

This article is licensed under a Creative Commons Attribution-NonCommercial NoDerivatives 4.0 International License.

miR-325-3p Promotes the Proliferation, Invasion, and EMT of Breast Cancer Cells by Directly Targeting S100A2

Huiling Wang,* Xin Hu,† Feng Yang,‡ and Hui Xiao*

*Department of Surgery, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), Changsha, P.R. China

†Department of Surgery, Hunan Children's Hospital, Changsha, P.R. China

‡Department of Pharmacy, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), Changsha, P.R. China

This study was designed to investigate the precise mechanisms of miR-325-3p/S100A2 axis in breast cancer (BC). In this study, we found that the level of miR-325-3p was dramatically increased in BC tissues and cell lines, and the expression of S100A2 was significantly decreased. Also, the high level of miR-325-3p was closely associated with low expression of S100A2 in BC tissues. Moreover, introduction of miR-325-3p significantly promoted proliferation, invasion, and EMT of BC cells. Bioinformatics analysis predicted that the S100A2 was a potential target gene of miR-325-3p. Luciferase reporter assay demonstrated that miR-325-3p could directly target S100A2. In addition, miR-325-3p overexpression had similar effects with knockdown of S100A2 on BC cells. Overexpression of S100A2 in BC cells partially reversed the promoted effects of miR-325-3p mimic. Overexpression of miR-325-3p promoted cell proliferation, invasion, and EMT of BC cells by directly downregulating S100A2 expression.

Key words: Breast cancer (BC); MicroRNA-325-3p; S100A2; Proliferation; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Breast cancer (BC) is the most common cause of cancer-related mortality among females with more than 508,000 deaths, and it is the second most common cancer with nearly 1.67 million new cases diagnosed in 2012 worldwide^{1,2}. BC is a heterogeneous disease characterized by many kinds of pathological features³. Despite many great advances made in the early diagnosis and treatment strategies, the long-term survival in patients, especially in patients with metastatic BC, remains ungratified⁴. Due to tumor metastasis, the mortality of BC is increased⁵. It has been illustrated that tumor metastasis is a complicated process⁶. Therefore, it is critical to study the precise mechanisms that cause primary tumors to metastasize.

The S100 gene family encodes for low-molecular weight calcium-binding proteins and comprises 20 members^{7,8}. A number of evidences have reported that aberrant expressions of the S100 family genes contribute to tumorigenesis, tumor progression, and metastasis by

promoting cancer cell proliferation, apoptosis, migration, and invasion⁹. For example, S100A4 promoted invasion of lung cancer cells by upregulating NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2)¹⁰. Overexpression of S100A6 promoted cell proliferation and migration in hepatocellular carcinoma¹¹. A previous study reported that knockdown of S100A7 reduced epithelial ovarian cancer cell proliferation, migration, and invasion¹². However, little is known about the functional roles of S100A2 in BC. Therefore, the mechanism of S100A2 in BC deserves further studies.

MicroRNAs (miRNAs), small noncoding RNAs that regulate the translation of many genes by binding to the untranslated region (3'-UTR) of target mRNAs, are involved in a variety of physiological and pathological processes, including cancer development^{13,14}. Accumulating evidence shows that miRNAs are aberrantly expressed in many types of cancers, including BC, and some of these miRNAs function as tumor suppressor genes or oncogenes during tumor development and

Address correspondence to Huiling Wang, Department of Surgery, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), No. 61, Jiefang Road, Changsha 410000, Hunan Province, P.R. China. Tel: +86 0731-84762702; Fax: +86 0731-84762702; E-mail: wanghuilinghenan@163.com

progression^{15,16}. Distinct miRNAs have been reported to be directly involved in BC carcinogenesis and progression by regulation of cell proliferation, apoptosis, invasion, or drug sensitivity¹⁷. For example, Du et al. showed that miR-137 alleviates doxorubicin resistance in breast cancer through inhibition of epithelial–mesenchymal transition by targeting DUSP4¹⁸. Wei et al. showed that miR-223 functioned as tumor suppressor and inhibited breast cancer cell proliferation, invasion and epithelial–mesenchymal transition (EMT) by directly targeting FOXO1¹⁹. Xiao et al. suggested that miR-425-5p acted as an oncogene and promoted the proliferation and migration of BC cell lines by downregulating PTEN expression²⁰. However, the expression and role of miR-325-3p in BC remain unclear.

In this study, we found significant downregulation of S100A2 in BC tissues and cells. Knockdown of S100A2 promoted the proliferation, invasion, and EMT of BC cells. Up to now, the relationship between S100A2 and miRNA processing in tumor progression is little known. We found that S100A2 was a direct target of miR-325-3p in BC cells. Overexpression of miR-325-3p promoted the proliferation, invasion, and EMT of BC cells. Moreover, upregulation of S100A2 reversed the promoted effects of miR-325-3p overexpression. Therefore, our outcomes showed critical roles for miR-325-3p/S100A2 axis in the pathogenesis of BC and suggested its possible application in tumor treatment.

MATERIALS AND METHODS

Patients and Tissue Samples

This study included 30 female patients with breast invasive ductal carcinoma who underwent surgical treatment at our hospital between January 2018 and December 2018. Primary cancer tissues and paired adjacent non-cancerous tissues were snap-frozen in liquid nitrogen immediately after resection and then stored at -80°C for RNA extraction. This study was conducted according to the principles of the Helsinki Declaration. The study protocol was approved by the Ethics Committee of Hunan Provincial People's Hospital. All participants had signed written informed consents in advance.

Cell Culture and Transient Transfection

The human breast cell line MCF10A and the BC cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-20, and MCF7 were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM medium containing 10% fetal bovine serum (FBS, GIBCO), ampicillin, and streptomycin at 37°C in a humidified cell incubator with an atmosphere of 5% CO_2 at 37°C .

The miR-325-3p mimics, miR-NC, miR-325-3p inhibitor, anti-miR-NC, siRNA for S100A2 (si-S100A2,

5'-CAGGAAAACAGCAUACUCCTG-3'), and siRNA-negative control (si-NC) were synthesized and purified by Gene-Pharma (Shanghai, China). The S100A2 overexpression plasmid was generated by inserting S100A2 cDNA into a pcDNA3.1 vector. This plasmid was sequence confirmed by Gene-Pharma. miR-325-3p mimics (50 nM), miR-325-3p inhibitors (100 nM), si-S100A2 (100 nM), and S100A2-overexpression plasmid (100 nM) were transfected by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Total RNA and protein were collected 48 h after transfection.

RNA Isolation and qRT-PCR

Total RNA was isolated using TRIzol according to the manufacturer's instructions. The first chain of cDNA was synthesized by reverse transcription with TaqMan MicroRNA Reverse Transcription kit according to the manufacturer's protocol. RT-PCR reaction was conducted in an ABI 7500 Real-Time PCR instrument (Applied Biosystems, Inc., Foster City, CA, USA). U6 was taken as internal control. Primer sequences used for S100A2, p21, CDK2, cyclin E1, MMP-2, MMP-9, TIMP-1, and GAPDH detection are shown in Table 1. Each experiment was done in triplicate.

