Knockdown of REV7 Inhibits Breast Cancer Cell Migration and Invasion

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REV7 (also known as MAD2L2) is a multifunctional protein involved in DNA damage tolerance, cell cycle regulation, gene expression, and carcinogenesis. Although its expression is reportedly associated with poor prognosis in several kinds of human cancers, the significance of REV7 expression in breast malignancies is unclear. In this study, REV7 was found to be increased in breast cancer. We found that knockdown of REV7 inhibited the migration, invasion, and epithelial–mesenchymal transition (EMT) of breast cancer cells. Meanwhile, overexpression of REV7 promoted the migration, invasion, and EMT of breast cancer cells. As shown by Western blot, knockdown of REV7 can promote TGF-β1 expression. Western blot analysis indicated that TGF-β1 may play a role as a downstream factor of REV7. Moreover, interference of TGF-β1 can also inhibit the cell's ability for migration, invasion, and EMT, as well as in a cell line whose REV7 is overexpressed. Taken together, these results contributed to a recognition of the oncogene functions of REV7 in breast cancer cells and provided a novel direction to treat breast cancer.

Key words: REV7; Breast cancer; Epithelial–mesenchymal transition (EMT);
Transforming growth factor-β1 (TGF-β1)

INTRODUCTION

Breast cancer is one of the most common malignant cancers occurring in women. The rate of breast cancer's incidence accounts for 7–10% compared with other varied malignant cancers, only second to uterine cancer in females (1). Although there are significant advances in diagnosis and treatment, breast cancer is still the second leading cause of cancer-related deaths worldwide (2,3). Therefore, there is a great need for development of a novel direction to treat breast cancer.

REV7 encodes a subunit of polymerase ζ (Polζ), one of the error-prone DNA polymerases involved in translation DNA synthesis (TLS) polymerases to repair DNA damage during DNA replication (4,5). When the replication fork is encountered at DNA damage sites during DNA replication, the replication fork stalls and this arrest of DNA replication causes genome instability and cell death; however, cells can relieve this arrest of DNA replication by bypassing DNA damage sites using the TLS polymerases (6,7). Therefore, the TLS polymerases play an essential role in maintaining genome integrity and tolerance to DNA damage in mammalian cells, and most of the reported targeted mutations of the TLS polymerases show defects in DNA integrity and stability (8,9). On the

basis of the above, REV7 was found to be associated with immunologic response, regulation, and control of mitotic cycle and tumor cell's drug resistance. Nevertheless, the function and mechanism that REV7 plays in breast cancer are still unknown to scientists.

In the present study, the roles of REV7 were investigated in breast cancer genesis. The expression of REV7 was found to be increased in a majority of human breast cancer tissues examined compared with normal tissues. In MCF7, one of the human breast cancer cell lines, we found that overexpression of REV7 significantly enhanced cell migration, invasion, and epithelial–mesenchymal transition (EMT). Further study found that overexpression of REV7 increased the protein levels of transforming growth factor-β1 (TGF-β1). Conversely, knockdown of REV7 in the MDA-MB-231 cell line inhibited breast cancer cell migration, invasion, and EMT. Collectively, these results indicate an important role for REV7 in breast cancer cell migration, invasion, and EMT.

MATERIALS AND METHODS

Samples, Cells, and Antibodies

Human normal breast tissue samples and breast cancer tissue samples were provided by Affiliated Hospital

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of Qingdao University. Human breast cancer cell lines MCF10A, BT549, MDA-MB-231, HCC1428, MCF7, and SK-BR-3 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen).
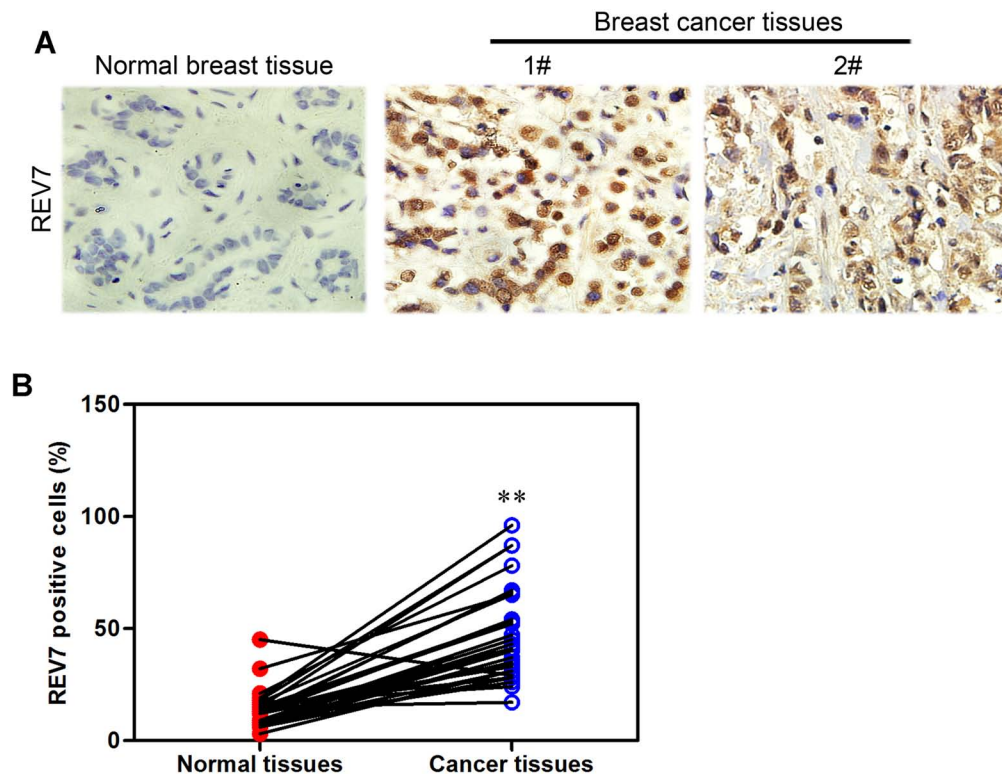
Immunohistochemistry Staining

Immunohistochemical Envision two-step method was carried out to detect the REV7 expression of breast cancer tissue samples and normal cancer tissue samples. Tissues were first blocked by paraffin and then sliced so as to obtain 3-μm-thickness paraffin sections. Paraffin sections were deparaffinized by dimethylbenzene, hydrated by alcohol in different concentrations, washed by phosphate-buffered saline (PBS) one by one, and then incubated with 1:200 anti-REV7 antibodies at 4°C overnight. Goat-radish peroxidase-conjugated secondary antibody (1:500) was then added and further incubated for 1 h at room temperature. The sections were developed using a 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit at room temperature for 1–5 min and then counterstained with hematoxylin.

