Downregulation of MicroRNA-449 Promotes Migration and Invasion of Breast Cancer Cells by Targeting Tumor Protein D52 (TPD52)

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Our study aimed to investigate whether microRNA-449 (miR-449) plays a key role in regulating the migration and invasion of breast cancer cells via targeting tumor protein D52 (TPD52). The results of the qRT-PCR and Western blotting showed that, in comparison with normal breast tissues and cells, miR-449 was significantly downregulated in breast cancer tissues and cells, while TPD52 was markedly upregulated. After transfection with an miR-449 inhibitor, suppression of miR-449 significantly promoted cell migration and invasion. Also, when miR-449 was overexpressed by transfection with miR-449 mimics, E-cadherin expression significantly increased, and the expression of N-cadherin and vimentin were markedly decreased, whereas the opposite effects were obtained when miR-449 was suppressed by transfection with an miR-449 inhibitor. TPD52 was also confirmed as the direct target of miR-449 via luciferase reporter analysis. Knockdown of TPD52 significantly alleviated the effects of miR-449 overexpression on cell migration and invasion, as well as the expression of E-cadherin, N-cadherin, and vimentin. Our results indicate that downregulation of miR-449 may promote the migration and invasion of breast cancer cells by targeting TPD52. miR-449 may serve as a potential target in the therapy of breast cancer.

**Key words: Breast cancer; MicroRNA-449 (miR-449); Cell proliferation; Cell migration;
Cell invasion; Tumor protein D52 (TPD52)**

INTRODUCTION

Breast cancer is the most common malignant cancer in women[1,2]. Metastatic breast cancer is currently untreatable with primary surgery[3]; thus, it is considered to be incurable. Therefore, a better understanding of the molecular mechanisms controlling the metastasis of breast cancer has great significance for the development of a promising therapeutic approach.

MicroRNAs (miRNAs), endogenous short noncoding RNAs, can bind to the coding or untranslated regions of mRNAs, thus regulating their target genes negatively[4]. The dysregulation of miRNAs is widely found in the progression of breast cancer[5,6]. In a previous study, miR-10b was shown to play a key role in initiating tumor migration and invasion in breast cancer[7]. It was also reported that the levels of circulating miR-10b and miR-373 are strongly associated with lymph node metastasis in breast cancer[8]. Moreover, miR-31 was found to regulate the expression of key metastatic genes associated with breast cancer, such as radixin, integrin α5, and RhoA[9]. miR-139-5p has also

been verified to be a key regulator in metastatic pathways in breast cancer[10]. Therefore, identification of key miRNAs associated with the metastasis of breast cancer has aroused more and more interest. Recently, miR-449 has been identified in the progression of several cancers. For instance, miR-449 can suppress cell proliferation in hepatocellular carcinoma (HCC)[11]. miR-449 can also inhibit cell migration and invasion in non-small cell lung cancer (NSCLC)[12]. In breast cancer, miR-449a has been found to be a potent player in regulating the viability of breast cancer cells[13]. However, the role of miR-449 in regulating the migration and invasion of breast cancer cells has not been fully investigated.

It is reported that the progression of primary tumors to metastatic cancer depends on the migration and invasion capacities of the cells[14]. In the present study, we detected whether miR-449 was dysregulated in breast cancer tissues and cells. We further investigated whether overexpression and suppression of miR-449 changed the migration and invasion capacities of breast cancer cells

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and explored the possible regulatory mechanism of miR-449. Our findings are expected to provide a theoretical basis and new insight for the treatment of breast cancer.

MATERIALS AND METHODS

Patients and Samples

From September 2015 to March 2016, 49 patients who were diagnosed with breast cancer in our hospital were enrolled in this study. Each patient gave informed consent before participation, and the procedures were approved by the human ethics committee of Shanxi Provincial People's Hospital. The diagnosis of breast cancer was pathologically confirmed in accordance with the classification of the World Health Organization. Cancer tissues and their adjacent normal tissues were obtained, snap frozen with liquid nitrogen, and stored at −80°C for subsequent experiments.

Cell Culture and Transfection

Human breast epithelial MCF-10 and breast cancer MDA-MB-231 cell lines were obtained from the European Collection of cell cultures (Wiltshire, UK). These cells were cultured in the DMEM (Dulbecco's modified Eagle's medium; Gibco, USA) containing 10% FBS (fetal bovine serum), 100 U/ml penicillin, and streptomycin (Gibco) at 37°C in an incubator with 5% CO_2. Cells (5×10^5) were seeded in six-well plates and continued to culture for 24 h before transfection. The miR-449 mimics, miR-449 inhibitor, negative scramble RNA, and TPD52-siRNA, which were synthesized by Sangon Biotech (Shanghai, P.R. China), were transfected into cells by Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. After transfection, the expression of miR-449 was determined by quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