Cell Proliferation Assay

To study the role of miR-325-3p mimic or inhibitor in proliferation of BC cells, 5×10^3 cells were seeded in a 96-well plate and then incubated overnight in complete medium. After removing the medium, the cells were transfected with miR-325-3p mimic or inhibitor for 48 h

Table 1. Sequences of Primers for qRT-PCR

Gene	Primer Sequence
S100A2	F: 5'-TGCCAAGAGGGCGACAAGTTCA-3' R: 5'-AAGTCCACCTGCTGGTCACTGT-3'
CDK2	F: 5'-ATGGATGCCCTGCTCTCACTG-3' R: 5'-CCCGATGAGAATGGCAGAAAGC-3'
Cyclin E1	F: 5'-TGTGTCTGGATGTTGACTGCC-3' R: 5'-CTCTATGTCGCACCACTGATACC-3'
p21	F: 5'-AGGTGGACCTGGAGACTCTCAG-3' R: 5'-TCCTCTGGAGAAGATCAGCCG-3'
MMP-2	F: 5'-TACAGGATCATTGGCTACACACC-3' R: 5'-GGTCACATCGCTCCAGACT-3'
MMP-9	F: 5'-TGTACCGCTATGGTTACTACTCG-3' R: 5'-GGCAGGGACAGTTGCTTCT-3'
TIMP-1	F: 5'-CTTCTGCAATCCGACCTCGT-3' R: 5'-ACGCTGGTATAAGGTGGTCTG-3'
miR-325-3p	F: 5'-GTAGGTGTCCAGTAAGTG-3' R: 5'-GAACATGTCTGCGTATCTC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTACGAATTTGCGT-3'
GAPDH	F: 5'-GAGTCAACGGATTTGGTTCGTATTG-3' R: 5'-CCTGGAAGATGGTGTGGGATT-3'

at 37°C. Cell Proliferation ELISA-BrdU (colorimetric) kit (Roche Diagnostics, Indianapolis, IN, USA) was used to detect the cell proliferation according to the manufacturer's protocols.

In Vitro Invasion Assay

For invasion assays, 1×10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). A medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were removed, whereas cells that had migrated or invaded to the lower membrane were stained with 0.1% crystal violet. The membranes were then rinsed with 30% glacial acetic acid. Finally, the washing solution was examined at 540 nm for the counting of the number of BC cells.

Protein Extraction and Western Blot Analysis

The cells were lysed in RIPA lysis buffer (Sigma-Aldrich). Protein lysates were prepared, and protein concentration was measured using the BCA Protein Assay kit (Beyotime, Shanghai, China). Equal quantities of protein samples were loaded on 10% SDS-PAGE and transferred onto PVDF membranes, and blocking was performed with 5% nonfat milk. The membrane was incubated overnight with anti-S100A2 (ab109494), anti-p21 (ab109520), anti-CDK2 (ab32147), anti-cyclin E1 (ab33911), anti-E-cadherin (ab40772), anti-N-cadherin (ab76011), anti-vimentin (ab92547), and anti- α -tubulin (ab7291) (Abcam, Cambridge, MA, USA). After washing and incubation with horseradish peroxidase-conjugated antibody (Sigma-Aldrich) for 1.5 h at room temperature, blotted proteins were detected using an enhanced chemiluminescence (ECL) system following the manufacturer's protocol.

Luciferase Reporter Assay

The 3'-UTR of human S100A2 was amplified and cloned into the pGL3-luciferase reporter plasmid (Promega, Madison, WI, USA). The pGL3-S100A2-mut vector was built by PCR. Cells were cotransfected with wild-type pGL3-S100A2 or mutated pGL3-S100A2-mut vector, and miR-325-3p mimics, inhibitor, or negative control, using Lipofectamine 2000 for 24 h. The luciferase activity was measured using the commercial Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Statistical Analysis

The data were expressed as the mean \pm standard error of the mean (SEM). The number of independent experiments was represented by *n*. Correlations between miR-325-3p and S100A2 levels were analyzed using Pearson's correlation coefficient. Two-tailed Student's *t*-test was

used for other comparisons. A value of $p < 0.05$ was considered to have a statistically significant difference.

RESULTS

Low Expression of S100A2 Was in BC Specimens and its Effects on Cell Proliferation, Invasion, and EMT of BC Cells

It has been reported that S100A2 expression is closely associated with many kinds of cancers. In this study, we detected the mRNA level of S100A2 in BC tissues. Our result showed that mRNA level of S100A2 was significantly decreased in BC tissues compared with the adjacent tissues (Fig. 1A). To investigate the functional roles

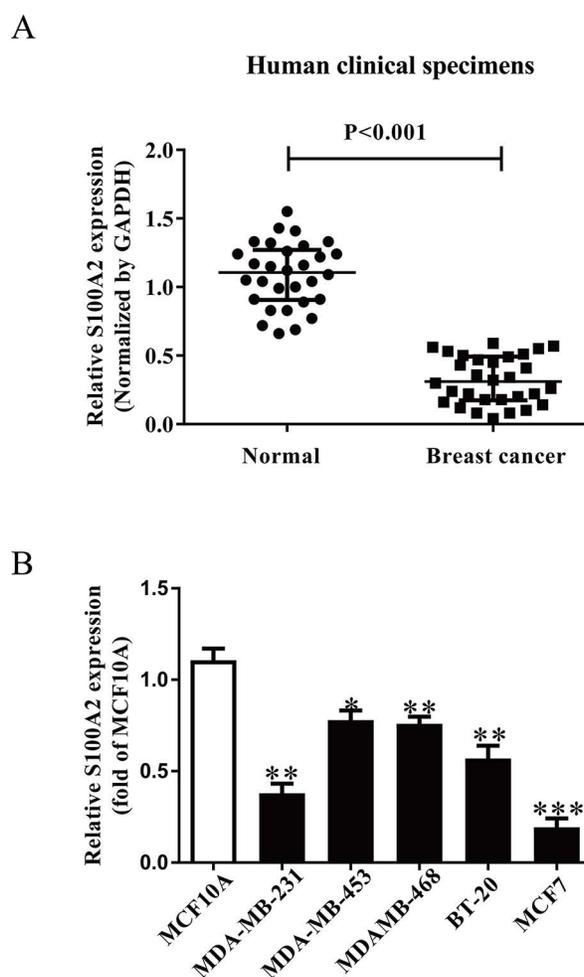


Figure 1. The expressions and its effects of S100A2 in BC tissues and cell lines. (A) qRT-PCR analysis of S100A2 expression in breast cancer (BC) tissues and the adjacent tissues ($n = 30$). Transcript levels were normalized by GAPDH expression. (B) Relative S100A2 expression analyzed by qRT-PCR in five BC cell lines including MDA-MB-231, MDA-MB-453, MDAMB-468, BT-20, MCF7, and a human breast cell line MCF10A were normalized with GAPDH. All data are presented as mean \pm SEM, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus normal or MCF10A.