Western Blot

Cells were quantified after washing and scraping in a lysis buffer. Protein (20 mg) was loaded on an SDS-PAGE and then transferred onto a 0.2-μm nitrocellulose membrane. Membranes were incubated with a primary antibody overnight after being blocked with 5% nonfat dry milk in Tris-buffered saline with 1% Tween-20 (TBS-T). The membrane was incubated for 1 h with the horseradish peroxidase (HRP)-conjugated secondary antibody after washing. Membranes were then washed thrice with TBS-T, and proteins were visualized with Super Signal (Pierce, Bonn, Germany) enhanced chemiluminescence.

RT-PCR

By using the TRIzol reagent (Invitrogen), total RNA was extracted from breast cancer tissues and cell lines. Reverse transcription (RT) was performed by using the ThermoScript RT System (Invitrogen). Polymerase chain reaction (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 (for REV7) or 26 cycles [for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)]. The primers used in the study were as follows: REV7, 5'-AGAGTTTCATCCCCAAAGACAA-3' (sense) and

Figure 1. REV7 is highly expressed in human breast cancer samples. (A) REV7 protein expression was analyzed by immunohistochemistry in 21 breast cancer tissues and 15 normal breast tissues. (B) Immunohistochemical results were summarized by statistical software. **$p < 0.001$ is based on Student's *t*-test. Error bars, SD.

5′-AGTTCAGGCAGTAGGCAAAGTC-3′ (antisense); GAPDH, 5′-TGCCTCCTGCACCACCAACT-3′ (sense) and 5′-CCCGTTCAGCTCAGGGATGA-3′ (antisense).

Plasmids Construct

According to previous reports, human cDNA of REV7 was cloned. The full-length cDNAs were subcloned into the multiple cloning sites of the vector plasmid forming the vector-REV7 expression plasmids. Short hairpin RNA (shRNA) targeting REV7 (sense: 5′-TCGACGCG AATCTCTCTTTGGCAAGTTCAAGAGACTTGCCA AAGAGAGATTCGTTTTTTGGAAT-3′; antisense: 5′-CTAGATTCCAAAAAACGAATCTCTCTTTGGCAA GTCTCTTGAACTTGCCAAAGAGAGATTCGCG-3′) was initially inserted into the *Sal*I and *Xba*I sites of vector plasmid, forming the vector-shREV7 plasmids.

Establishment of Stable Expression and Knockdown Cell Lines

According to the manufacturer's instructions (Invitrogen), the MDA-MB-231 cell was transfected with the vector, vector-shREV7 plasmid by using Lipofectamine 2000. Meanwhile, the MCF7 cell was transfected with the vector, vector-REV7 plasmid similarly using Lipofectamine 2000. Stable transfectants were obtained after selection by puromycin (10 µg/ml; Invitrogen) for 2 weeks. Expression of REV7 mRNA and protein in stable cell lines was analyzed by RT-PCR and Western blot, respectively.

Cell Invasion and Motility Assay

Invasion of cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA)-coated Transwell inserts (6.5 mm; Costar, Manassas, VA, USA) containing poly-carbonate filters with 8-µm pores as detailed previously (10). According to the manufacturer's recommendations, the inserts were coated with 50 µl of 1 mg/ml Matrigel matrix. Cells (2×10^5) were plated in the upper chamber with 200 µl of serum-free medium, while 600 µl of medium with 10% FBS was added to the lower well. Top cells were removed, and bottom cells were counted after 24-h incubation. Cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet after migration to the lower surface of the membrane. Five random fields were counted at 10× magnification for each membrane. The mean was calculated from three independent experiments done in triplicate. Motility assays were similar to the Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

