

# miR-542-3p targets sphingosine-1-phosphate receptor 1 and regulates cell proliferation and invasion of breast cancer cells

H.-X. WU<sup>1</sup>, G.-M. WANG<sup>2</sup>, X. LU<sup>3</sup>, L. ZHANG<sup>4</sup>

<sup>1</sup>Department of Nursing, Binzhou Central Hospital, Binzhou, Shandong, China

<sup>2</sup>Department of Traumatology, Binzhou Central Hospital, Binzhou, Shandong, China

<sup>3</sup>Department of Infection Management, Binzhou Central Hospital, Binzhou, Shandong, China

<sup>4</sup>Department of Clinical Laboratory, Jinan Infectious Disease Hospital, Jinan Shandong, China

*Hong-Xia Wu and Guang-Min Wang are co-first author*

**Abstract.** – **OBJECTIVE:** MicroRNAs (miRs) regulate the proliferation and metastasis of numerous cancer cell types. This study aimed to reveal the role of microRNA-542-3p (miR-542-3p) in breast cancer (BC) cell proliferation and its potential mechanisms.

**MATERIALS AND METHODS:** MiR-542-3p expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) and sphingosine-1-phosphate receptor 1 (S1PR1) protein expression was measured by Western blotting. TargetScan was used to predict the downstream target genes of miR-542-3p, which were confirmed by luciferase and RNA immunoprecipitation assays. Biological functions of miR-542-3p and S1PR1 were analyzed using CKK-8, colony formation, migration, and invasion.

**RESULTS:** It was demonstrated that the expression of miR-542-3p was downregulated in BC tissues and cell lines. We also showed that the expression of S1PR1 was upregulated in BC tissues and cell lines. Furthermore, we found that the expression level of miR-542-3p was negatively correlated with the expression level of S1PR1 in BC tissues. Over-expression of miR-542-3p inhibited BC cell proliferation, colony formation, migration and invasion. The dual luciferase reporter experiments showed that miR-542-3p regulated the expression of S1PR1 by combining with its 3'UTR. Finally, we showed that down-expression of miR-542-3p inhibited BC cell proliferation, colony formation, migration and invasion.

**CONCLUSIONS:** Our study provides the new insight that miR-542-3p inhibited colon cancer cells via targeting S1PR1, suggesting that miR-542-3p/S1PR1 can serve as a potential therapeutic target for BC.

Key Words:

Breast cancer, miR-542-3p, Sphingosine-1-phosphate receptor 1.

## Introduction

Breast cancer (BC) is the most common malignant cancer and the major cause of mortality due to cancer in females worldwide<sup>1,2</sup>. Although the marked progress in diagnostic instruments and the development of standardized systematic therapy, including genome-wide expression profiling technologies, various combinations of surgery hormone therapy and ultrasound<sup>3,4</sup>, the stage of detection and the ability of cancer cells to metastasize to distant organs have been the major challenges. Thus, studying the molecular mechanisms of breast cancer cells in the process of invasion and metastasis has meaningful significance for the pathogenesis of breast cancer.

Several studies<sup>5,6</sup> have identified that MicroRNAs (miRNAs) are a class of small noncoding RNAs that typically suppress gene expression by binding to 3' untranslated regions (3'UTRs) of their target mRNAs for degradation. Many miRNAs have been demonstrated as key regulators of many cellular biological processes in BC such as cell proliferation, metastasis, and epithelial-mesenchymal transformation<sup>7-9</sup>. MiR-542-3p is a novel member of these miRNAs, which has been characterized as a tumor suppressor in human cancers by targeting critical cancer-related pathways<sup>10,11</sup>. However, little is known about the expression of miR-542-3p or its role in BC.

In the present work, we examined the expression of miR-542-3p in human BC tissues and cell lines, and investigated the regulation of miR-542-3p and sphingosine-1-phosphate receptor 1 (S1PR1) on the proliferation and migration of BC cells. Subsequent investigations revealed that

miR-542-3p directly inhibited the expression of S1PR1.

## Materials and Methods

### *Tissue Samples and Cell Lines*

BC and adjacent normal tissues were collected from 25 patients undergoing surgery at our hospital. After surgical resection, tumor tissues and adjacent normal tissues were collected and stored at  $-80^{\circ}\text{C}$  until use. Written informed consent was obtained from all participants involved in this study. Human breast cancer cell lines (MDA-MB-231, MCF-7, T-47D and BT-474) and normal mammary epithelial cell line (HBL-100) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained at  $37^{\circ}\text{C}$  in a humidified chamber supplemented with 5 %  $\text{CO}_2$ .