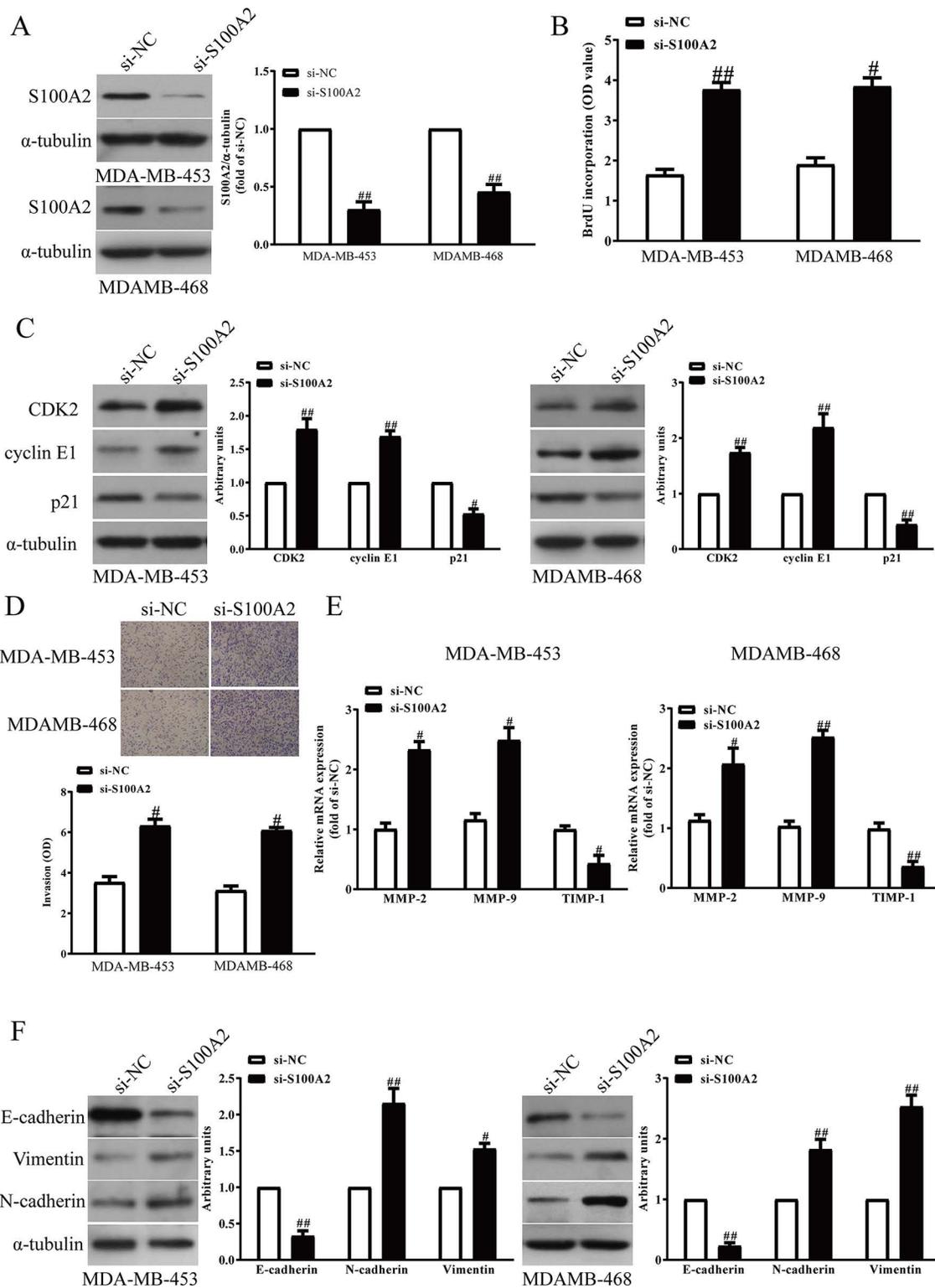


Figure 2. The effects of S100A2 knockdown on the proliferation, invasion, and EMT in BC cells. MDA-MB-453 and MDA-MB-468 cells were transfected with si-S100A2 or si-NC for 48 h. (A) The protein expression of S100A2 was determined via Western blot. (B) Cell proliferation was assessed by a BrdU assay. (C) The protein expressions of CDK2, cyclin E1, and p21 were determined by Western blot. (D) The invasion of BC cells was assessed by Transwell assay. (E) The mRNA levels of MMP-2, MMP-9, and TIMP-1 were detected by quantitative RT-PCR assay. (F) The expressions of E-cadherin, vimentin, and N-cadherin were detected by Western blot. All data are presented as means \pm SEM, $n = 6$. # $p < 0.05$, ## $p < 0.01$ versus si-NC.

of S100A2 in BC, several BC cell lines were determined. Subsequently, we determined the level of S100A2 in five BC cell lines including MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-20, MCF7, and a human breast cell line MCF10A. Compared with MCF10A, the expressions of S100A2 in BC cell lines were lower than those in other BC lines (Fig. 1B). We used MDA-MB-453 and MDA-MB-468 cells in the following experiments for further study because its S100A2 expression is exceptionally high.

Next, to investigate the functional roles of S100A2 in BC cells, the proliferation and invasion and EMT of BC cells were detected after transfection with si-NC or si-S100A2. Transfection with si-S100A2 significantly decreased the S100A2 expression in MDA-MB-453 and MDA-MB-468 cells compared with the si-NC group (Fig. 2A). Then we found that silencing S100A2 evidently increased the proliferation of MDA-MB-453 and MDA-MB-468 cells (Fig. 2B). Moreover, downregulation of S100A2 enhanced the expressions of CDK2 and cyclin E1, and reduced the expression of p21 (Fig. 2C). Next, Transwell assay revealed that decreased S100A2 expression promoted invasion of MDA-MB-453 and MDA-MB-468 cells (Fig. 2D). For further study, knockdown of S100A2 markedly upregulated expressions of MMP-2 and MMP-9, and downregulated expression of TIMP-1 in MDA-MB-453 and MDA-MB-468 cells (Fig. 2E). Next, the EMT of BC cells was promoted after silencing S100A2 expression, by reducing E-cadherin expression and enhancing N-cadherin and vimentin expressions (Fig. 2F). Consequently, silencing S100A2 remarkably promoted BC cell proliferation, invasion, and EMT.

miR-325-3p Directly Targeted S100A2 3'-UTR

To further study S100A2 expression regulated by which miRNA, we detected the levels of miR-325-3p predicted by online database TargetScan 7.2 in BC tissues. We found that miR-325-3p levels were significantly upregulated in BC tissues (Fig. 3A) and BC cells (Fig. 3B) compared with the adjacent tissues and MCF10A, respectively. Moreover, the Pearson's correlation analysis revealed a significant inverse correlation between miR-325-3p and S100A2 in BC tissues (Fig. 3C). To elucidate the mechanisms through which miR-325-3p acts on BC, we predicted the target gene of miR-325-3p with TargetScan databases. S100A2 3'-UTRs was found to have binding sequences for miR-325-3p (Fig. 3D). To further confirm S100A2 as an miR-325-3p target, we determined the expression of S100A2 in miR-325-3p overexpression or knockdown BC cells. The S100A2 expression was downregulated at protein level in MDA-MB-453 and MDA-MB-468 cells by miR-325-3p mimic (Fig. 3E), while the S100A2 expression was upregulated at protein level in MDA-MB-231 and MCF7 cells by miR-325-3p

inhibitor (Fig. 3E). To verify the effects of miR-325-3p on S100A2, luciferase activity assay was performed. Here, we found that miR-325-3p significantly inhibited the luciferase activity of the 3'-UTR S100A2-Wt group, compared with that in the S100A2-Mut group, indicating that S100A2 is one of the direct targets of miR-325-3p in BC (Fig. 3F). Taken together, those findings strongly suggested that S100A2 is a direct target of miR-325-3p in BC.

Upregulation of miR-325-3p Promoted BC Cell Proliferation

Next, we studied the effects of miR-325-3p on the proliferation of BC cells. After transfection with miR-325-3p mimic and miR-NC, the qRT-PCR analysis showed that the level of miR-325-3p was significantly upregulated in the miR-325-3p mimic group compared with the miR-NC group (Fig. 4A). To study the effect of miR-325-3p on the proliferation of BC cells, the results from BrdU assay showed that increased level of miR-325-3p markedly promoted the proliferation of MDA-MB-231 and MCF7 cells (Fig. 4B). To further confirm the above results, we detected the effects of miR-325-3p on several proliferation and cell cycle-related proteins and genes. Overexpression of miR-325-3p increased the protein and mRNA levels of CDK2, cyclin E1, and decreased p21 expression in BC cells (Fig. 4C). However, knockdown of miR-325-3p (Fig. 5A) significantly inhibited the proliferation of BC cells (Fig. 5B), and decreased the protein and mRNA levels of CDK2 and cyclin E1 and increased p21 expression in BC cells (Fig. 5C).

Introduction of miR-325-3p Increased the Invasion and EMT of BC Cells

Next, we transfected miR-325-3p mimic or inhibitor into MDA-MB-231 and MCF7 cells, and the invasive ability of BC cells was assessed by Transwell assay. Increased miR-325-3p level significantly increased the number of invading BC cells compared with the miR-NC group (Fig. 6A). However, cell invasion ability in the miR-325-3p inhibitor group was weaker than that of the miR-NC inhibitor group (Fig. 7A). In addition, the expressions of MMP-2, MMP-9, and TIMP-1 were determined. Our findings demonstrated that expressions of MMP-2 and MMP-9 were markedly increased by increasing the miR-325-3p level in MDA-MB-231 and MCF7 cells (Fig. 6B), while the expression of TIMP-1 was dramatically decreased by increasing the miR-325-3p level (Fig. 6B). However, downregulation of miR-325-3p had the reversed effects on the expressions of MMP-2, MMP-9, and TIMP-1 (Fig. 7B).