Statistical Analysis

Data were described as the mean ± standard deviation (SD). Student's two-tailed *t*-test was used to compare the data between different groups. SPSS11.0 (SPSS Inc.,
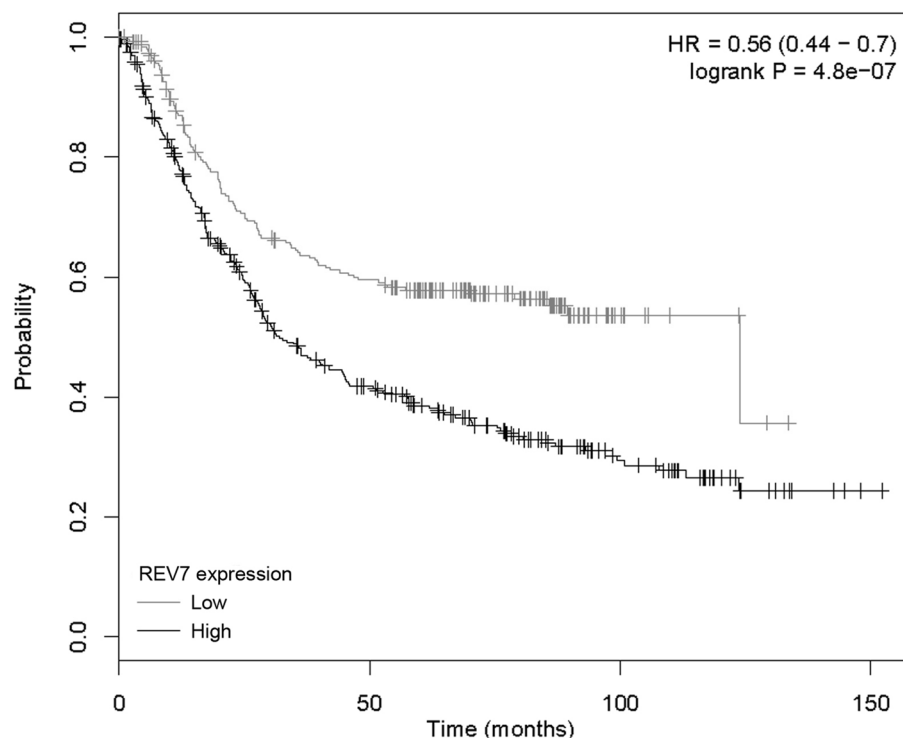
Figure 2. Survival rate and REV7 expression have a negative correlation through follow-up visit. Survival analysis of patients with breast cancers was based on the Kaplan–Meier survival analysis in TCGA.
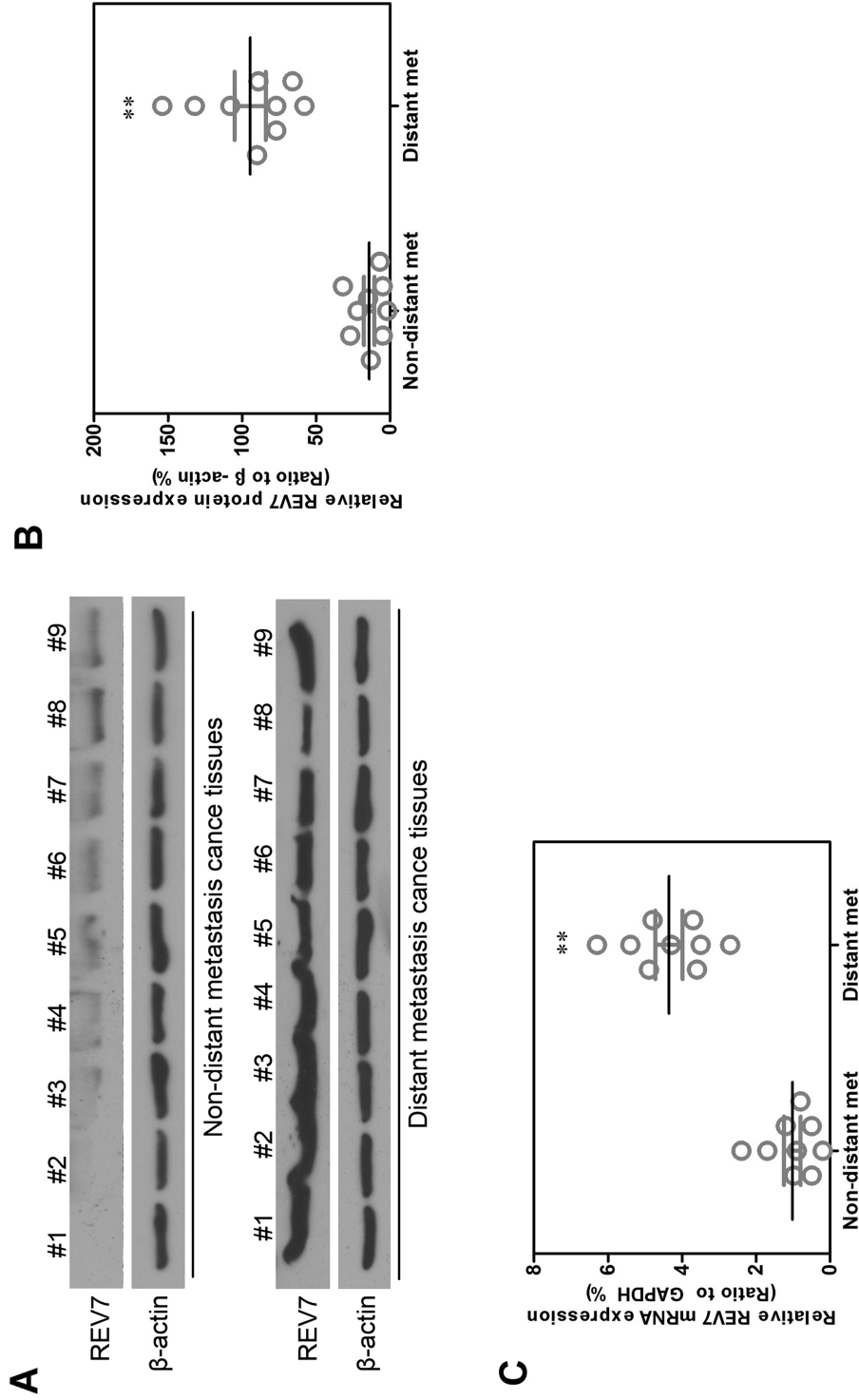
Figure 3. A high level of REV7 is expressed in distant metastasis breast cancer tissues. (A) REV7 protein expression was analyzed by Western blot assay in nine nondistant metastasis breast cancer tissues and nine distant metastasis breast cancer tissues. (B) REV7 protein expression detected by Western blot assay was summarized by statistical software. (C) REV7 mRNA expression detected by quantitative RT-PCR assay was summarized by statistical software in these tissues. **$p<0.001$ is based on Student's t-test. Error bars, SD.

Chicago, IL, USA) was used to analyze the statistical significance of the difference between mean values determined by a value of $p < 0.05$.

RESULTS

A High Level of REV7 Is Expressed in Human Breast Cancer Samples

REV7's protein level was analyzed by immunohistochemistry staining in 15 normal breast samples and 21 breast cancer samples (Fig. 1A). Statistical software was used to analyze the data (Fig. 1B). A curve graph was drawn to describe the correlation of one's survival time and REV7 expression using The Cancer Genome Atlas (TCGA) database. We found that patients with a higher REV7 level in breast cancer tissues had a shorter life span than those with lower REV7 expression level (Fig. 2), suggesting that a negative correlation between REV7 and long survival exists.

Furthermore, Western blot assay was performed to examine the protein levels of REV7 in nine nondistant metastasis breast cancer samples and nine distant metastasis breast cancer samples (Fig. 3A), and the data were analyzed by statistical software (Fig. 3B). Semiquantitative RT-PCR was implemented to detect the mRNA levels of REV7 in nine nondistant metastasis breast cancer samples and nine distant metastasis breast cancer samples; the results were shown in the form of statistical analysis (Fig. 3C). Compared with normal breast cells, REV7 showed a significantly high level of expression in breast cancer cells, especially in distant metastasized breast cancer cells.