### *Cell Transfection*

MiR-542-3p mimic, scrambled RNAs, and S1PR1 siRNA duplexes were from GenePharma (Pudong, Shanghai, China). Transfection was performed when cells were grown to 80 % confluence, using the Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### *Quantitative Real-Time PCR and RT-PCR*

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from tissues or cells. cDNA was synthesized using the Taqman miRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA), and expression levels of miR-542-3p were quantified using miRNA-specific TaqMan MicroRNA Assay Kit (Applied Biosystems, Carlsbad, CA, USA). RT-PCR was performed with SYBR Green Premix Ex Taq (TaKaRa, Dalian, Liaoning, China). The relative expression levels of miRNAs were normalized to that of internal control U6 by using  $2^{-\Delta\Delta\text{Ct}}$  cycle threshold method.

### *Dual-Luciferase Reporter Assay*

The wild-type 3'UTR and the mutant 3'UTR of S1PR1 were synthesized in vitro and were cloned into the downstream of pMIR-REPORT luciferase vector by Spe-1 and Hind III enzyme. MCF-7 cells were co-transfected with miR-542-3p mimic and wild-type S1PR1 3'UTR or the mutant 3'UTR. Forty-eight hours after transfection, luciferase activity of each experiment was measured

by a dual-luciferase reporter assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

### *Western Blotting*

Protein concentration was measured by use of the BCA reagent kit (Merck, Darmstadt, Germany). The protein was resolved by SDSPAGE and transferred to a PVDF membrane, which was probed with specific primary antibody against S1PR1 (1:200). GAPDH was blotted to show equal protein loading.

### *Cell Proliferation and Colony-Forming Analysis*

CCK-8 assay was performed to evaluate the cell proliferation according to manufacturer's instructions. Optical density (OD) was measured at 450 nm with an enzyme immunoassay instrument (BioRad, Hercules, CA, USA). For colony formation experiment, cells were cultured in six-well plates and cultured for 7 days. Then, they were fixed in 4% formaldehyde for 20 min and stained with 1.0% crystal violet.

### *Migration and Invasion Assays*

Cell invasion and migration assays were performed using Transwell inserts (Corning, New York, NY, USA) according to the manufacturer's instructions. An inverted microscope was used to observed cell invasion. The invasion assay was terminated when the cells crossed into the lower well. After the Matrigel was scraped off, the number of cells in the bottom well was counted. Images of cells stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained. Each treatment was performed in triplicate.

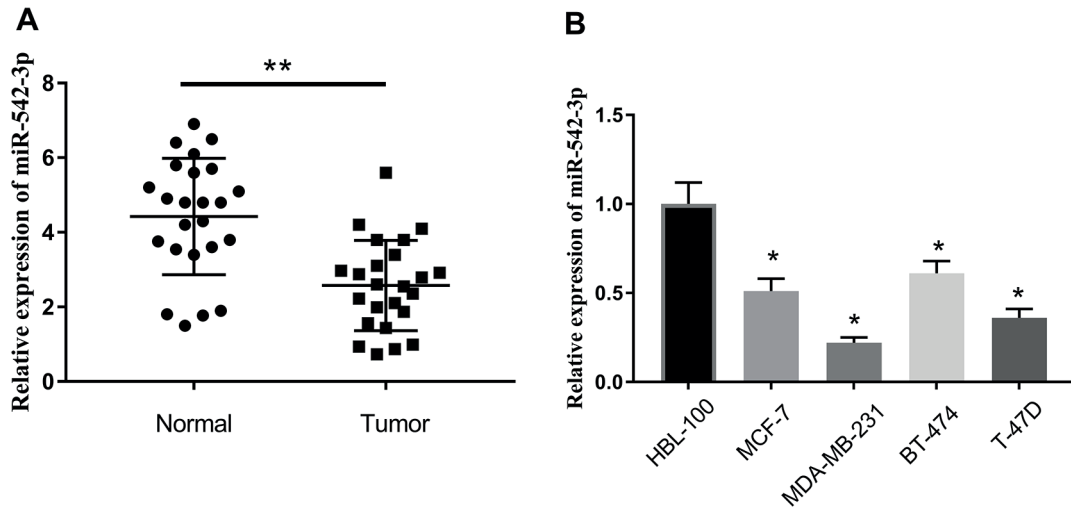
### *Statistical Analysis*

All experiments were performed independently at least three times. GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used for all data analysis. Differences between groups were assessed by unpaired, two-tailed Student's *t*-test.  $p < 0.05$  was considered to indicate a statistically significant difference.