EMT is one of the key initiation steps in the metastasis process, which provides cancer cells with motility, invasion, and migration properties. Therefore, we explored

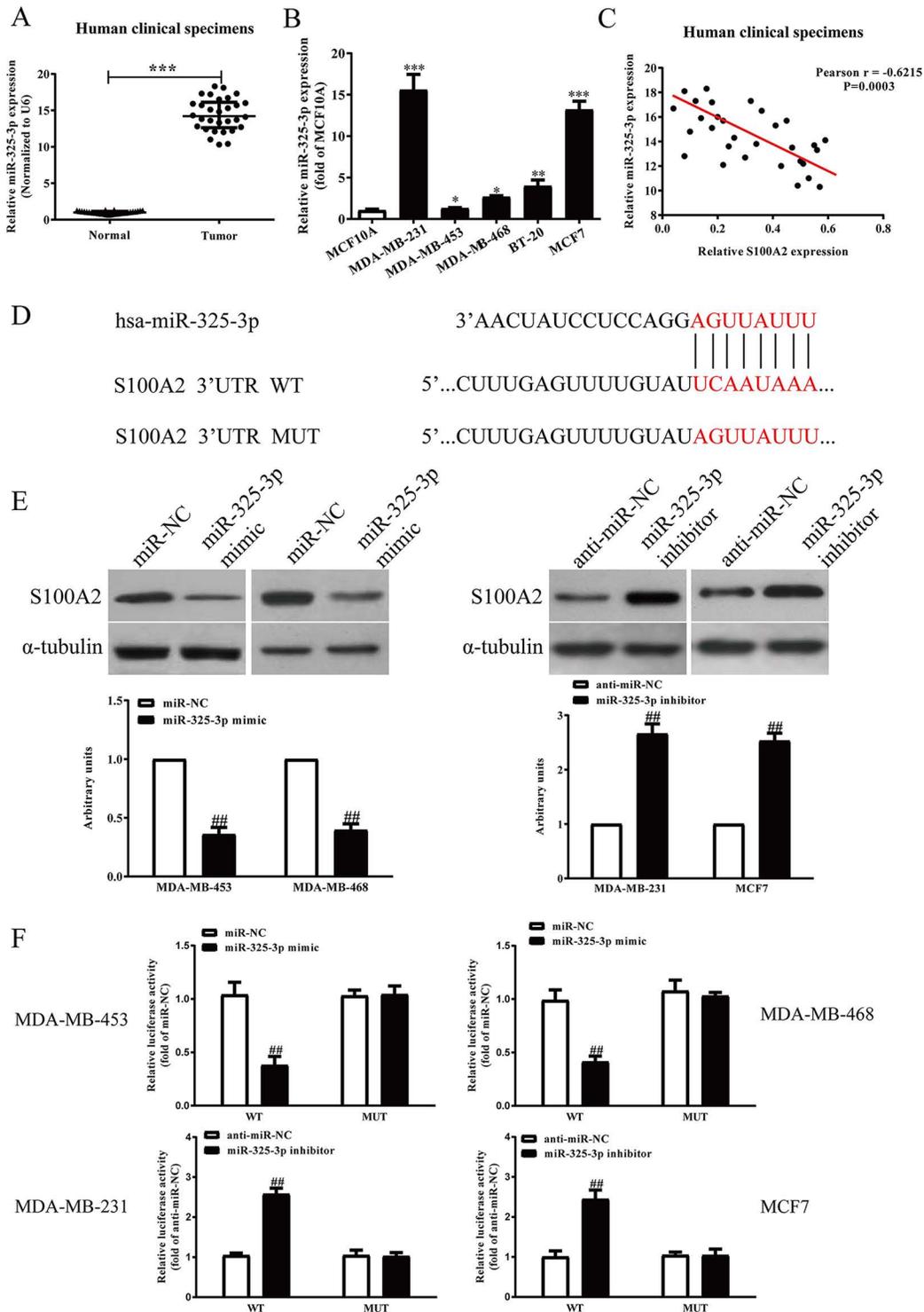


Figure 3. S100A2 was a direct target of miR-325-3p. (A) qRT-PCR analysis of miR-325-3p level in BC tissues and the adjacent tissues ($n = 30$). Transcript levels were normalized by U6 level. (B) qRT-PCR analysis of miR-325-3p level in five BC cell lines including MDA-MB-231, MDA-MB-453, MDAMB-468, BT-20, MCF7, and a human breast cell line MCF10A. (C) Pearson's correlation analysis of the relative expression levels of miR-325-3p and the relative S100A2 mRNA levels in BC tissues ($n = 30$). (D) Schematic representation of S100A2 3'-UTRs showing putative miRNA target site. BC cells were transfected with miR-325-3p mimic or inhibitor for 48 h. (E) The protein expression of S100A2 was determined by Western blot. (F) The analysis of the relative luciferase activities of S100A2-WT and S100A2-MUT. All data are presented as mean \pm SEM, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus normal or MCF10A; ## $p < 0.01$ versus miR-NC or anti-miR-NC.