Stable Cell Lines of Downregulated and Upregulated Expression of REV7 Are Constructed

REV7 protein expression in six different breast cancer cell lines (MCF10A, MCF7, SK-BR-3, HCC1428, BT549, and MDA-MB-231) was detected by Western blot assay (Fig. 4A). The expression of REV7 was knocked down by shRNAs in MDA-MB-231 cells, and REV7 expression in the MDA-MB-231-shREV7s cell line construct was verified by Western blot (Fig. 4B). REV7 mRNA's low expression in the MDA-MB-231-shREV7 cell line construct was verified by quantitative RT-PCR (Fig. 4C). The expression of REV7 was upregulated in SK-BR-3 cells, and REV7 expression in the SK-BR-3-REV7 cell line construct was verified by Western blot assay (Fig. 4D). REV7 mRNA's high expression in the SK-BR-3-REV7 cell line construct was verified by quantitative RT-PCR assay (Fig. 4E).

Knockdown of REV7 Inhibits Capacities of Migratory and Invasion of Breast Cancer Cells

Transwell migration and Matrigel invasion assays were performed on the established MDA-MB-231 stable transfectants to determine whether knockdown of REV7 would influence the cell capacity of migratory and invasion. The results showed that MDA-MB-231-vector-shREV7 cells' migratory activity decreased more significantly than that of control cells (i.e., MDA-MB-231-vector) (Fig. 5A). The results were shown more intuitively using a statistical column diagram (Fig. 5B). As shown in the Matrigel invasion assay, MDA-MB-231-vector-shREV7 cells exhibited less ability for invasion than that of control cells (Fig. 5C). Similarly, the statistical column diagram showed us this more intuitively (Fig. 5D).

Overexpression of REV7 Promotes the Capacity of Migration and Invasion in Breast Cancer Cells

SK-BR-3-REV7, whose REV7 protein was overexpressed, was found to increase more significantly in cell migratory activity than in its control cells (i.e., SK-BR-3-vector) using the same assays (Fig. 6A). Meanwhile, the results were shown more intuitively using a statistical column diagram (Fig. 6B). As showed in Matrigel invasion assay, SK-BR-3 -REV7 cells exhibited more a significant ability of invasion than that of control cells (Fig. 6C). At the same time, the results were shown intuitively by a statistical column diagram (Fig. 6D).

Knockdown of REV7 Inhibits EMTs

EMT progression between the MDA-MB-231-shREV7 cell line and its control was detected by Western blot. The expression of four proteins related to EMT (E-cadherin, α-cadherin, N-catenin, and vimentin) was examined by Western blot assay in the MDA-MB-231-shREV7 cell line and its control cells. Knockdown of REV7 upregulated the expression of E-cadherin and α-cadherin, while it downregulated the expression of N-cadherin and vimentin when compared with the control (Fig. 7A).

Overexpression of REV7 Promotes EMTs

EMT progression was detected by Western blot between the SK-BR-3-REV7 cell line and its control. Expression of four proteins (E-cadherin, α-cadherin, N-catenin, and vimentin) was examined by Western blot assay between the SK-BR-3 -REV7 cell line and its control cells. Overexpression of REV7 downregulated the expression of E-cadherin and α-cadherin, while it upregulated the expression of N-cadherin and vimentin compared with the control (Fig. 7B).

REV7 Can Regulate the Expression of TGF-β1

Western blot assay was performed to characterize the mechanisms by which REV7 engaged in the development and progression of breast cancer cells, using the MDA-MB-231-shREV7 and SK-BR-3-REV7 cell lines and their control with the empty vector plasmids, respectively. The results identified TGF-β1, whose protein level expression
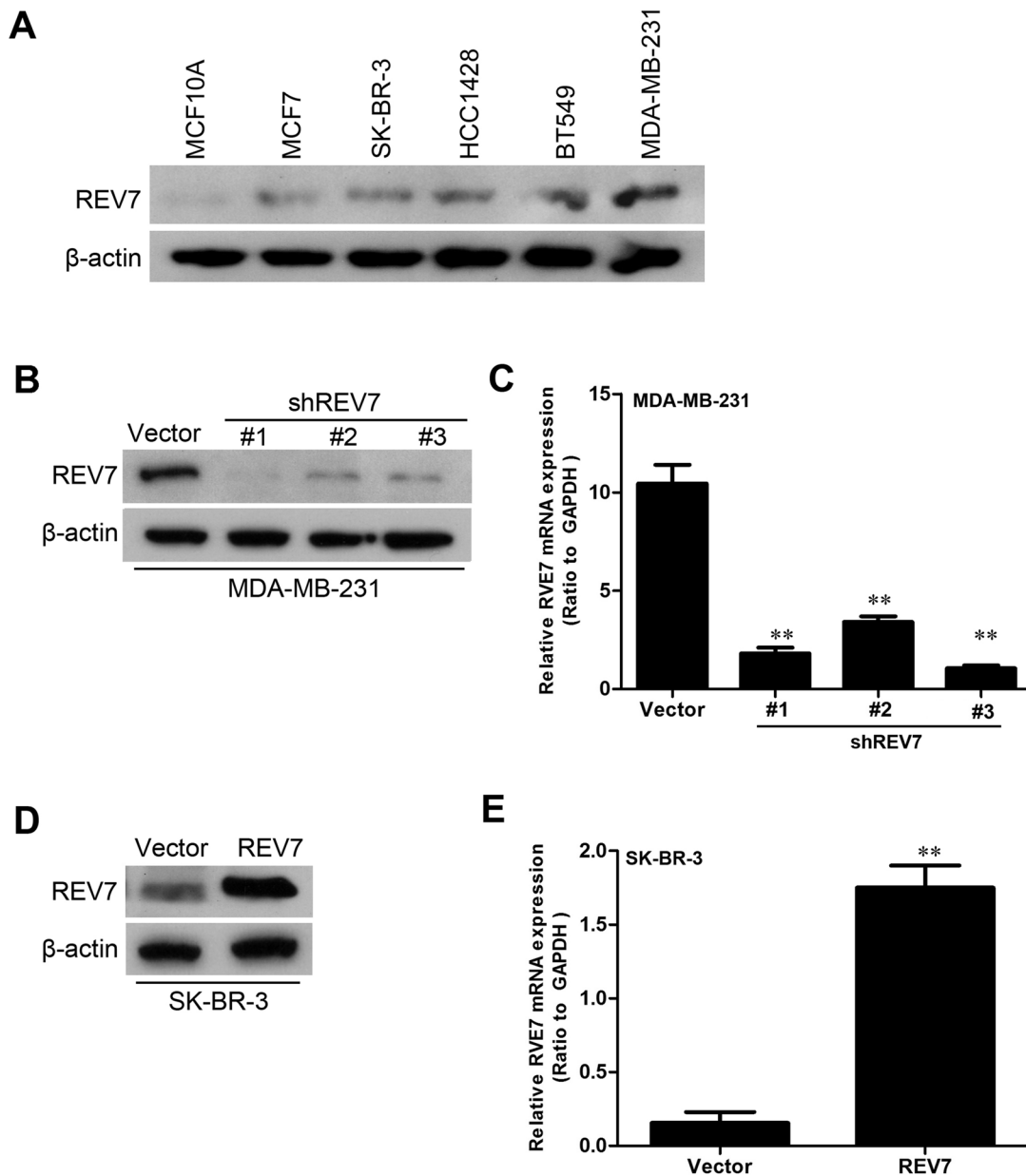
Figure 4. Stable cell lines of downregulated and upregulated expression of REV7 are constructed. (A) REV7 protein expression in six different breast cancer cell lines was detected by Western blot assay. (B) REV7 protein's low expression in the MDA-MB-231-shREV7 cell line constructed was verified by Western blot assay compared with the control cells. (C) REV7 mRNA's low expression in the MDA-MB-231-shREV7 cell line constructed was verified by quantitative RT-PCR assay compared with the control cells. (D) REV7 protein's high expression in the SK-BR-3-REV7 cell line constructed was verified by Western blot assay compared with the control. (E) REV7 mRNA's high expression in the SK-BR-3-REV7 cell line constructed was verified by quantitative RT-PCR assay compared with the control. $**p < 0.001$ is based on Student's t-test. Error bars, SD.