RNA Extraction and qRT-PCR Analysis

Total RNA from tissues and cells was extracted with TRIzol reagent (Invitrogen) as per the manufacturer's protocols. The concentration and purity of isolated RNA were assessed using SMA 400 UV–VIS (260/280 ratio: 1.8–2.0; Merinton, Shanghai, P.R. China). cDNA synthesis was then performed using a miScript II reverse transcription kit (Qiagen, Valencia, CA, USA). miR-449 expression was detected using an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA), and TPD52 mRNA expression was determined using a SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA). Melting curve analysis was conducted at the end of each PCR to confirm that only one product was amplified. The relative expression of miR-449 and TPD52 mRNA was respectively normalized to U6 and GAPDH and was then calculated using the $2^{-\Delta\Delta CT}$ method as previously described[15]. The forward primer for miR-449 was 5′-TGGCAGTGTATTGTTAGCTGGT-3′, and for U6 was 5′-CGCAAGGATGACACGCAAATTC-3′. The primers for TPD5, E-cadherin, N-cadherin, vimentin, and GAPDH are displayed in Table 1.

MTT Assay

Cell viability was assessed using an MTT assay according to previously described methods. Cells (5×10^3) in different transfected groups were cultured and placed into 96-well plates. After being cultured for 24 h, the supernatant was removed by centrifugation, and 20 μl of MTT was added into each well to culture cells for another 4 h. Dimethyl sulfoxide (DMSO) (150 μl) was then added to mix with the cells for 10 min. Consequently, the absorbance at 570 nm was observed under an absorption spectrophotometer (Olympus, Tokyo, Japan) to assess the cell viability of each well.

Colony Assay

Colony assay was also performed to detect the ability of cell proliferation. In brief, after cell transfection, cells (100 cells/dish) were plated into culture dishes containing RPMI-1640 medium supplemented with 10% FBS and incubated for 14 days. Cells were then fixed and stained with Diff-Quick. After air drying, colonies were counted under a microscope (IX83; Olympus).

Transwell Assay

Cell invasion and migration were assessed by Transwell assay. For the invasion assay, the upper layer of the Transwell chamber (8-μm pore size; Corning, Corning,

Table 1. Primers Used for Target Amplification

Name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
TPD52	GGAAGAGGAGCAGGAAGAGC	GATGACTGAGCCAACAGACG
E-cadherin	AACGCATTGCCACATACAC	AACGCATTGCCACATACAC
N-cadherin	AACTCCAGGGGACCTTTTC	CAAATGAAACCGGGCTATC
Vimentin	TCCAAGTTGCTGACCTCTC	TCAACGGCAAAGTTCTCTTC
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

NY, USA) was enveloped with serum-free DMEM containing 50 mg/L Matrigel, while the upper layer of the Transwell chamber remained without Matrigel for the migration assay. Briefly, 48 h after transfection, cells (5×10^4) were seeded in the upper chamber of the Transwell chamber and incubated in serum-free medium for 24 h. Then the lower Transwell chamber was filled with DMEM mixed with 10% FBS as a chemoattractant. After 48 h, the Transwell chamber in each group was washed with PBS buffer and fixed in ice-cold alcohol. Cells were then stained with 0.1% crystal violet for 30 min and decolorated with 33% acetic acid, and the number

of migrated and invaded cells was respectively counted using light microscopy (Leica, Wetzlar, Germany). Each experiment was performed in triplicate.