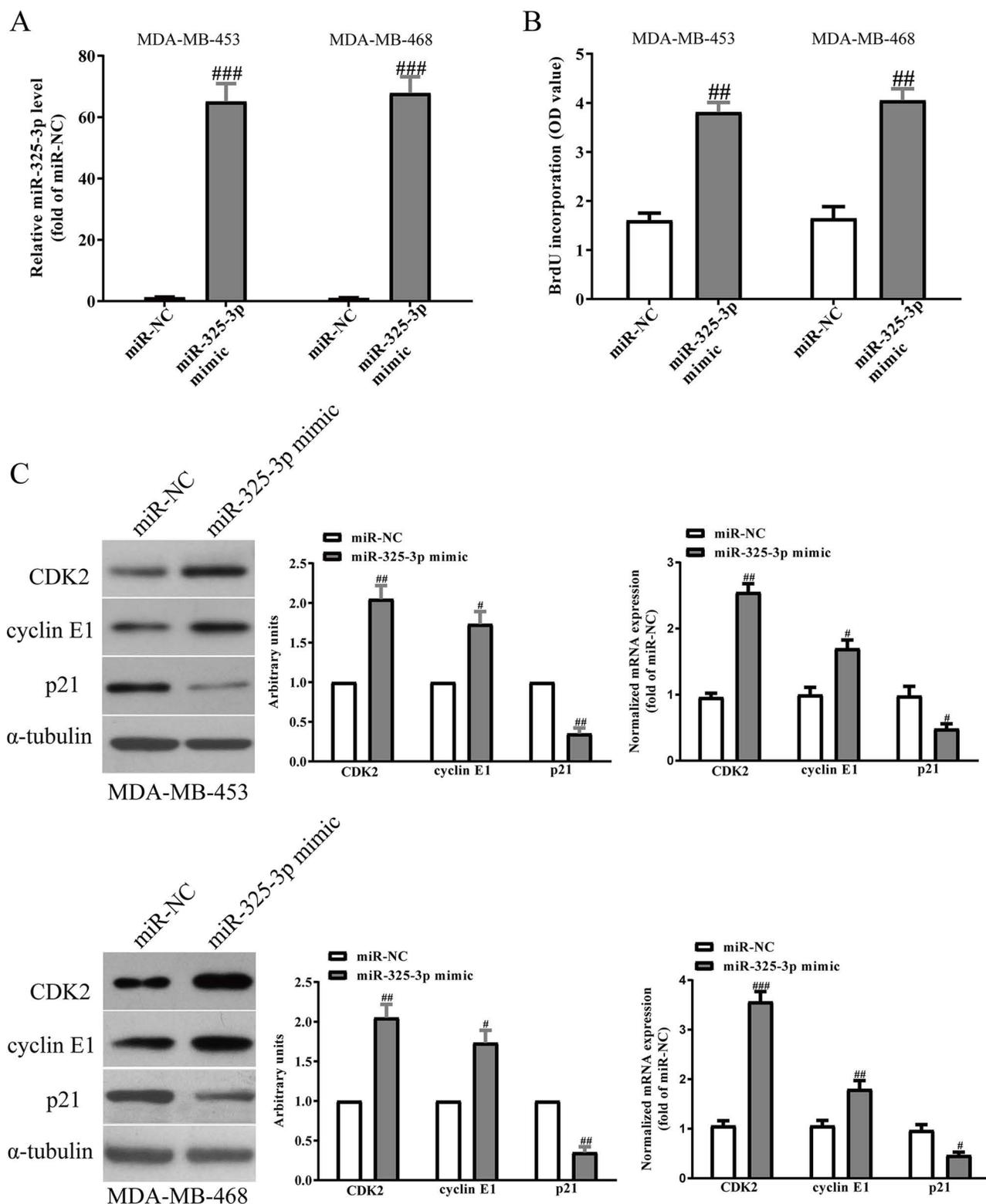


Figure 4. Effects of miR-325-3p overexpression on the proliferation and related gene expressions in BC cells. MDA-MB-453 and MDA-MB-468 cells were transfected with miR-325-3p mimic or miR-NC for 48 h. (A) The levels of miR-325-3p in BC cells were determined by qRT-PCR. (B) Cell proliferation was assessed by BrdU assay. (C) The protein and mRNA expressions of CDK2, cyclin E1, and p21 were determined by Western blot and qRT-PCR, respectively. All data are presented as mean \pm SEM, $n=6$. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus miR-NC.

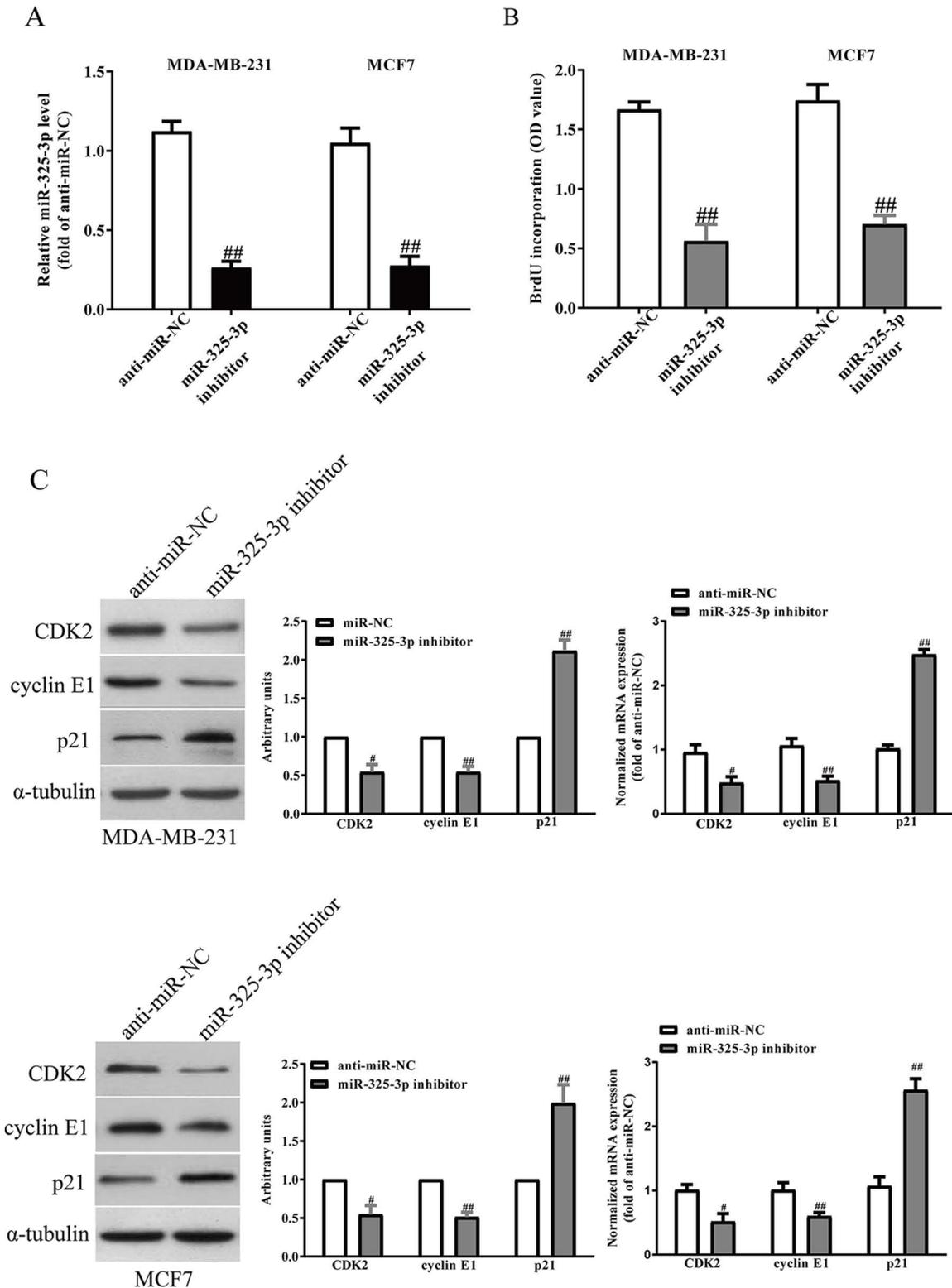


Figure 5. Effects of miR-325-3p knockdown on the proliferation and related gene expressions in BC cells. MDA-MB-231 and MCF7 cells were transfected with miR-325-3p inhibitor or miR-NC inhibitor for 48 h. (A) The levels of miR-325-3p in BC cells were determined by qRT-PCR. (B) Cell proliferation was assessed by BrdU assay. (C) The protein and mRNA expressions of CDK2, cyclin E1, and p21 were determined by Western blot and qRT-PCR, respectively. All data are presented as mean \pm SEM, $n=6$. [#] $p < 0.05$, ^{##} $p < 0.01$ versus anti-miR-NC.