was strongly and differentially affected by the down-regulated expression (Fig. 8A) or upregulated expression of REV7 (Fig. 8B). These results suggested that REV7 may regulate breast cancer's functions through TGF-β1.

To further determine the correlation between REV7 and TGF-β1, direct interference of TGF-β1 in the SK-BR-3-REV7 cell line was done. As we can see, the expression

of TGF-β1 expression was downregulated after interference of TGF-β1 in the SK-BR-3-REV7 cell lines (Fig. 9A). EMT progression was detected by Western blot between the SK-BR-3-REV7 cell line with successful interference of TGF-β1 and its control with interference of Ctrl. Similarly, we used Western blot to examine four proteins (E-cadherin, α-cadherin, N-catenin, and vimentin), which
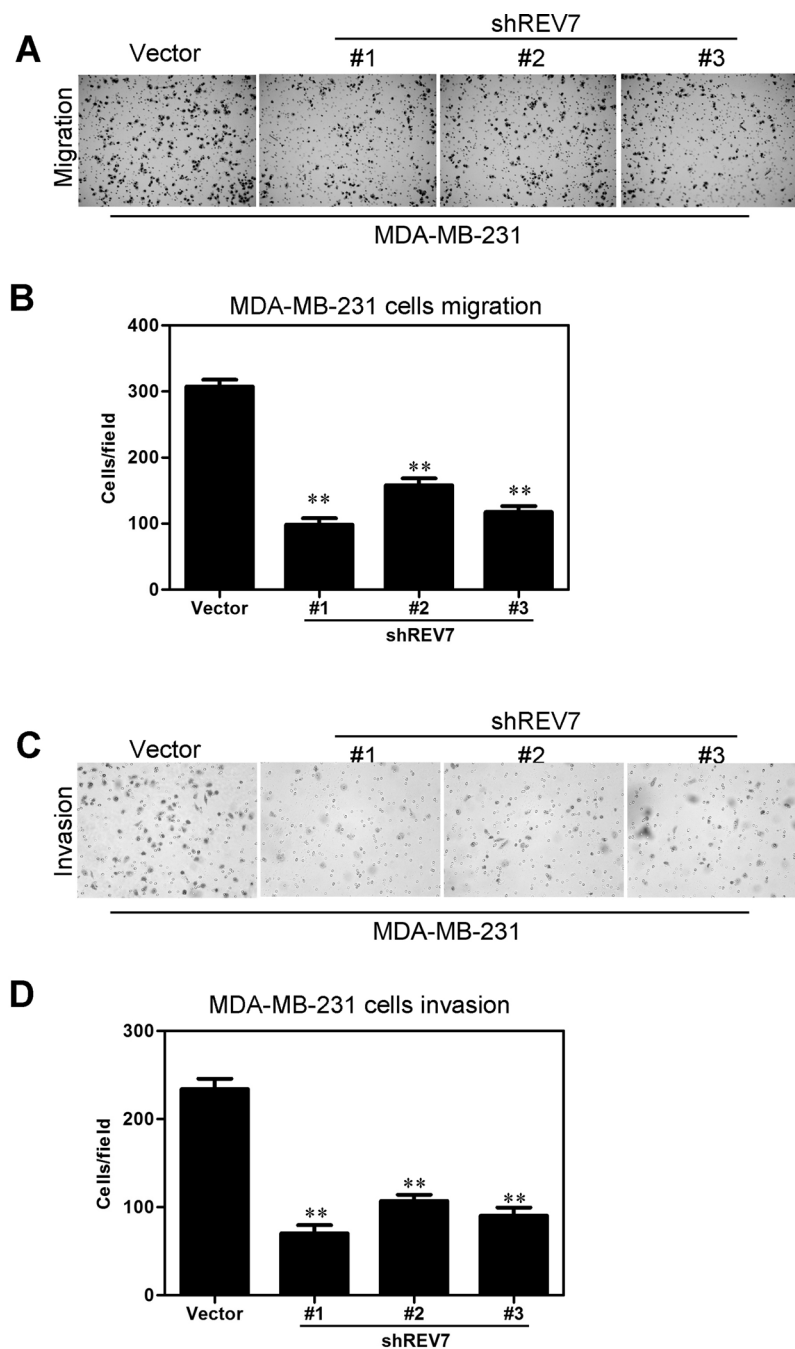
Figure 5. Knockdown of REV7 inhibits breast cancer cells' migration and invasion in vitro. (A) MDA-MB-231-shREV7 and its control cells were subjected to Transwell migration. (B) Quantification of migrated cells through the membrane is shown as proportions of their vector controls in a column diagram for (A). (C) MDA-MB-231-shREV7 and its control cells were subjected to Matrigel invasion assay. (D) Quantification of invaded cells through Matrigel is shown as proportions of their vector controls in a column diagram for (C). $**p < 0.001$ is based on Student's t-test. Error bars, SD.