## Results

### *Reduced Expression of miR-542-3p in BC Cell Lines and Clinical Tumors*

To determine the expression of miR-542-3p in clinical tissues, we collected 25 pairs of human BC samples and their adjacent, non-tumorous tis-



**Figure 1.** MiR-542-3p is frequently downregulated in BC cells and tissue specimens. **(A)** qRT-PCR analysis of miR-542-3p expression in 25 pairs of BC tissues and their corresponding adjacent tissues. **(B)** miR-542-3p expression in BC MCF-7, MDA-MB-231, BT-474 and T-47D cells was detected by qPCR. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

sues. Our results showed that expression levels of miR-542-3p in BC tissues were significantly lower than those in non-tumor tissues ( $p < 0.01$ , Figure 1A). We also demonstrated that miR-542-3p expression was decreased in the BC cell lines (MCF-7, MDA-MB-231, BT-474, and T-47D) compared with normal breast cells line (HBL-100) (All  $p < 0.01$ , Figure 1B).

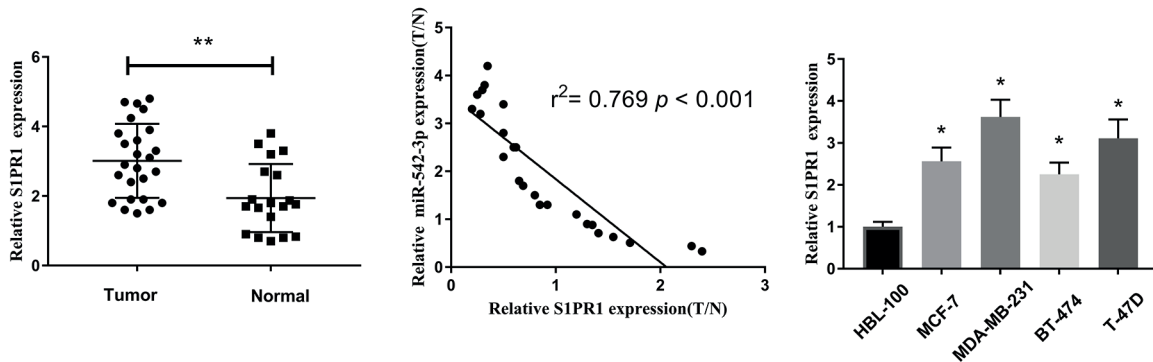
**Increased Expression of miR-542-3p in BC cell lines and Clinical Tumors**

S1PR1 was significantly downregulated in BC tissues compared with the corresponding controls ( $p < 0.01$ , Figure 2A). Moreover, there was a negative relationship between the expression level of