Luciferase Reporter Analysis

Vectors of TPD52-3′-UTR were synthesized by Sangon Biotech. The dual luciferase reporter plasmids TPD52-WT and TPD52-Mut (containing the wild-type and mutant TPD52 putative 3′-UTR-binding site, respectively) were constructed. Forty-eight hours after transfection, luciferase activity was then measured using the dual-luciferase reporter assay system (Promega, Madison,
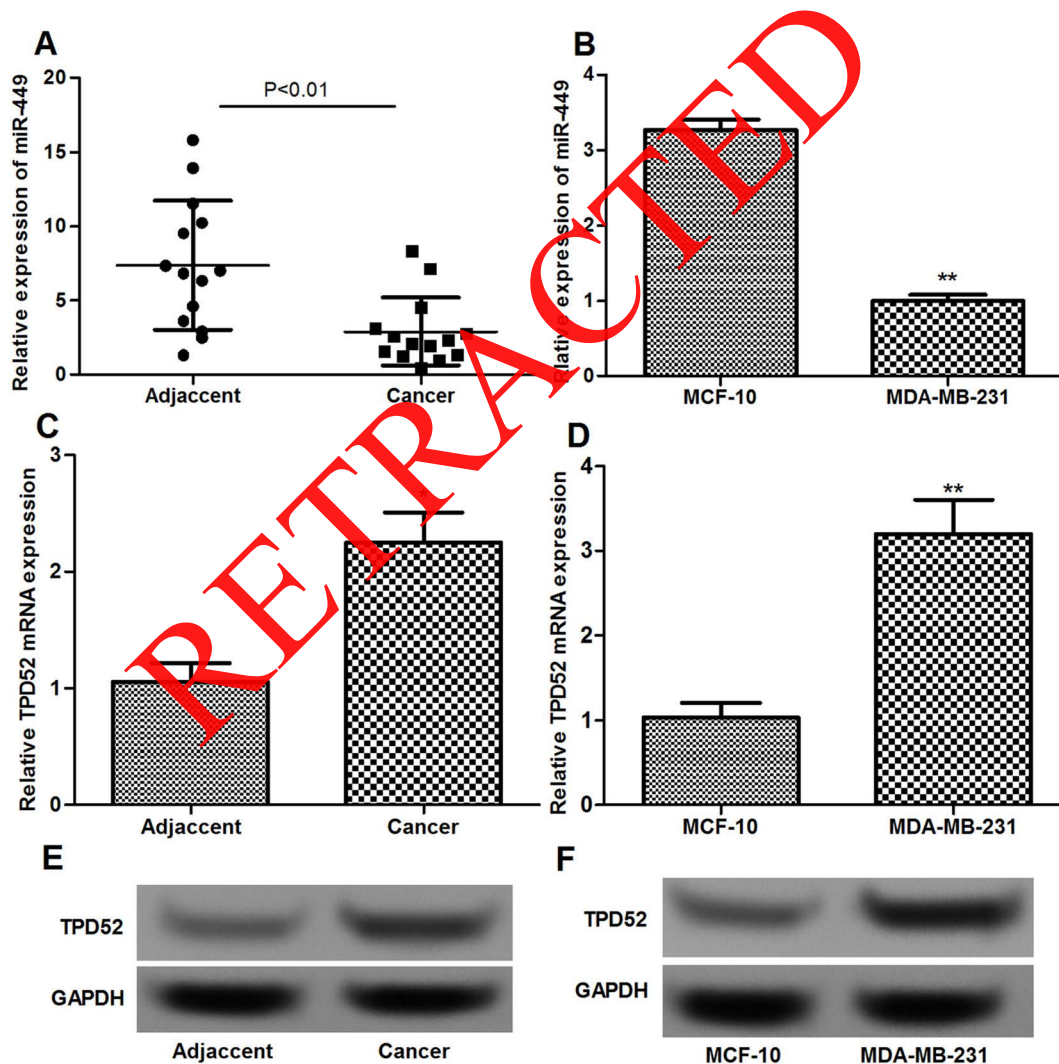
Figure 1. qRT-PCR and Western blotting show the expression of miR-449 and TPD52 in breast cancer tissues and cells. (A) qRT-PCR shows the expression levels of miR-449 in breast cancer tissues and normal adjacent tissues. (B) qRT-PCR shows the expression levels of miR-449 in MDA-MB-231 cells and MCF-10 cells. (C) qRT-PCR shows the expression levels of TPD52 in breast cancer tissues and normal adjacent tissues. (D) qRT-PCR shows the expression levels of TPD52 in MDA-MB-231 cells and MCF-10 cells. (E) Western blotting shows the expression levels of TPD52 in breast cancer tissues and normal adjacent tissues. (F) Western blotting shows the expression levels of TPD52 in MDA-MB-231 cells and MCF-10 cells. Error bars indicate means±SD. *$p < 0.05$, **$p < 0.01$.

WI, USA). The relative reporter activity was normalized to r-luc activity.

Western Blotting

Cells were lysed with RIPA lysate (radioimmuno-precipitation; Sangon Biotech) for 10 min at 4°C. Protein supernatants were collected, and their concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples (50 μg per lane) were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes. Primary antibodies (1:1,000 for TPD52, E-cadherin, N-cadherin, and vimentin, and 1:5,000 for GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to incubate the members at 4°C overnight, followed by appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology). The immunoreactive protein bands were developed by enhanced chemiluminescence and then analyzed with a densitometer.

Statistical Analysis

All data in our study were presented as mean±SD. The difference between two groups was calculated using independent sample *t*-test in GraphPad prism 5.0 software (GraphPad Prism, San Diego, CA, USA). The differences for more than three groups were detected using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was defined as statistically significant.