are involved in EMT progression. Results suggested that interference of TGF-β1 in the SK-BR-3-REV7 cell line upregulated the expression of E-cadherin and α-cadherin, while it downregulated the expression of N-cadherin and vimentin compared with the control (Fig. 9B).

Transwell migration and Matrigel invasion assays were also performed on the SK-BR-3-vector-REV7 cell line with successful interference of TGF-β1 and its control with interference of Ctrl to determine whether interference of TGF-β1 would influence the cell capacity of migration and
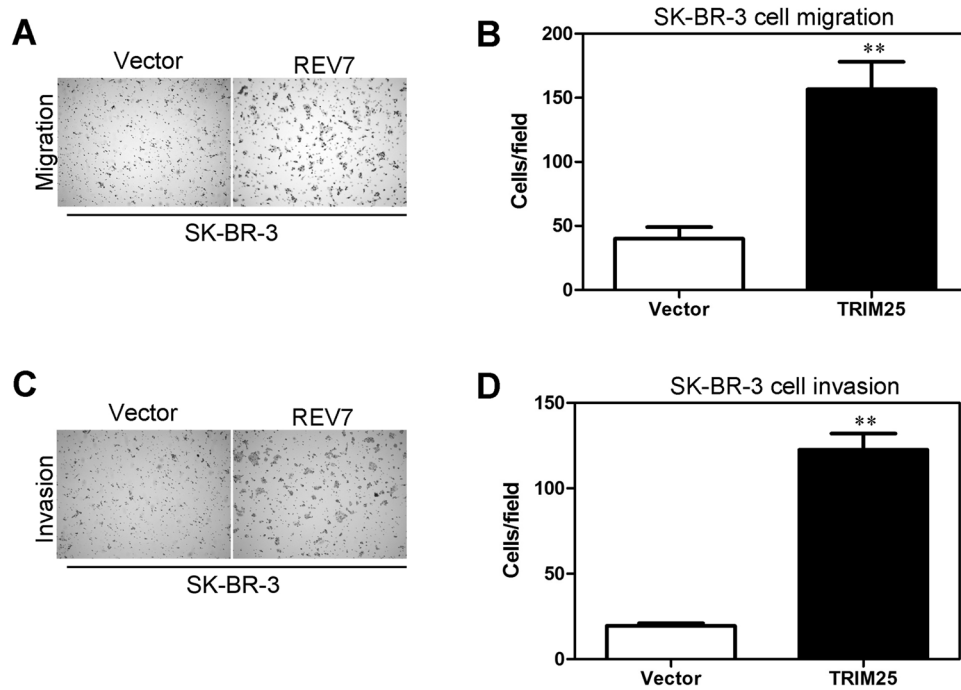
Figure 6. Overexpression of REV7 promotes breast cancer cells' migration and invasion in vitro. (A) SK-BR-3-REV7 and its control cells were subjected to Transwell migration. (B) Quantification of migrated cells through the membrane is shown as proportions of their vector controls in a column diagram for (A). (C) SK-BR-3-REV7 and its control cells were subjected to Matrigel invasion assay. (D) Quantification of invaded cells through Matrigel is shown as proportions of their vector controls in a column diagram for (C). **$p < 0.001$ is based on Student's t-test. Error bars, SD.
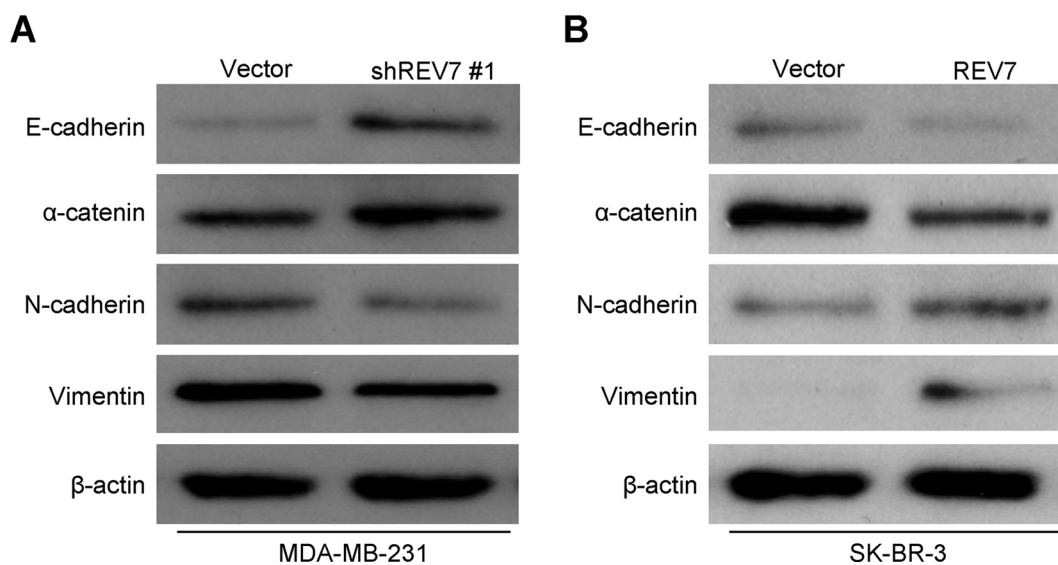
Figure 7. REV7 modulates breast cancer cells EMT in vitro. (A) Western blot assay was applied to detect expression of four proteins (E-cadherin, α-catenin, N-cadherin, and vimentin), which are related to EMT between the MDA-MB-231-shREV7 cell line and its control. (B) Western blot assay was applied to detect expression of four proteins (E-cadherin, α-catenin, N-cadherin, and vimentin), which are related to EMT between SK-RB-3-REV7 cell line and its control.
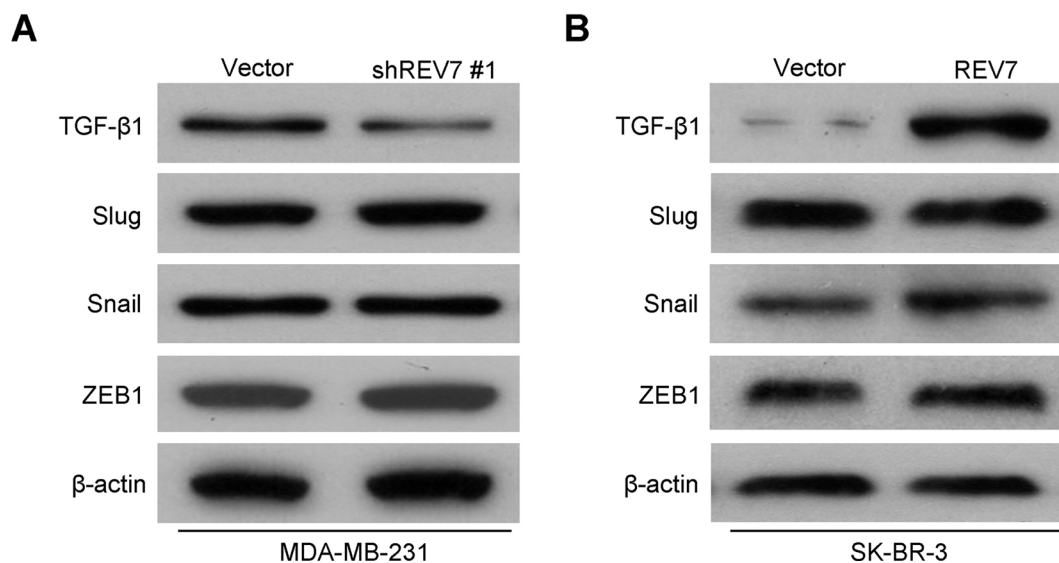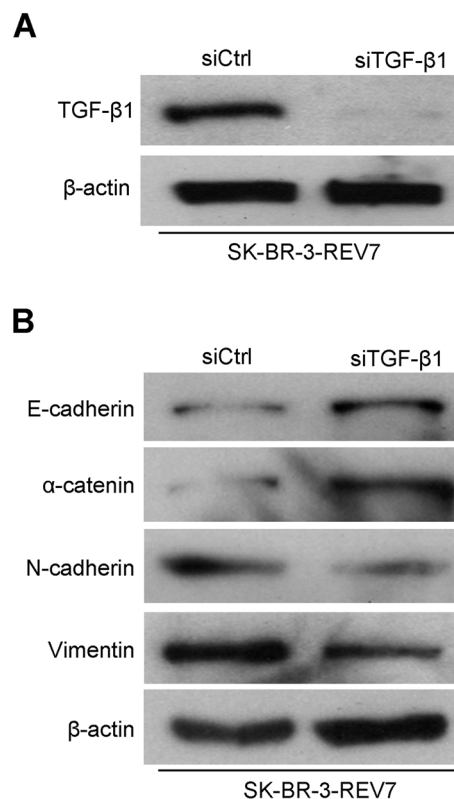
Figure 8. REV7 regulates the expression of TGF-β1. (A) Expression of signal proteins (TGF-β1, Slug, Snail, and ZEB1) was detected by Western blot assay between the MDA-MB-231-shREV7 cell line and its control. (B) Expression of signal proteins (TGF-β1, Slug, Snail, and ZEB1) was detected by Western blot assay between the SK-BR-3-REV7 cell line and its control.