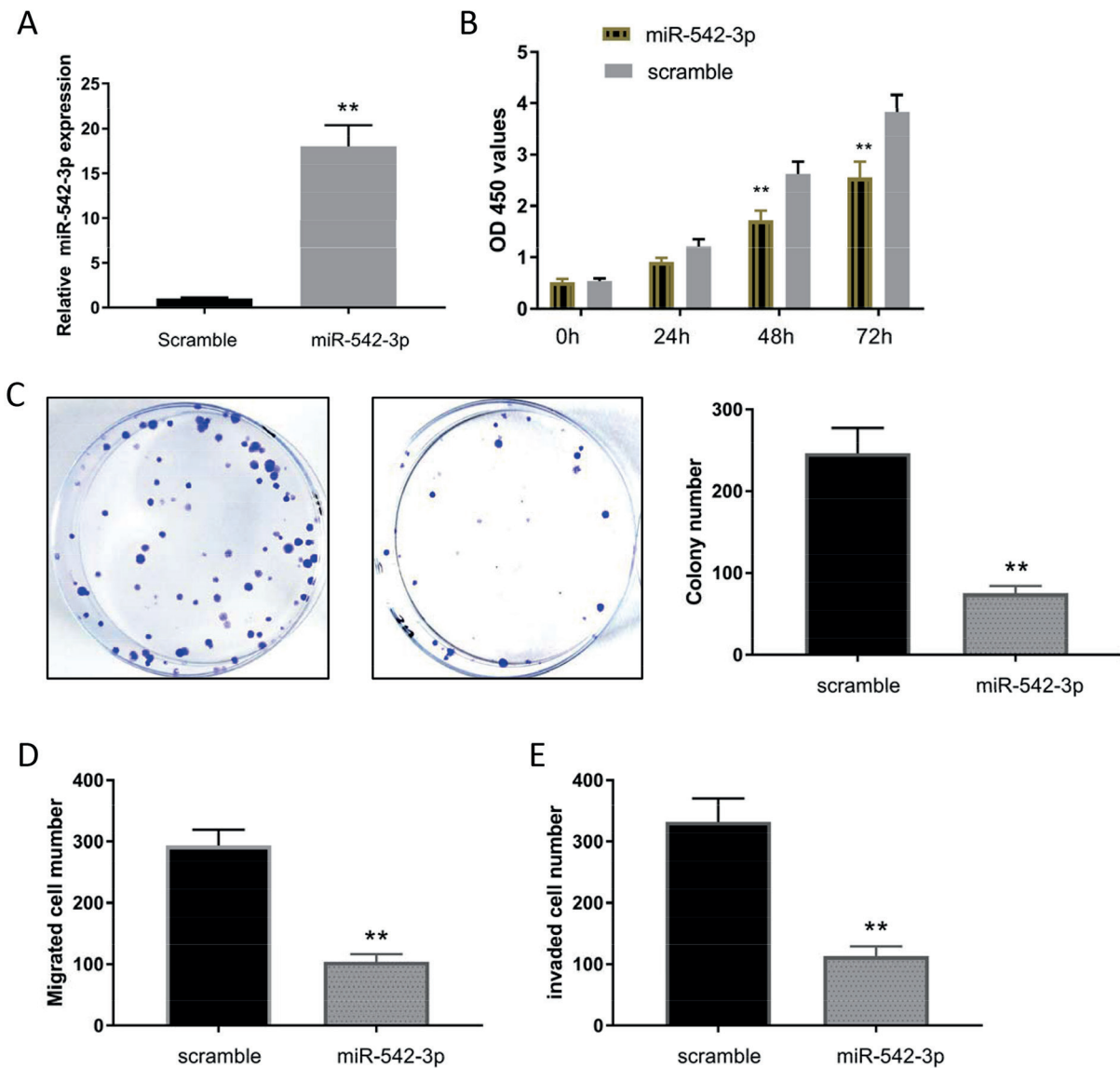
miR-542-3p and S1PR1 in the BC tissues (Figure 2B). We also demonstrated that S1PR1 expression was increased in the BC cell lines (MCF-7, MDA-MB-231, BT-474, and T-47D) compared with normal breast cells line (HBL-100) (All  $p < 0.01$ , Figure 1B).

**miR-542-3p Inhibits the BC Cell Proliferation, Colony Formation, Migration and Invasion**

Due to the down-regulated expression of miR-542-3p in BC clinical tissues and cell lines, we speculated that miR-542-3p might act as a negative tumor role in the development of BC. We transfected miR-542-3p or scramble miR-



**Figure 2.** S1PR1 is frequently upregulated in BC cells and tissue specimens. **(A)** Expression of S1PR1 was detected by qRT-PCR in 25 BC patient tissues. **(B)** There is a negative relation between the expression of miR-542-3p and expression of S1PR1 in the BC tissues. **(C)** Expression of S1PR1 in four BC cell lines and a normal breast cell line (HBL-100). (\* $p < 0.05$ , \*\* $p < 0.01$ ).



**Figure 3.** MiR-542-3p inhibits cell proliferation, colony formation, migration and invasion in human BC cells. (A) The expression level of miR-542-3p was detected in miR-542-3p mimics transfected cells by qRT-PCR. (B-E) Cell proliferation, colony formation, migration and invasion were determined in MCF-7 cells transfected with miR-542-3p mimics or scramble ( $p < 0.05$ ,  $**p < 0.01$ ).