RESULTS

Inverse Expression of miR-449 and TPD52 in Breast Cancer Tissues and Cells

The expression levels of miR-449 and TPD52 mRNA in breast cancer tissues and cells were determined using qRT-PCR and Western blotting. miR-449 expression in breast cancer tissues and MDA-MB-231 cells was significantly lower than that in adjacent normal tissues and epithelial MCF-10 cells ($p < 0.01$) (Fig. 1A and B).
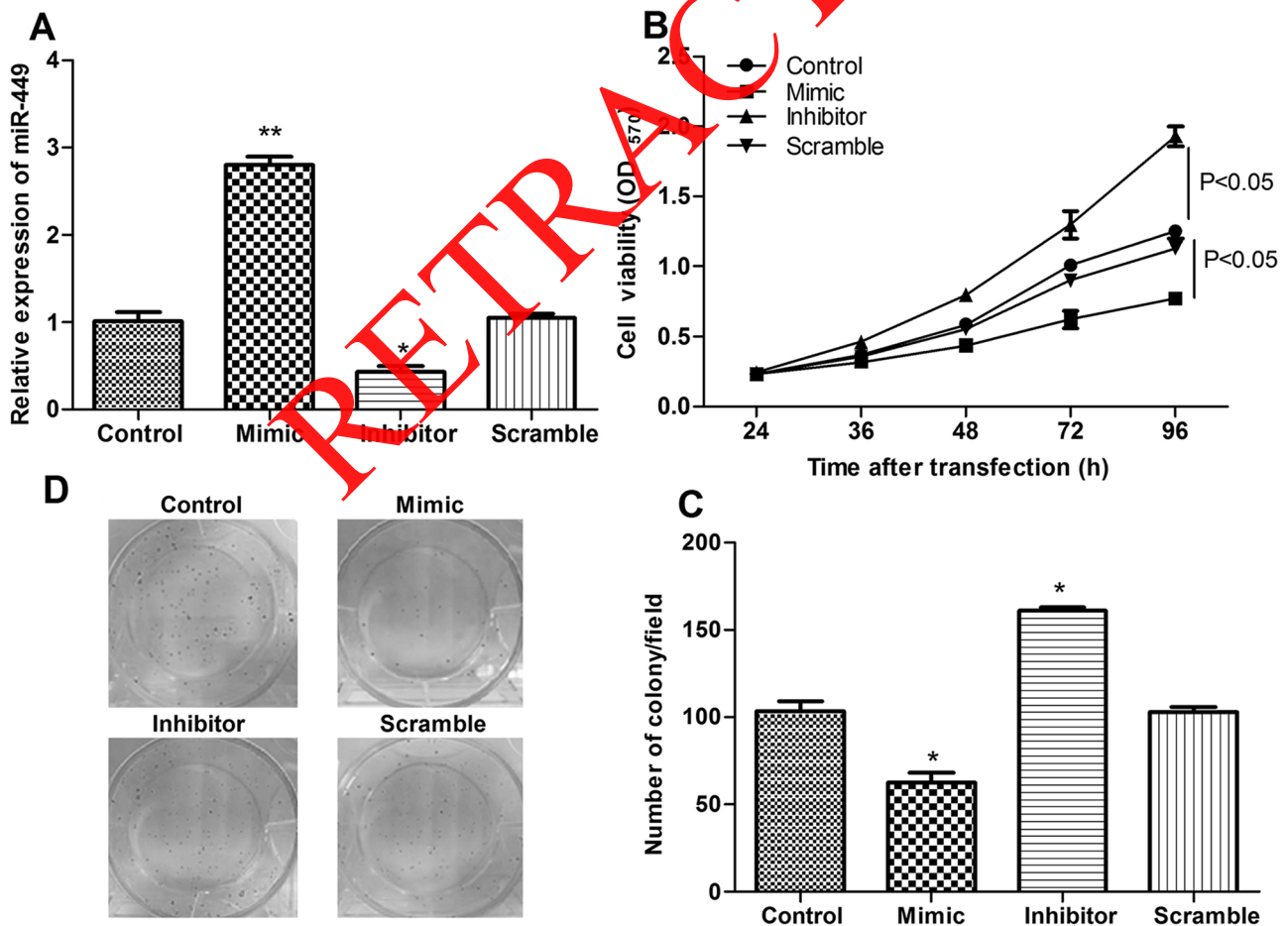
Figure 2. The effects of miR-449 on cell proliferation. (A) qRT-PCR shows the expression levels of miR-449 in different transfected groups. (B) MTT assay shows the role of miR-449 in cell proliferation. (C, D) Colony assay shows the role of miR-449 in cell proliferation. Error bars indicate means±SD. *$p < 0.05$, **$p < 0.01$.

In addition, mRNA and protein expression levels of TPD52 in breast cancer tissues and cells were significantly higher than that in normal breast tissues and cells ($p < 0.05$) (Fig. 1C–F).

