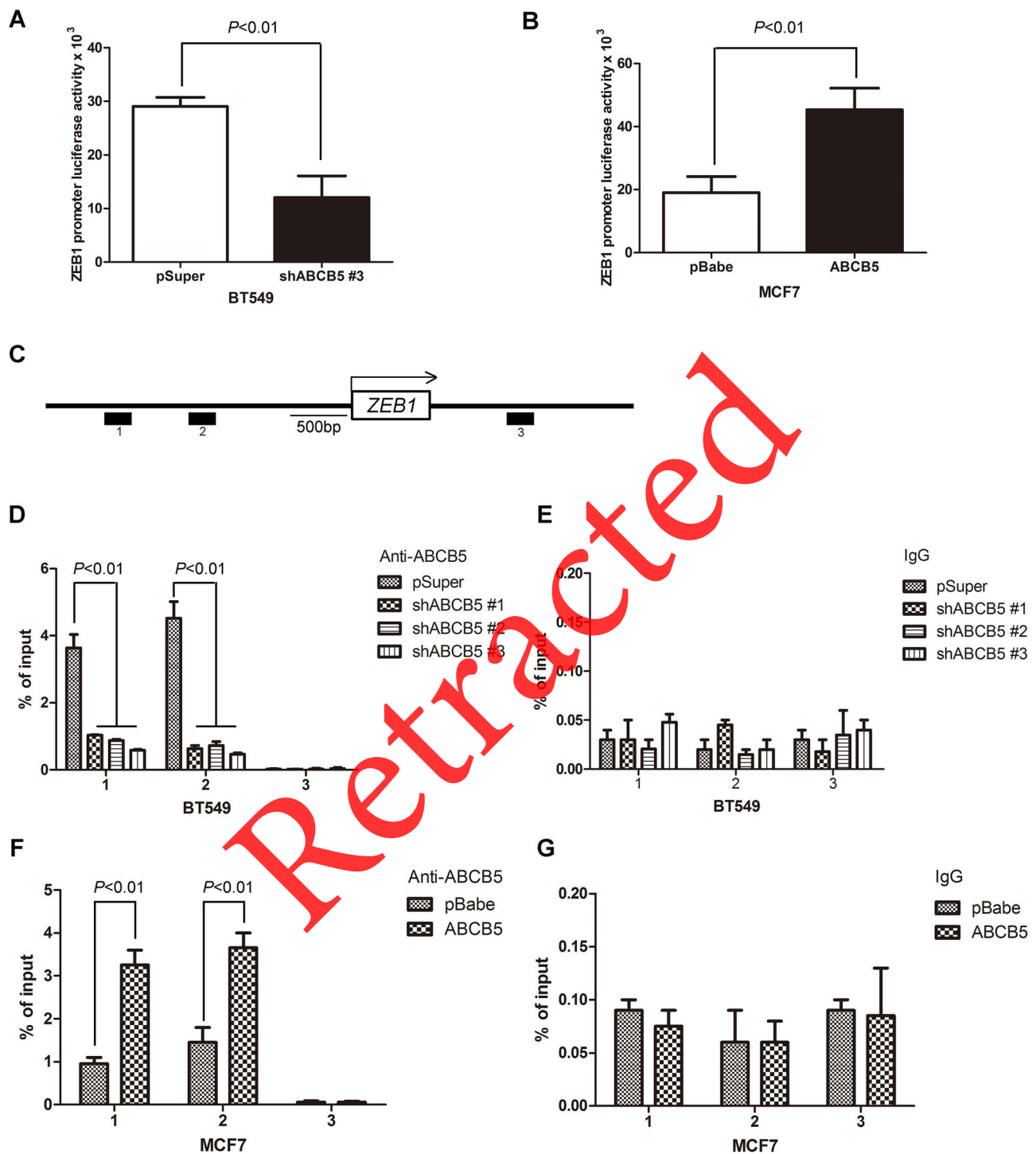
EMT happens gradually in the process of tumor development and is extremely complex<sup>15</sup>. In this process, polarity and adhesion to surrounding epithelial cells and