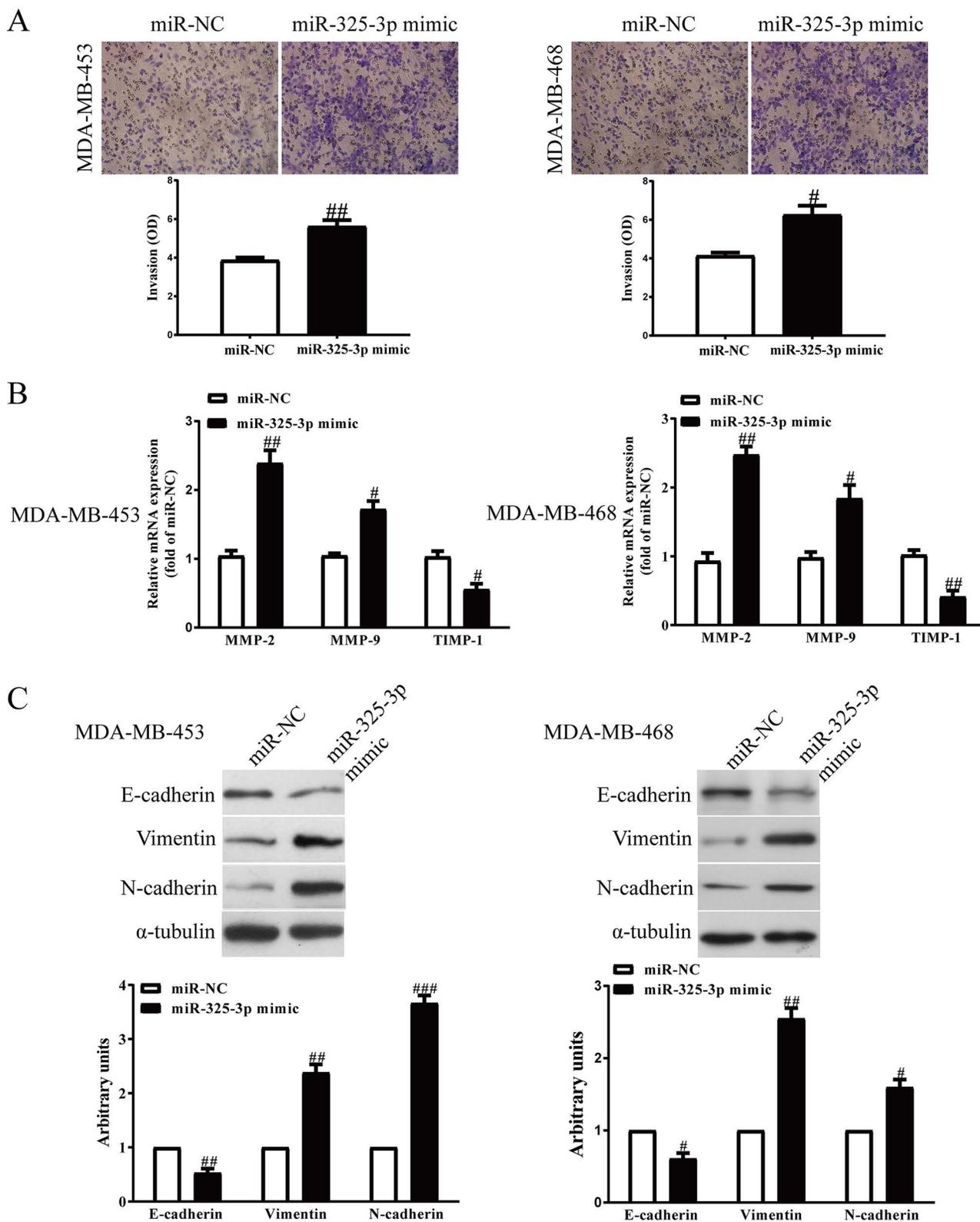


Figure 6. Effects of miR-325-3p overexpression on the invasion and EMT of BC cells. MDA-MB-453 and MDA-MB-468 cells were transfected with miR-325-3p mimic or miR-NC for 48 h. (A) The invasion of BC cells was assessed by Transwell assay. (B) The mRNA levels of MMP-2, MMP-9, and TIMP-1 were detected by PCR assays. (C) The expressions of E-cadherin, vimentin, and N-cadherin were detected by Western blot assays. All data are presented as mean \pm SEM, $n=6$. $\#p<0.05$, $\#\#p<0.01$, $\#\#\#p<0.001$ versus miR-NC.

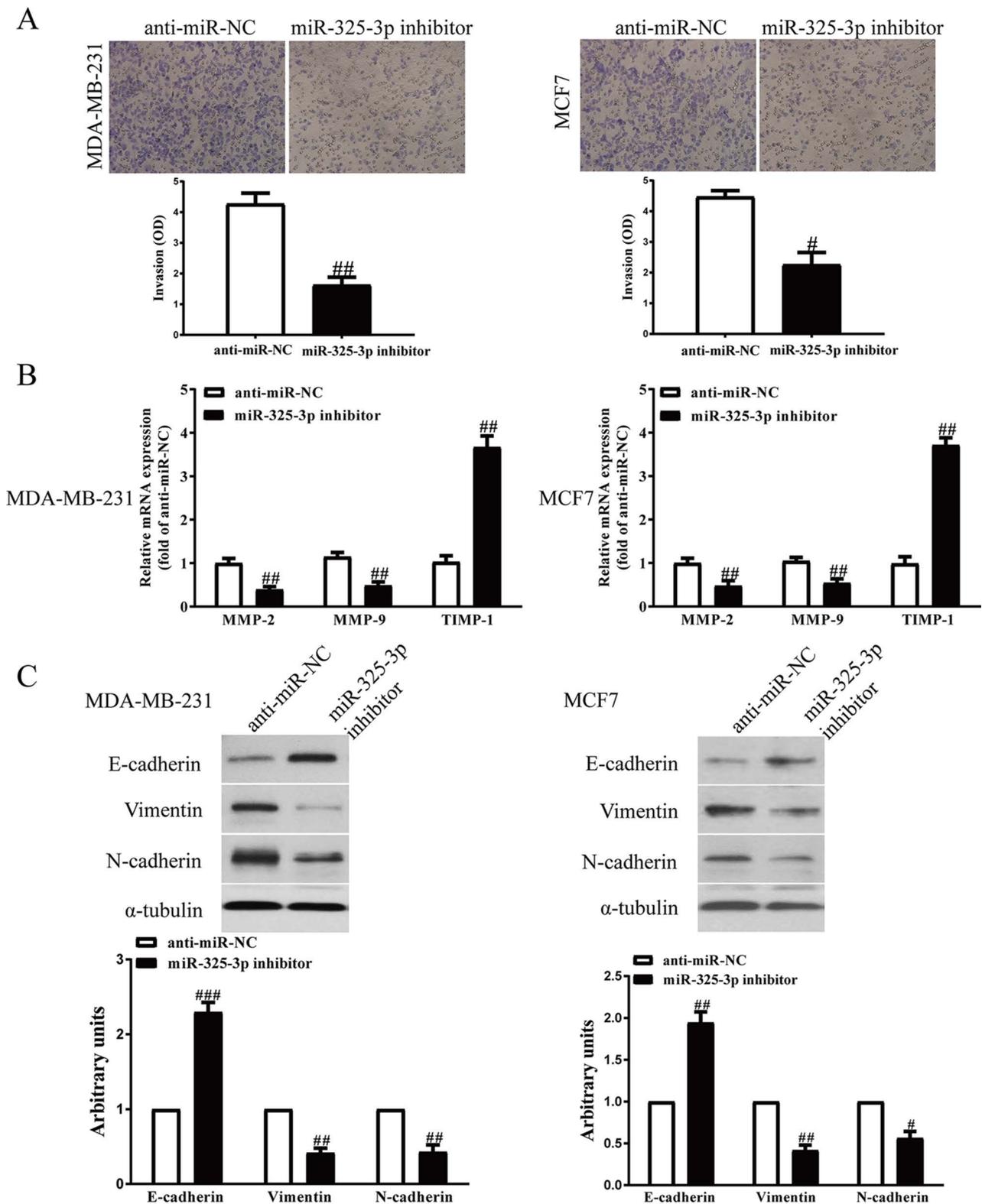


Figure 7. Effects of miR-325-3p knockdown on the invasion and EMT of BC cells. MDA-MB-231 and MCF7 cells were transfected with miR-325-3p inhibitor or anti-miR-NC for 48 h. (A) The invasion of BC cells was assessed by Transwell assay. (B) The mRNA levels of MMP-2, MMP-9, and TIMP-1 were detected by PCR assays. (C) The expressions of E-cadherin, vimentin, and N-cadherin were detected by Western blot assays. All data are presented as mean \pm SEM, $n = 6$. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus anti-miR-NC.

whether miR-325-3p promoting BC progression is mediated by the EMT process. Western blot showed that the epithelial marker E-cadherin was downregulated after miR-325-3p overexpression (Fig. 6C). By contrast, the mesenchymal markers N-cadherin and vimentin were upregulated in miR-325-3p-overexpressed MDA-MB-231 and MCF7 cells (Fig. 6C), while the miR-325-3p inhibitors showed the opposite effect (Fig. 7C). Thus, we demonstrated that miR-325-3p could promote the BC progression by promoting cell proliferation, invasion, and EMT.