Suppression of miR-449 Promoted Cell Proliferation

To further explore the relationship of miR-449 and breast cancer, miR-449 was successfully overexpressed and knocked down in breast cancer cells (Fig. 2A). Moreover, the role of miR-449 in cell proliferation was further assayed by MTT assay and colony assay. In comparison with the control group, miR-449 overexpression significantly inhibited cell viability, while suppression of miR-449 had the opposite effect ($p < 0.05$) (Fig. 2B). Similar results were obtained in the colony assay in that the number of colonies in the miR-449 mimic group decreased significantly compared with that in the miR-449 scramble or control groups while being markedly increased in the miR-449 inhibitor group ($p < 0.05$) (Fig. 2C and D).
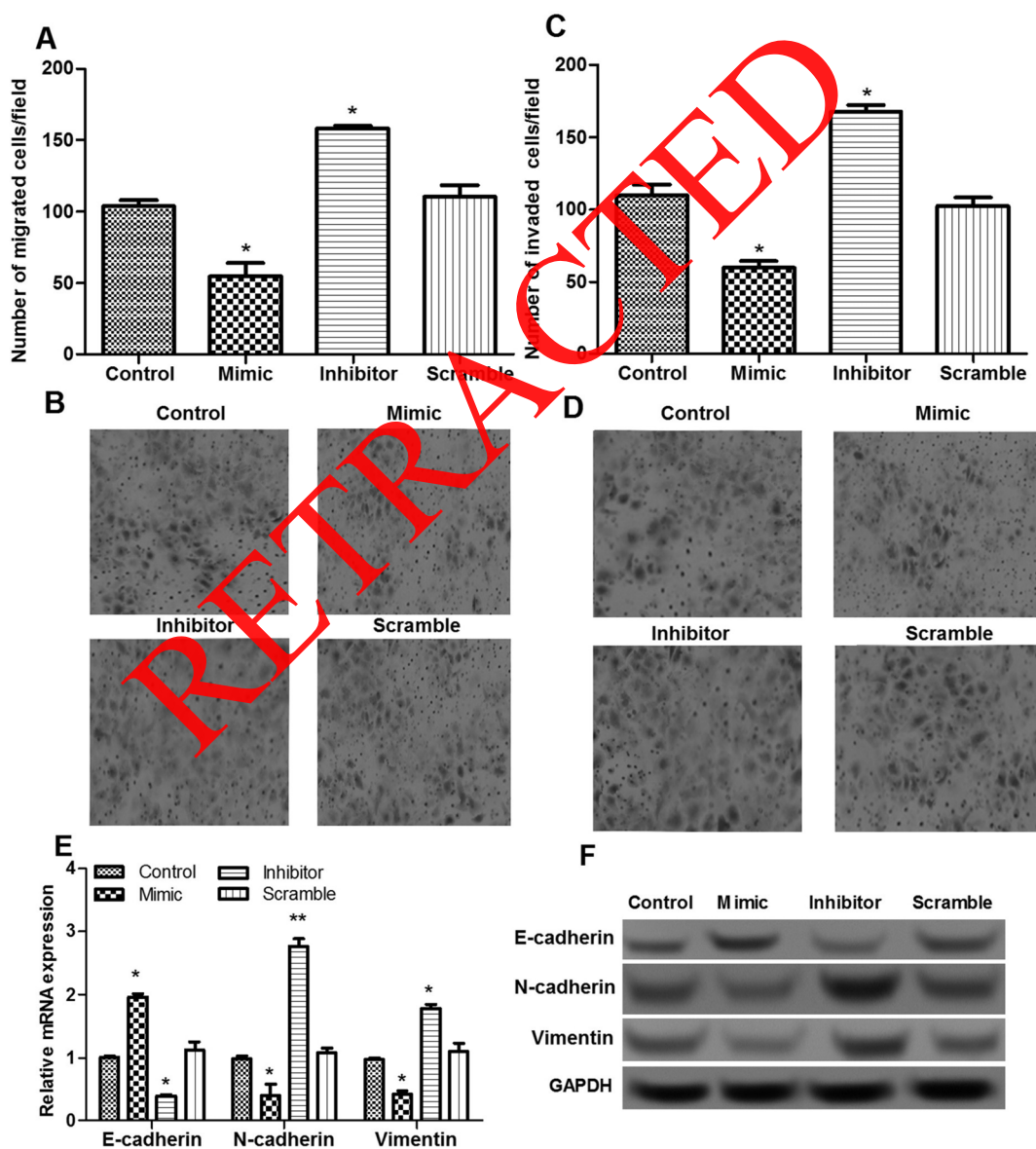
Figure 3. The effects of miR-449 on cell migration and invasion. (A, B) Transwell assay shows the number of migrated cells of different transfected groups. (C, D) Transwell assay shows the number of invaded breast cancer cells of different transfected groups. (E) qRT-PCR shows the expressions of N-cadherin, E-cadherin, and vimentin. (F) Western blotting shows the expressions of N-cadherin, E-cadherin, and vimentin. Error bars indicate means ± SD. *$p < 0.05$.