the matrix are reduced, and cells acquire a morphology similar to that of fibroblasts, concomitantly acquiring enhanced migratory ability<sup>16</sup>. EMT is characterized by reduced E-cadherin and  $\alpha$ -catenin expression as well as increased N-cadherin, vimentin, and fibronectin expression<sup>16</sup>. E-cadherin plays an important role in sustaining the polarity of epithelial cells as well as their structure and shape. As shown in many studies, reduced E-cadherin expression is closely related to relationship with epithelial cell<sup>17</sup>. Meanwhile, EMT progression usually occurs with higher expression of proteins associated with mesenchymal cells, especially vimentin<sup>18</sup>. In contrast with E-cadherin, N-cadherin expression is upregulated in EMT<sup>19</sup>.  $\alpha$ -Catenin can modify cell adhesion activity, and downregulation could lead to a loss of E-cadherin function, and thus a loss of contact inhibition among epithelial cells. Thus,  $\alpha$ -catenin downregulation is positively correlated with EMT progression. Previous studies have shown that EMT confers cancer cells with greater migratory and invasive abilities and thus induces distant metastases<sup>20</sup>. Our results show that overexpression of ABCB5 can promote the EMT process, while knockdown of ABCB5 can inhibit the EMT process. These results are consistent with the phenomenon that overexpression of ABCB5 in breast cancer cells significantly promotes tumor cell migration,