invasion. The results showed that SK-BR-3-REV7 cells' migratory activity decreased more significantly than that of control cells after successful interference of TGF-β1 (Fig. 10A). The results were shown more intuitively using a statistical column diagram (Fig. 10B). As shown in a Matrigel invasion assay, SK-BR-3-REV7 cells exhibited less ability for invasion than that of control cells after successful interference of TGF-β1 (Fig. 10A). Similarly, the statistical column diagram showed this more intuitively (Fig. 10B).

DISCUSSION

In this study, we characterized the role of REV7 in breast cancer. Compared to the normal breast tissues, we detected increased levels of REV7 mRNA and protein in a majority of the breast cancer tissues that were examined. We also found that knockdown of REV7 inhibited cell migration and invasion as well as accelerated the EMT progression. Similarly, overexpression of REV7 in breast cancer cells was found to significantly promote tumor cell migration, invasion, and EMT progression. As to mechanisms, it was found that REV7 affects the expression of TGF-β1 in breast cancer cells.

TLS is an important damage tolerance system that rescues cells from severe injury caused by DNA damage (11). This system functions depending on the kind of low-fidelity DNA polymerases, including DNA Polζ, which was first found in *Saccharomyces cerevisiae* (12). Polζ, which consists of Rev3 and Rev7 proteins, plays an important role in eucaryon (13). REV7 was originally identified as a gene that complements the reversionless phenotypes of yeast *S. cerevisiae* mutant strains (14).