*Suppression of miR-449 Promoted Cell Migration and Invasion Possibly via Regulating EMT-Related Proteins*

The role of miR-449 in the migration and invasion of breast cancer cells was also evaluated using a Transwell assay. The results showed that the number of migrated cells in the miR-449 mimic group decreased significantly in comparison with that in the scramble and control groups, while being markedly increased in the miR-449 inhibitor group ($p<0.05$) (Fig. 3A and B). Moreover, the number of invaded breast cancer cells had similar results after miR-449 overexpression and suppression ($p<0.05$) (Fig. 3C and D). To further investigate the possible mechanisms of miR-449 on cell invasion and migration, the expressions of epithelial-to-mesenchymal transition (EMT)-related proteins, including N-cadherin, E-cadherin, and vimentin, were measured. The results showed that when miR-449 was overexpressed, E-cadherin expressions significantly increased, and the expressions of N-cadherin and vimentin were markedly decreased when compared to the other two groups, whereas the opposite effect was obtained when miR-449 was suppressed ($p<0.05$) (Fig. 3E and F).

TPD52 Was the Direct Target of miR-449

The predicted sequences of TPD52 regulated by miR-449 were obtained (Fig. 4A). Moreover, we used luciferase reporter analysis to verify whether TPD52 was the target of miR-449. The results showed that the relative luciferase activities containing the wild-type 3′-UTR of TPD52 were significantly decreased in the miR-449 mimic-transfected cells compared to the control cells ($p<0.05$) (Fig. 4B). However, mutation of the predicted binding site of miR-449 on the TPD52 3′-UTR rescued the decreased luciferase activity ($p<0.05$) (Fig. 4B). Moreover, TPD52 expression in the miR-449 mimic group was significantly decreased compared with the scramble and control groups, while being obviously increased in the miR-449 inhibitor group ($p<0.05$) (Fig. 4C and D). These findings indicated that TPD52 was the direct target of miR-449.