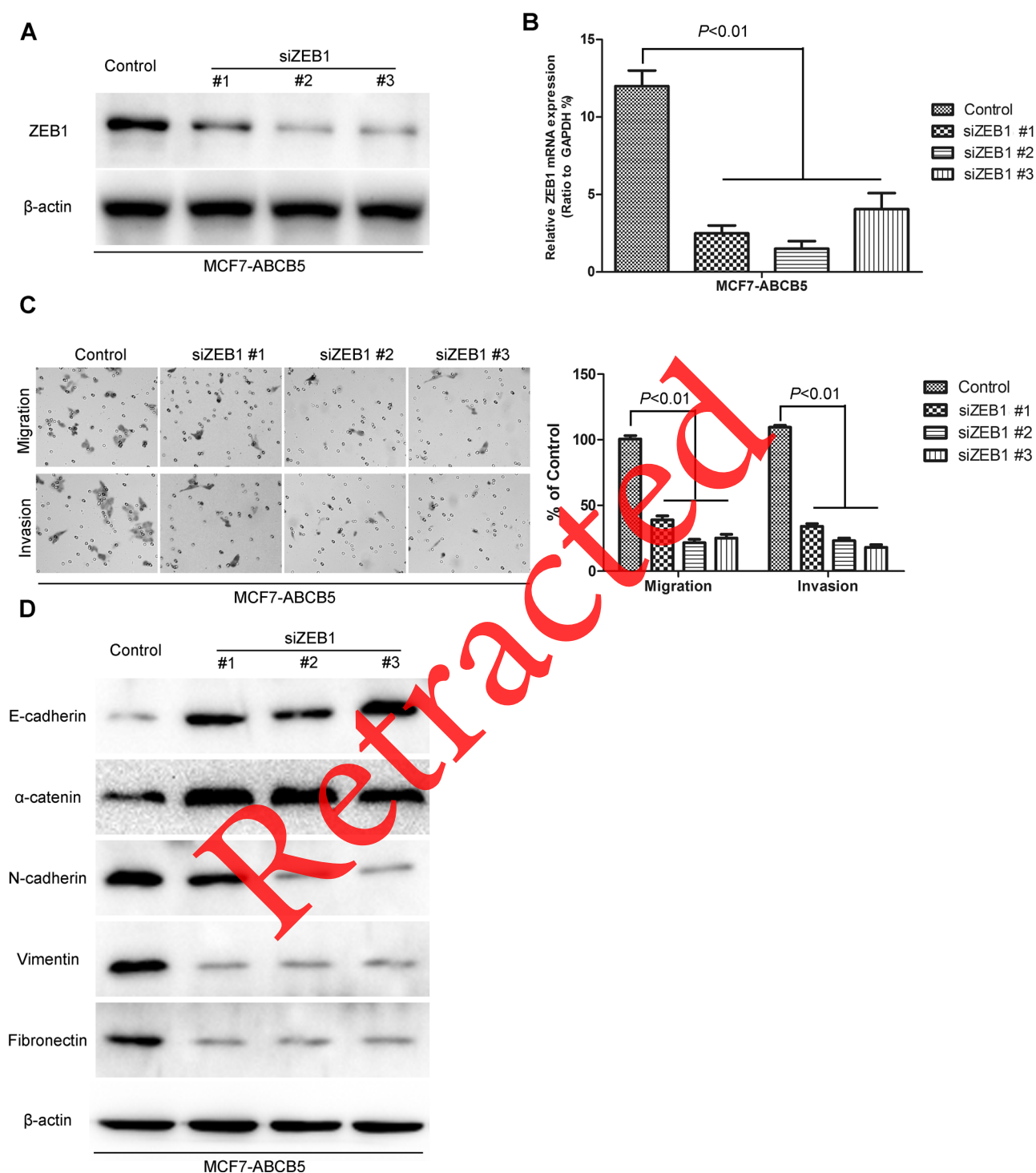




**Figure 6.** ZEB1 expression is affected by ABCB5. ZEB1 expression in an ABCB5-silenced cell line was assayed by Western blot (A) and qRT-PCR (B). Detection of ZEB1 in an ABCB5-overexpressing cell line using Western blot (C) and qRT-PCR (D). (E) ZEB1 was positively correlated with ABCB5 in breast cancer tissues.  $p < 0.01$  based on Student's *t*-test. Error bars, SD.



**Figure 7.** ABCB5 binds to the promoter of ZEB1. (A, B) ZEB1 promoter luciferase activity was detected by the luciferase assay in established cell lines. (C) Schematic diagram of the ZEB1 promoter region. qChIP was performed in ABCB5-silenced (D) and -overexpressing (F) cell lines. (E, G) IgG was used as the negative control.  $p < 0.01$  based on Student's *t*-test. Error bars, SD.



**Figure 8.** Silencing ZEB1 decreases the migration of cells ectopically expressing ABCB5. (A) Western blot analysis of ZEB1 in established breast cancer cell lines. (B) qRT-PCR analysis of ZEB1 expression in established breast cancer cell lines. (C) Silencing ZEB1 reverses ABCB5-induced migration. (D) Silencing ZEB1 reverses the ABCB5-induced changes in EMT markers.  $p < 0.01$  based on Student's  $t$ -test. Error bars, SD.

invasion in vitro, and distant metastasis in vivo. All results indicate that ABCB5 affects cell migration via regulating EMT progression.

Microarray was carried out to investigate the mechanism by which ABCB5 regulates the progression of breast cancer. ZEB1 was identified as an effective mediator of these ABCB5-induced phenomena<sup>21</sup>. ZEB1 binds to the E-box in gene promoters to repress gene expression<sup>22</sup>. This signaling has emerged in recent years as an attractive target for anticancer therapy because its aberrant activation is implicated in several cancers<sup>22</sup>. In this study, we found that silencing ZEB1 in ABCB5-overexpressing cell lines resulted in a phenomenon similar to that caused by ABCB5 knockdown.

In summary, we demonstrated that ABCB5 is highly expressed in metastatic breast cancer, and the knockdown of ABCB5 inhibits breast cancer cell migration. Therefore, these findings may shed light on potential targets in breast cancer prevention and therapy.

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