Overexpression of S100A2 Significantly Blocked the Effects of miR-325-3p Mimic on BC Cells

To determine whether miR-325-3p targeting S100A2 was responsible for the promotion of proliferation, invasion, and EMT of BC cells, we constructed an expression vector that encoded the entire S100A2 coding sequence but lacked the 3'-UTR. Then we cotransfected this vector (pcDNA-S100A2) or its negative control (pcDNA3.1) with miR-325-3p mimic or miR-NC into MDA-MB-231 cells (Fig. 8A). Cell proliferation assay data showed that concomitant overexpression of miR-325-3p and S100A2 abrogated the promoted effect of miR-325-3p mimic (Fig. 8B). The protein levels of CDK2 and cyclin E1 were decreased, and the levels of p21 were increased in miR-325-3p-overexpressing BC cells after exogenous introduction of S100A2 (Fig. 8C). Next, we found that increased S100A2 expression could reverse the promoted effect of the miR-325-3p mimic on invasion of BC cells (Fig. 8D), reduce the expressions of MMP-2 and MMP-9 upregulated by miR-325-3p mimic, and enhance the expression of TIMP-1 inhibited by miR-325-3p mimic (Fig. 8E). Moreover, miR-325-3p mimic-promoted EMT of BC cells was suppressed by overexpression of S100A2 (Fig. 8F). Therefore, the promoted effects of miR-325-3p were reversed by S100A2 overexpression. Taken together, all the above results clearly confirmed that miR-325-3p promoted cell proliferation, invasion, and EMT in BC cells by downregulation of S100A2, and miR-325-3p targeting S100A2 was responsible for promotion of the proliferation, invasion, and EMT of BC cells.

DISCUSSION

A severe manifestation of BC is metastasis, which is the main reason for the high mortality observed in BC patients²¹. In a previous study, Wicki et al. demonstrated that S100A2 was significantly downregulated in BC tissues compared with normal BC tissues²². However, its function in human BC is not fully understood. Accumulating evidence indicates that miRNAs play vital roles in regulating the occurrence and progression of many tumors^{23,24}. miRNAs are detectable in plasma or serum, and are therefore expected to have utility as novel biomarkers of

cancer prognosis²¹. Therefore, identification and confirmation of appropriate biomarkers are still urgent for evaluating the prognosis and guiding tumor treatment of BC. In the present study, we examined the functional roles of S100A2 and its regulation by an miRNA in BC cells.

Aberrant expressions of S100 family genes have been linked to tumorigenesis and tumor progression⁹. S100A2 was reported to play an important role in the development of cancers, including non-small cell lung cancer (NSCLC)²⁵, colorectal cancer²⁶, gastric cancer²⁷, oral cancer²⁸, bladder cancer, and head and neck cancer²⁹. Ectopic expression of S100A2 induces metastasis of NSCLC²⁵. Furthermore, high S100A2 level was a prognostic marker for patients with stages II and III colorectal cancer²⁶. Nevertheless, loss of S100A2 expression contributed to the development and progression of gastric cancer²⁷. However, the functional roles of S100A2 in BC have not been elucidated. Our study found that S100A2 mRNA level was downregulated significantly in BC tissues and cell lines, which was consistent with previous study²². Additionally, functional investigation revealed that siRNA-mediated knockdown of S100A2 led to promoting cell growth, invasion, and EMT of BC cells.

It is well known that miRNAs perform their function by regulating the expression of its target gene. Here, TargetScan 7.2 database predicted that only miR-325-3p could directly target S100A2, and luciferase reporter assay suggested that S100A2 might be the functional target gene of miR-325-3p. We found that the expression of miR-325-3p was increased in BC tissues and cell lines. In addition, we found that miR-325-3p expression was inversely correlated with S100A2 expression in BC. Furthermore, increased expression or knockdown of miR-325-3p significantly inhibited or promoted S100A2 expression, respectively. These results indicated that miR-325-3p was an oncogene in BC. Increasing studies revealed that dysregulated miRNAs contribute to tumor progression. It is reported that miR-325-3p can act as a tumor suppressor³⁰⁻³². For example, previous studies found that overexpression of miR-325-3p inhibited NSCLC cell invasion and proliferation by directly targeting high-mobility group box 1 (HMGB1)³⁰. Lin et al. showed that upregulation of miR-325-3p in bladder cancer had tumor-suppressive functions³¹. miR-325-3p inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma by downregulation of aquaporin 5³². The present study for the first time explored the function of miR-325-3p in the progression of BC. Increased expression of miR-325-3p significantly promoted BC cell proliferation, invasion, and EMT in vitro. Next, our data revealed that S100A2 overexpression partially abolished the promoted effects of miR-325-3p on BC cell progression and reversed miR-325-3p-caused EMT-related marker changes,

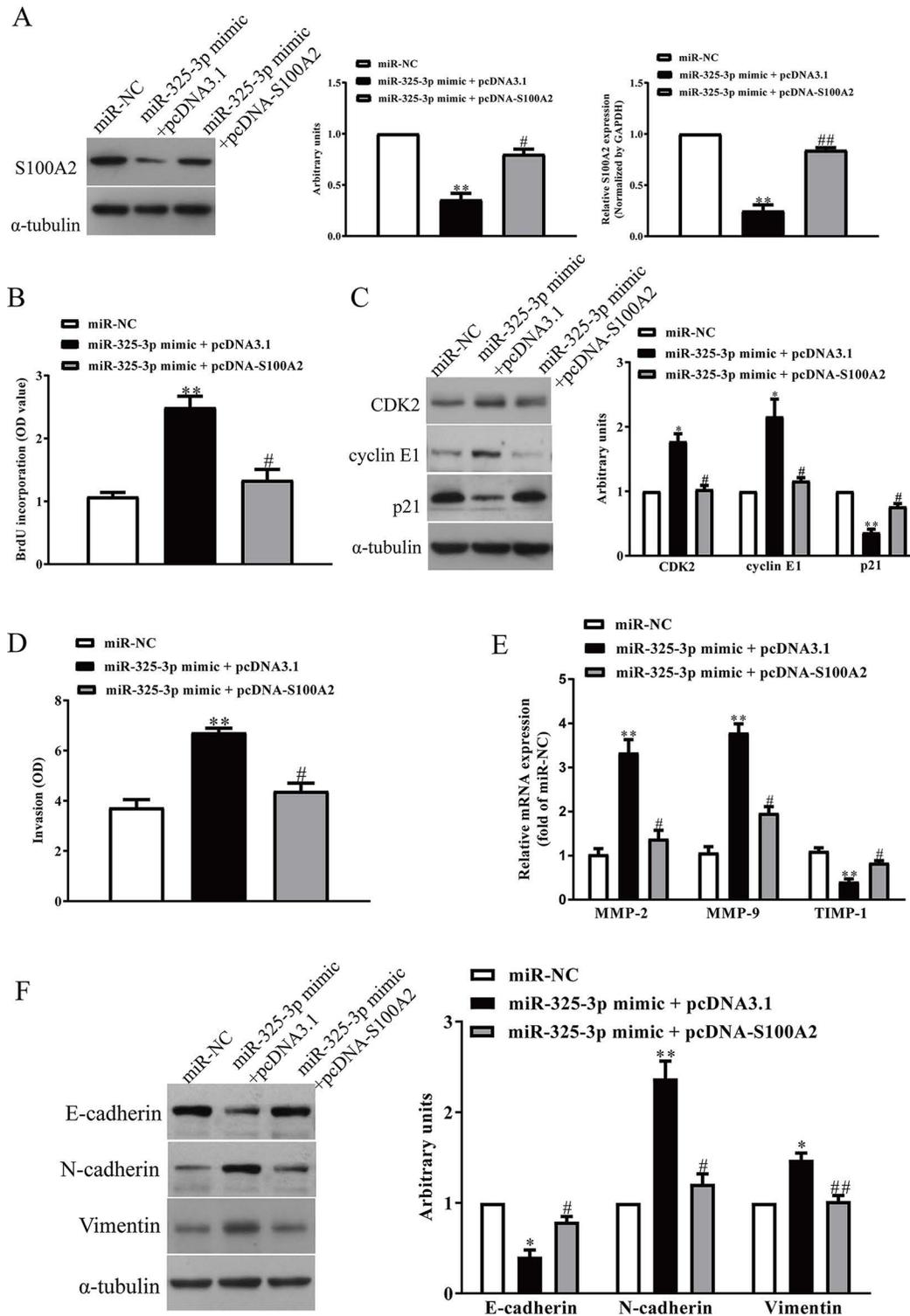


Figure 8. Overexpression of S100A2 partially promoted cell proliferation, invasion, and EMT in miR-325-3p-overexpressing BC cells. BC cells were transfected with either miR-325-3p mimic with or without pcDNA-S100A2 vector. (A) The protein expression of S100A2 was determined by Western blot. (B) Cell proliferation was assessed by CCK-8 assay. (C) The mRNA and protein expressions of PCNA, CDK2, cyclin E1, and p21 were determined by qRT-PCR and Western blot, respectively. (D) The invasion of BC cells was assessed by Transwell assay. (E) Total secretions of MMP-2, MMP-9, and TIMP-1 in the culture supernatants were detected by ELISA assays. (F) The expressions of E-cadherin, vimentin, and N-cadherin were detected by Western blot assays. All data are presented as mean \pm SEM, $n=6$. * $p<0.05$, ** $p<0.01$, versus miR-NC; # $p<0.05$, ## $p<0.01$ versus miR-325-3p mimic + pcDNA3.1.

suggesting that miR-325-3p could regulate BC progression via targeting S100A2.