Suppression of miR-449 Promoted Cell Migration and Invasion via Targeting TPD52

To further investigate whether miR-449 played a role in breast cancer cell migration and invasion via
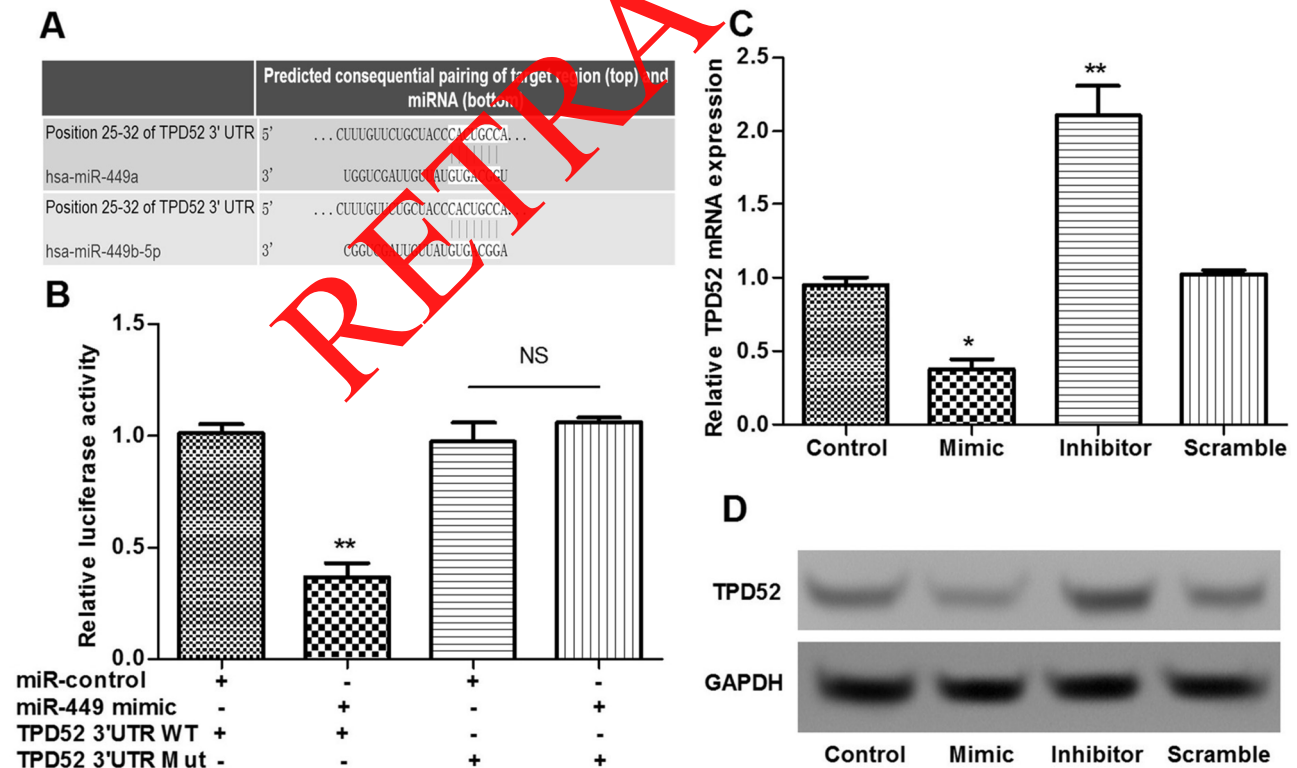
Figure 4. TPD52 was the direct target of miR-449. (A) The predicted sequences of TPD52 regulated by miR-449. (B) Luciferase reporter analysis shows that TPD52 was the target of miR-449. (C) qRT-PCR shows the TPD52 expression of different transfected groups. (D) Western blotting shows the TPD52 expression of different transfected groups. Error bars indicate means±SD. *$p<0.05$, **$p<0.01$.
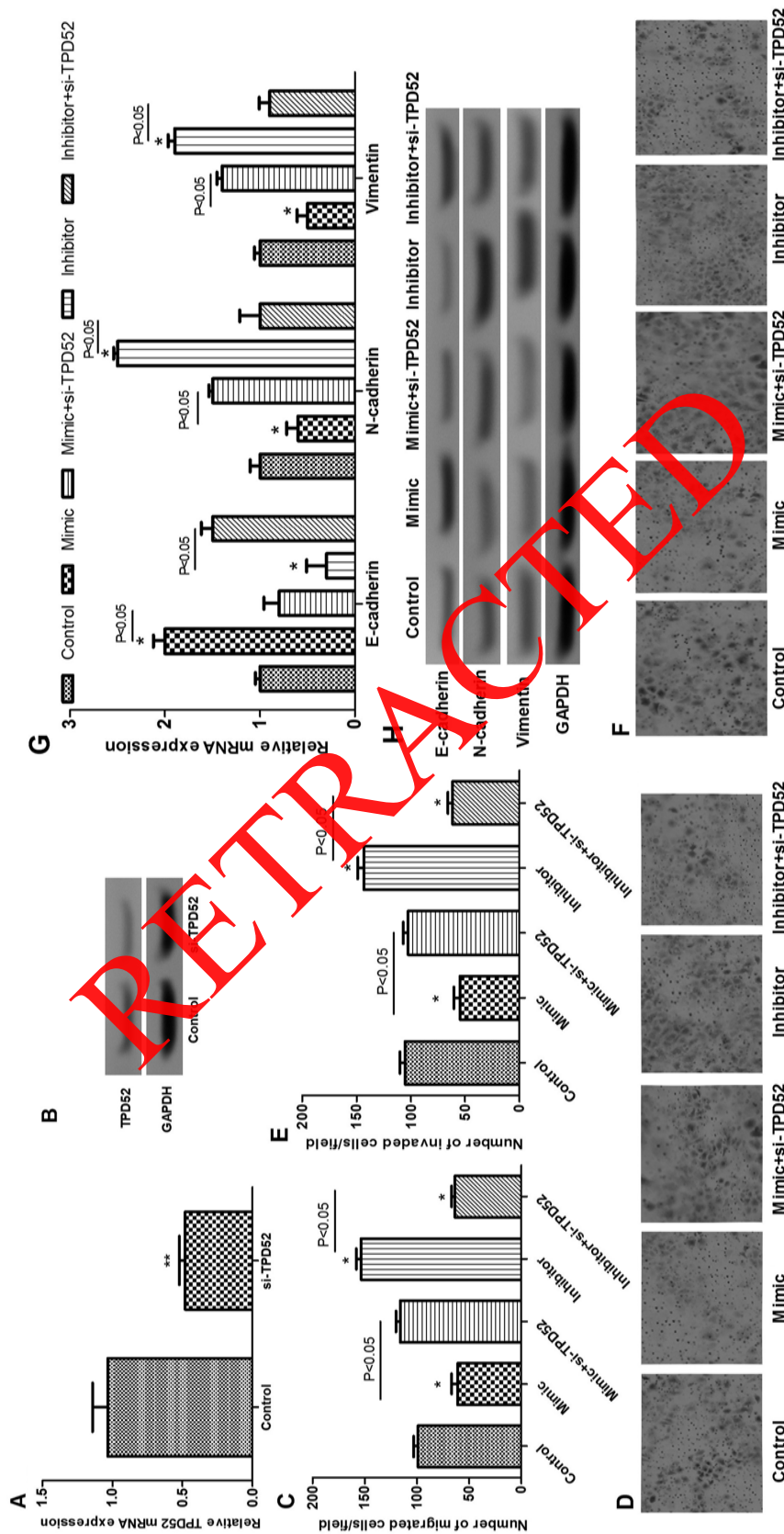
Figure 5. The effects of TPD52 knockdown on cell migration and invasion. (A) qRT-PCR shows TPD52 expression after si-TPD52 treatment. (B) Western blotting shows TPD52 expression after si-TPD52 treatment. (C, D) Transwell assay shows the number of migrated cells of different transfected groups. (E, F) Transwell assay shows the number of invaded breast cancer cells of different transfected groups. (G) qRT-PCR shows the expression of N-cadherin, E-cadherin, and vimentin. (H) Western blotting shows the expression of N-cadherin, E-cadherin, and vimentin. Error bars indicate means±SD. *$p < 0.05$, **$p < 0.01$.