NA into MCF-7 cells, and confirmed the up-regulated expression of miR-542-3p to more after 48 h (Figure 3A). CKK-8 and colony-formation assay showed that over-expressed miR-542-3p increased the ability of cell growth and colony formation (Figure 3B, C;  $p < 0.05$ ). Next, transwell assays were performed to evaluate the cell metastasis capacity. The results showed that cell migration and invasion capacity were significantly decreased when the BC cells were transfected with miR-542-3p (Figure 3 D-E). These results

indicated that miR-542-3p reduced the migration and invasion of BC cells.

#### **miR-542-3p Directly Targets S1PR1**

We performed bioinformatics analysis to identify the possible targets of miR-542-3p. Next, we searched the miRNA prediction database TargetScan and found that miR-542-3p was predicted to target S1PR1 (Figure 4A). The 3'-UTR of PTEN (200 bp) containing the potential miR-542-3p binding site was cloned for a firefly luciferase re-

porter assay (Figure 4A). Luciferase activity was reduced by approximately 50% in miR-542-3p-expressing cells compared with the control transfectants (Figure 4B). Furthermore, up-regulation of miR-542-3p suppressed the S1PR1 protein expression in the MCF-7 cell (Figure 4C). These data suggested that S1PR1 is a direct target of miR-542-3p.

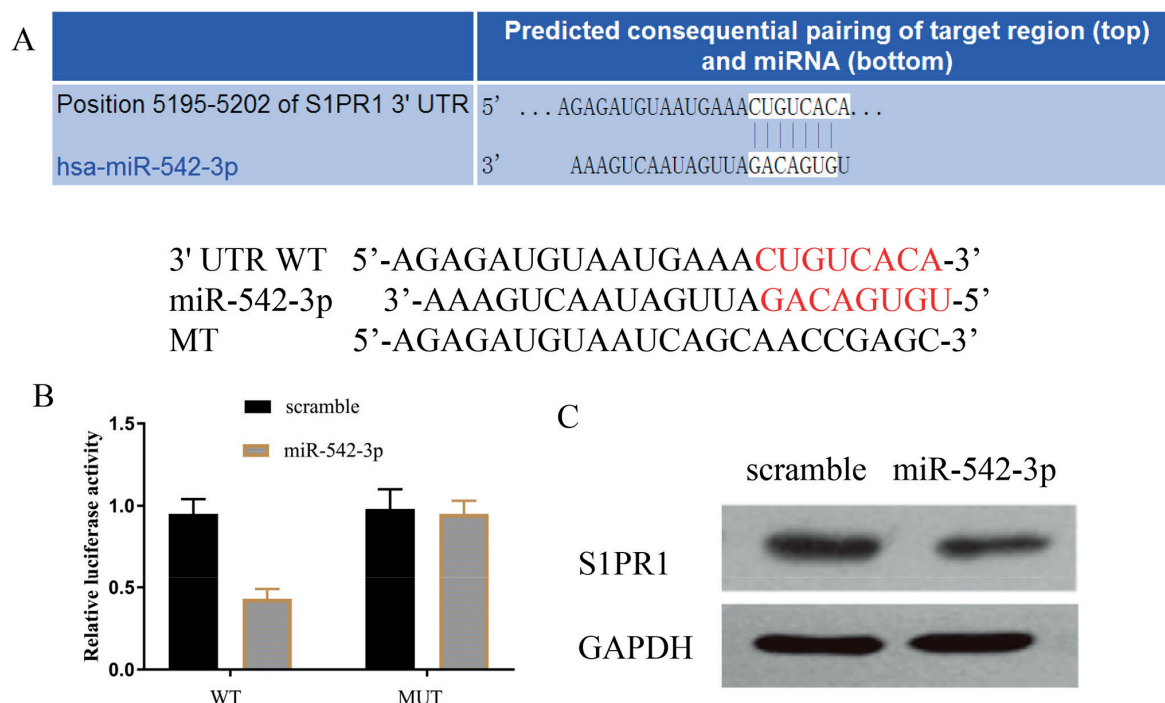
#### **Down Regulation of S1PR1 Exhibited Similar Effect with miR-542-3p Over Expression in BC Cells**