In summary, the current study provided that miR-325-3p could serve as a potential oncogene in BC progression. miR-325-3p promoted BC cell proliferation, invasion, and EMT by directly targeting S100A2.

ACKNOWLEDGMENTS: *The analyzed data sets generated during the study are available from the corresponding author on reasonable request. Author contributions: Huiling Wang, Xin Hu, Feng Yang and Hui Xiao conceived the project and designed the study. Huiling Wang and Xin Hu performed the experiments and wrote the manuscript; Huiling Wang, Xin Hu, Feng Yang, and Hui Xiao performed the data analysis and interpretation. All authors discussed the results and commented on the manuscript. The authors declare no conflict of interest.*

REFERENCES

- Bray F, Jemal A, Grey N, Ferlay J, Forman D. Global cancer transitions according to the human development index (2008–2030): A population-based study. *Lancet Oncol.* 2012;13:790–801.
- Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136: E359–86.
- Polyak K. Heterogeneity in breast cancer. *J Clin Invest.* 2011;121:3786–8.
- Jones SE. Metastatic breast cancer: The treatment challenge. *Clin Breast Cancer* 2008;8:224–33.
- Steeg PS. Tumor metastasis: Mechanistic insights and clinical challenges. *Nat Med.* 2006;12:895–904.
- Vanharanta S, Massague J. Origins of metastatic traits. *Cancer Cell* 2013;24:410–21.
- Donato R. S100: A multigene family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol.* 2001;33:637–68.
- Cmoch A, Groves P, Palczewska M, Piłkuła S. S100A proteins in propagation of a calcium signal in norm and pathology. *Postepy Biochem.* 2012;58(4):429–36.
- Salama I, Malone PS, Mihaimeed F, Jones JL. A review of the S100 proteins in cancer. *Eur J Surg Oncol.* 2008;34(4):357–64.
- Liu L, Qi L, Knifley T, Piccoro DW, Rychahou P, Liu J, Mitov MI, Martin J, Wang C, Wu J, Weiss HL, Butterfield DA, Evers BM, O'Connor KL, Chen M. S100A4 alters metabolism and promotes invasion of lung cancer cells by up-regulating mitochondrial complex I protein NDUFS2. *J Biol Chem.* 2019;294(18):7516–27.
- Li Z, Tang M, Ling B, Liu S, Zheng Y, Nie C, Yuan Z, Zhou L, Guo G, Tong A, Wei Y. Increased expression of S100A6 promotes cell proliferation and migration in human hepatocellular carcinoma. *J Mol Med. (Berl)* 2014;92(3):291–303.
- Lin M, Xia B, Qin L, Chen H, Lou G. S100A7 regulates ovarian cancer cell metastasis and chemoresistance through MAPK signaling and is targeted by miR-330-5p. *DNA Cell Biol.* 2018;37(5):491–500.
- Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
- Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med.* 2009;60:167–79.
- Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C, Zheng ZM. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS One* 2008;3:e2557.
- Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. *Dev Biol.* 2007;302:1–12.
- Mandujano-Tinoco EA, García-Venzor A, Melendez-Zajgla J, Maldonado V. New emerging roles of microRNAs in breast cancer. *Breast Cancer Res Treat.* 2018;171(2):247–59.
- Du F, Yu L, Wu Y, Wang S, Yao J, Zheng X, Xie S, Zhang S, Lu X, Liu Y, Chen W. miR-137 alleviates doxorubicin resistance in breast cancer through inhibition of epithelial-mesenchymal transition by targeting DUSP4. *Cell Death Dis.* 2019;10(12):922.
- Wei YT, Guo DW, Hou XZ, Jiang DQ. miRNA-223 suppresses FOXO1 and functions as a potential tumor marker in breast cancer. *Cell Mol Biol. (Noisy-le-grand)* 2017;63(5):113–8.
- Xiao S, Zhu H, Luo J, Wu Z, Xie M. miR-425-5p is associated with poor prognosis in patients with breast cancer and promotes cancer cell progression by targeting PTEN. *Oncol Rep.* 2019;42(6):2550–60.
- McGuire A, Brown JA, Kerin MJ. Metastatic breast cancer: The potential of miRNA for diagnosis and treatment monitoring. *Cancer Metastasis Rev.* 2015;34:145–55.
- Wicki R, Franz C, Scholl FA, Heizmann CW, Schäfer BW. Repression of the candidate tumor suppressor gene S100A2 in breast cancer is mediated by site-specific hypermethylation. *Cell Calcium* 1997;22(4):243–54.
- Bao S, Wang X, Wang Z, Yang J, Liu F, Yin C. MicroRNA-30 mediates cell invasion and metastasis in breast cancer. *Biochem Cell Biol.* 2018;96:825–31.
- Ren Z, Yang T, Ding J, Liu W, Meng X, Zhang P, Liu K, Wang P. MiR-520d-3p antitumor activity in human breast cancer via post-transcriptional regulation of spindle and kinetochore associated 2 expression. *Am J Transl Res.* 2018;10:1097–108.
- Hountis P, Matthaïos D, Froudarakis M, Bouros D, Kakolyris S. S100A2 protein and non-small cell lung cancer. The dual role concept. *Tumour Biol.* 2014;35(8):7327–33.
- Masuda T, Ishikawa T, Mogushi K, Okazaki S, Ishiguro M, Iida S, Mizushima H, Tanaka H, Uetake H, Sugihara K. Overexpression of the S100A2 protein as a prognostic marker for patients with stage II and III colorectal cancer. *Int J Oncol.* 2016;48(3):975–82.
- Liu YF, Liu QQ, Wang X, Luo CH. Clinical significance of S100A2 expression in gastric cancer. *Tumour Biol.* 2014;35(4):3731–41.
- Kumar M, Srivastava G, Kaur J, Assi J, Alyass A, Leong I, MacMillan C, Witterick I, Shukla NK, Thakar A, Duggal R, Roychoudhury A, Sharma MC, Walfish PG, Chauhan SS, Ralhan R. Prognostic significance of cytoplasmic S100A2 overexpression in oral cancer patients. *J Transl Med.* 2015;13:8.
- Lee J, Wysocki PT, Topaloglu O, Maldonado L, Brait M, Begum S, Moon D, Kim MS, Califano JA, Sidransky D, Hoque MO, Moon C. Epigenetic silencing of S100A2 in bladder and head and neck cancers. *Oncoscience* 2015;2(4):410–8.
- Yao S, Zhao T, Jin H. Expression of MicroRNA-325-3p and its potential functions by targeting HMGB1 in non-small cell lung cancer. *Biomed. Pharmacother.* 2015;70:72–9.

31. Lin T, Zhou S, Gao H, Li Y, Sun L. MicroRNA-325 is a potential biomarker and tumor regulator in human bladder cancer. *Technol Cancer Res Treat*. 2018;17:15330338 18790536.
32. Zhang Z, Han Y, Sun G, Liu X, Jia X, Yu X. MicroRNA-325-3p inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma by down-regulation of aquaporin 5. *Cell Mol Biol Lett*. 2019;24:13.