targeting TPD52, TPD52 was knocked downed by si-TPD52 (Fig. 5A and B). In addition, the results showed that when cells were transfected with the miR-449 mimic and si-TPD52 simultaneously, the inhibitory effect of miR-449 overexpression on cell migration and invasion was rescued (Fig. 5C–F). Furthermore, knockdown of TPD52 significantly inhibited the increased expression of E-cadherin and the decreased expressions of N-cadherin and vimentin ($p < 0.05$) (Fig. 5G and H). These findings indicated that suppression of miR-449 promoted cell migration and invasion via targeting TPD52.

DISCUSSION

miRNAs have been implicated in a very wide range of biological and pathological processes, including tumorigenesis[16]. In the present study, we found that miR-449 was significantly downregulated in breast cancer tissues and cells. Suppression of miR-449 promoted cell migration and invasion. Also, suppression of miR-449 altered the expression of EMT-related proteins, including N-cadherin, E-cadherin, and vimentin. TPD52 was confirmed as the direct target of miR-449. Knockdown of TPD52 significantly alleviated the effects of miR-449 overexpression on cell migration and invasion, as well as the expressions of E-cadherin, N-cadherin, and vimentin. Our findings show the key roles and possible mechanism of miR-449 in regulating cell migration and invasion in breast cancer.

In previous studies, miR-499 has been suggested to be expressed at a low level in several cancer tissues and cell lines, including lung[17,18], gastric[19,20], and bladder cancers[21]. In our study, miR-449 expression in breast cancer tissues and cells was significantly lower than in normal breast tissues and cells. Thus, our results are in line with previous findings and suggest that downregulated miR-449 may contribute to the development of breast cancer.

Accumulating evidence has shown that metastasis formation in breast cancer is linked to the migratory and invasive phenotype of cancer cells[22]. Thus, miR-449 mimic and inhibitor were transfected into breast cancer cells in our study to detect the association between miR-449 and cell migration and invasion. The results confirmed that overexpression of miR-449 exerted an inhibitory effect on cell migration and invasion of breast cancer cells, while miR-449 suppression had the opposite effect. Consistent with those findings, miR-449 is reported to negatively regulate cell migration and invasion in NSCLC by targeting c-Met[12]. Our results showed that the expression of EMT-related proteins, including E-cadherin, N-cadherin, and vimentin, was dysregulated after miR-449 was overexpressed and then suppressed. EMT is a critical event in the progression toward cancer metastasis, in which EMT-related proteins play a key role[23]. In addition, aberrant expression of E-cadherin is shown to be correlated with the invasion and metastasis in various human cancers[24]. The reduced expression of E-cadherin plays a crucial role in the invasion and metastasis of human breast cancer[25]. N-cadherin is also reported to be associated with tumor metastasis in breast cancer[26]. Vimentin can also regulate EMT induction and migration in breast cancer[27]. Taken together, our results are consistent with previous findings and indicate that miR-449 may regulate cell migration and invasion in breast cancer, possibly via regulating EMT-related proteins.

Furthermore, TPD52 was confirmed to be the direct target of miR-449. TPD52 was originally identified to be overexpressed in human breast carcinoma nearly 20 years ago[28]. In addition, TPD52 is found to be frequency overexpressed and present as a gene amplification target at chromosome 8q21 in a variety of human cancers[29], such as ovarian cancer[30] and breast cancer[31]. Altered expression of TPD52 is also shown to regulate the migration and apoptosis of prostate cancer cells[32]. In addition, miR-22 is confirmed to inhibit cancer cell migration and invasion in prostate cancer by targeting TPD52[33]. In our study, when cells were transfected with the miR-449 mimic and si-TPD52 simultaneously, the inhibitory effect of miR-449 overexpression on cell migration and invasion was rescued. Also, knockdown of TPD52 significantly reversed the expression trend of EMT-related proteins caused by miR-449 overexpression. All these findings prompt us to speculate that miR-449 may control the migration and invasion of breast cancer cells via targeting TPD52. Further studies are needed to investigate our observation.

In conclusion, our findings indicate that downregulation of miR-449 may promote the migration and invasion of breast cancer cells by targeting TPD52. miR-449 may serve as a potential target in the therapy of breast cancer. Our findings will provide a broader perspective to explore a new therapy for this disease.

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