To investigate whether knockdown of S1PR1 has effects on MCF-7 cells, we performed CCK-8 assay and Transwell assay. After S1PR1 siRNA transfection for 48 h, the protein expression of S1PR1 was decreased (Figure 5A), indicating that S1PR1 expression was successfully knocked down. As shown by the cell proliferation and colony formation, we observed that ectopic S1PR1 expression significantly inhibited cell proliferation and colony formation compared to control cells (Figure 5B, C). Furthermore, transwell assay demonstrated that cell migration and invasion capacity were significantly decreased when the

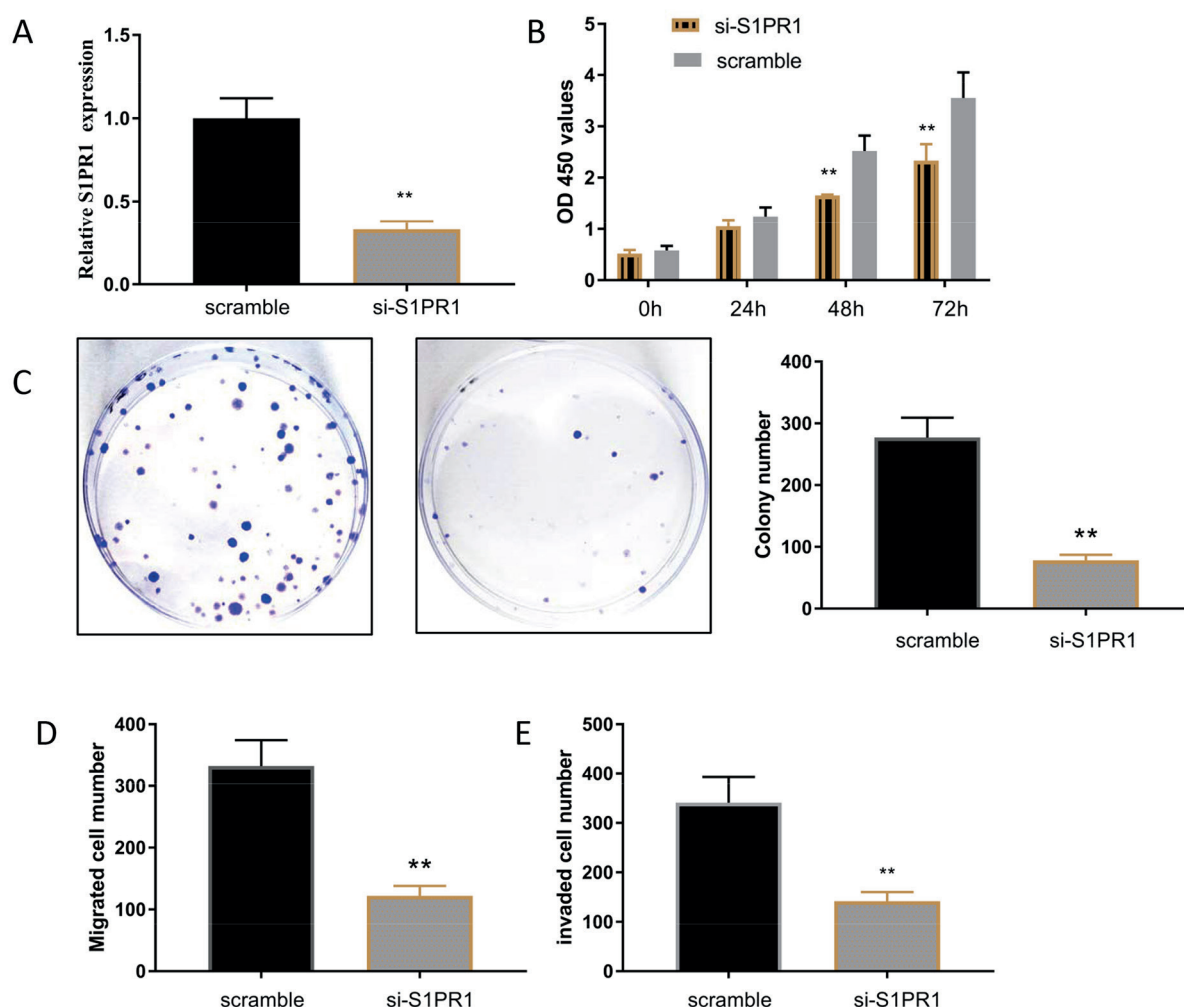
BC cells were transfected with si-S1PR1 (Figure 5D, E). As expected, down-expression of S1PR1 significantly suppressed cell proliferation, migration and invasion abilities.