Figure 9. REV7 regulates breast cancer cells' function through modifying TGF-β1. (A) TGF-β1 protein expression was downregulated in the SK-BR-3-REV7 cell line after interference of TGF-β1 through Western blot assay. (B) The expression of proteins related to EMT progression was downregulated in the SK-BR-3-REV7 cell line after interference of TGF-β1 through Western blot assay.
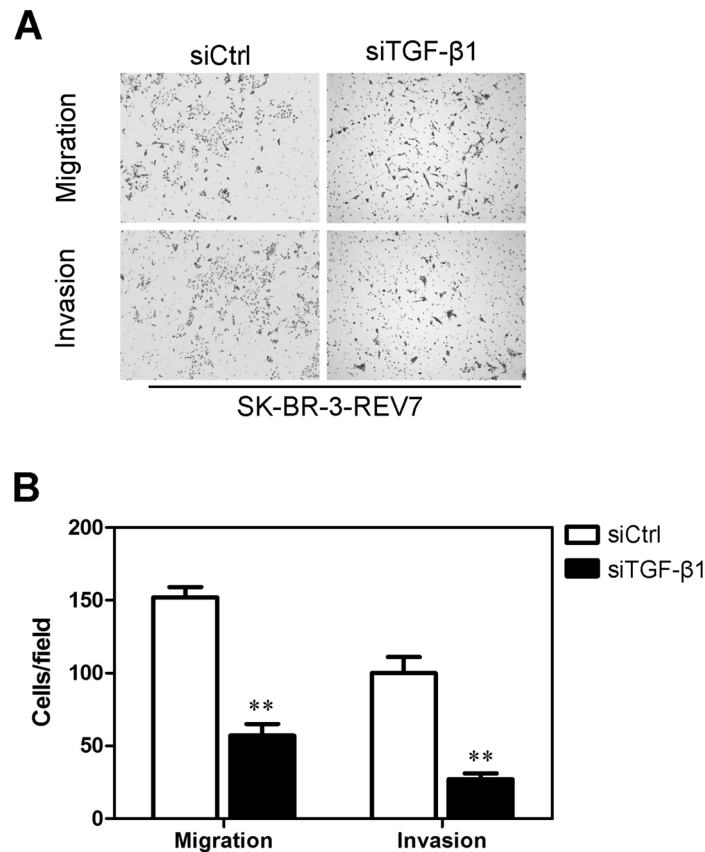
Figure 10. Interference of TGF-β1 inhibited migration and invasion in the SK-BR-3-REV7 cell lines. (A) Transwell migration and Matrigel invasion assays were performed in SK-BR-3-REV7 after interference of TGF-β1. (B) The results from Transwell migration and Matrigel invasion assays of SK-BR-3-REV7 after interference of TGF-β1 were summarized by statistical software. $**p < 0.001$ is based on Student's t-test. Error bars, SD.

Human REV7 was identified as a binding partner of human REV3 in the two-hybrid screening using human REV3 as bait (15). The REV7 gene encodes a protein of 245 amino acid residues with a molecular mass of 29 kDa (8). The amino acid sequence of human REV7 displays 23% identity and 53% similarity with the yeast Rev7 protein, and also it displays 23% identity and 54% similarity with the human spindle assembly checkpoint protein MAD2 (8). On the basis of its sequence similarity with the MAD2 protein, human REV7 is also called MAD2B or MAD2L2 (5). It has a HORMA (for Hop1, Rev7, and Mad2) domain just as yeast Rev7. This domain is also present in the eukaryotic spindle assembly checkpoint protein Mad2, and *S. cerevisiae* Hop1, a protein involved in meiotic–synaptonemal complex assembly (7). The HORMA domain is suggested to be responsible for protein–protein interaction and oligomerization, and to recognize chromatin status (16).

The REV7 gene was found to be associated with immunologic response, regulation and control of mitotic cycle, and tumor cell drug resistance. Nevertheless, the

function and mechanism that REV7 plays in breast cancer are still unknown to scientists. We detected the protein expression of REV7 in breast cancer and then investigated the relationships between REV7 expression and the clinicopathologic characteristics of patients with breast cancer. Our results showed that REV7 was more highly expressed in breast cancer tissues than in normal breast tissues, and highly expressed in a majority of the high-grade breast cancer tissues examined.

Next, we found that overexpression of REV7 promoted breast cancer cells' ability for migration and invasion as well as EMT progress, while knockdown of REV7 induced an inhibitory effect on migration and invasion as well as EMT progress. There were four proteins (E-cadherin, α-catenin, N-cadherin, and vimentin) involved in the EMT progress that were examined as an index to recognize EMT. EMT happens gradually in the process of tumor development and is extremely complex. As a feature of aggressive tumors, EMT is characterized by reduced E-cadherin and α-catenin, and increased N-cadherin and vimentin expression. E-cadherin plays an

important role in sustaining completeness and polarity of epithelial cells' structure and shape. As has been shown in many research studies, downregulation of E-cadherin protein expression is in a close relationship with epithelial cells. Meanwhile, cells that show EMT progression were usually accompanied by upregulated expression of protein originating from mesenchymal cells, especially vimentin. In contrast with E-cadherin, N-cadherin protein's upregulated expression was found to be positive in EMT. α-Catenin can modify cell adhesion activity, whose downregulated expression could lead to E-cadherin's deprivation of function, and contact inhibition among epithelial cells immediately followed. Thus, α-catenin protein's downregulated expression has a positive correlation with EMT progression.

To investigate the mechanism of REV7 modifying the development of cancer, Western blot assay was carried out. We identified TGF-β1 as an effective mediator of these REV7-induced phenomena. The secreted factor TGF-β1 has been proposed to play an important role in epithelial carcinogenesis, since it is known to be both a potent negative epithelial cell growth regulator and a targeted expression of TGF-β1. In cell lines whose REV7 was knocked down, the expression level of TGF-β1 decreased significantly when compared with the control empty vector plasmid cells, whereas overexpression of REV7 increased the TGF-β1 expression. Via constructing interference of TGF-β1 in SK-BR-3-vector-REV7 cell line, in which REV7 was knocked down, we found that interference of TGF-β1 in the SK-BR-3-vector-REV7 cell line upregulated the expression of E-cadherin and α-cadherin, while it downregulated the expression of N-cadherin and vimentin when compared with the control. Similarly, SK-BR-3-vector-REV7 cells' migratory activity decreased more significantly than that of control cells after successful interference of TGF-β1. Therefore, we further identify that REV7 regulates oncogenic activities through TGF-β1.

In conclusion, we have demonstrated that REV7 is expressed at a high level in breast cancer tissues, and the knockdown of REV7 inhibited breast cancer cells' ability for migration, invasion, and EMT. Therefore, these findings may shed light on potential targets in breast cancer prevention and therapy.

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