## **Discussion**

MiR-542-3p, located on chromosome Xq26.3 in humans, was reported to serve as a tumor suppressor in tumors<sup>12</sup>. Some articles have reported that miR-542-3p can regulate cell migration and invasion. For instance, Wu et al<sup>13</sup> showed that miR-542-3p was downregulated in melanoma tissues and cell lines and over-expression of miR-542-3p could inhibit the growth of melanoma cells by suppresses invasion and metastasis by targeting the proto-oncogene serine/threonine protein kinase, PIM1. Cai et al<sup>10</sup> also reported that Exogenous miR-542-3p suppressed glioblastoma cell invasion through targeting integrin-linked kinase and PIK3R1. Long et al<sup>14</sup> found miR-542-3p may act as a tumor suppressor in conferring tumorigenic features such as growth and invasion of malignant colorectal cancer. Notably, two pre-



**Figure 4.** MiR-542-3p directly targeted S1PR1. (A) The potential miR-542-3p binding sites of S1PR1 3'-UTR and the mutant (Mut). (B) Relative luciferase activity of the indicated S1PR1 reporter construct in MCF-7 cells is shown. Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity. (C) The protein level of S1PR1 was detected by western blot after transfected with miR-542-3p mimics or scramble in MCF-7 cells. (\* $p < 0.05$ , \*\* $p < 0.01$ ).



**Figure 5.** Downregulation of S1PR1 reduces tumorigenesis. (A) Validation of S1PR1 expression levels after transfection by western blot. (B-E) cell proliferation, colony formation, migration and invasion were determined in MCF-7 cells transfected with si-S1PR1 or scramble ( $*p < 0.05$ ,  $**p < 0.01$ ).

vious studies<sup>12,15</sup> reported that miR-542-3p plays a suppressive role in breast cancer angiogenesis. Those results suggested that miR-542-3p may be an important regulation biomarker. However, the underlying mechanism was largely unknown. Sphingosine-1-phosphate receptor 1 (S1PR1) has been reported to be engaged in the regulation of cancer growth, invasion, migration and radio-resistance<sup>16</sup>. For instance, Li et al<sup>17</sup> showed that S1PR1 has been found to mediate Wilms tumor cell migration and invasion. Bao et al<sup>18</sup> found that overexpression of S1PR1 could promote hepatocellular carcinoma cell migration and invasion. A similar result was found in BC<sup>15</sup>. More importantly, Zhang et al<sup>19</sup> identified that miR-148a played an inhibitory role in the regulation of hepatocel-

lular carcinoma cell invasion by directly targeting S1PR1. These results supported our current findings and suggested a potential mechanism for the tumor suppressor role of miR-542-3p mediated by the downregulation of S1PR1.

In the present work, we found a significant low-expression of miR-542-3p, both in human BC tissues and BC cell lines by qRT-PCR. We also showed that the expression of sphingosine-1-phosphate receptor 1(S1PR1) was upregulated in BC tissues and cell lines. Furthermore, we found that the expression level of miR-542-3p was negatively correlated with the expression level of S1PR1 in BC tissues. Additionally, over-expression of miR-542-3p could elevate the ability of BC cells invasion and migration. These

results indicate that miR-542-3p may play critical roles in the regulation of BC tumor metastasis. To further explore the mechanism by which miR-542-3p exhibited tumor suppressive function in BC, we performed a computational search for the potential targets for miR-542-3p, and we found S1PR1 may be targeted gene of 542-3p. Also, we used dual luciferase system to identify that S1PR1 is a direct target gene of 542-3p. Finally, knock-down of S1PR1 markedly suppressed BC cell proliferation, colony formation, and invasion. These results provide a mechanistic explanation for the suppressive growth activity of miR-542-3p in BC.

### Conclusions

Our findings showed that miR-542-3p functioned as a tumor suppressor. Also, S1PR1 was identified as a crucial target gene of miR-542-3p. Our data highlighted the importance of miR-542-3p in the cell proliferation and progression of BC, and suggested that miR-542-3p may be a useful therapeutic target for BC.

### Conflicts of interest

The authors declare no conflicts of interest.

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