

# miR-3188 Regulates Cell Proliferation, Apoptosis, and Migration in Breast Cancer by Targeting TUSC5 and Regulating the p38 MAPK Signaling Pathway

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This study intended to investigate the effects of miR-3188 on breast cancer and to reveal the possible molecular mechanisms. miR-3188 was upregulated and TUSC5 was downregulated in breast cancer tissues and MCF-7 cells compared to normal tissue and MCF-10 cells. After MCF-7 cells were transfected with miR-3188 inhibitor, cell proliferation and migration were inhibited, whereas apoptosis was promoted. Luciferase reporter assay suggested that TUSC5 was a target gene of miR-3188. In addition, miR-3188 overexpression increased the p-p38 expression, while miR-3188 suppression decreased the p-p38 expression significantly. miR-3188 regulated breast cancer progression via the p38 MAPK signaling pathway. In conclusion, miR-3188 affects breast cancer cell proliferation, apoptosis, and migration by targeting TUSC5 and activating the p38 MAPK signaling pathway. miR-3188 may serve as a potential therapeutic agent for the treatment of breast cancer.

**Key words: Breast cancer; miR-3188; Cell proliferation; Cell migration; Apoptosis**

## INTRODUCTION

Breast cancer is a phenotypically and genetically complex disease<sup>1</sup>, which is the most commonly occurring female cancer worldwide, accounting for 25% of all cases. According to the World Health Organization, it resulted in 1.68 million cases and 522,000 deaths in 2012<sup>2</sup>. Presently, it is an increasingly serious public health problem in developing countries<sup>3</sup>. Although extensive research on the molecular mechanisms involved in breast cancer has been done, challenges still prevail in the early diagnosis and treatment of this disease.

Studies have found that breast cancer results from abnormalities in certain gene products, including coding and noncoding genes<sup>4</sup>. MicroRNAs (miRNAs) are small (~21–23 nt in size) noncoding RNAs that are capable of modulating gene expression at the posttranscriptional level<sup>5</sup>. They play important roles in a broad range of cellular physiological and pathological processes<sup>6</sup>. They are suggested to be involved in almost all aspects of cancer biology, including cell proliferation, apoptosis, invasion, and metastasis<sup>7</sup>. The miRNA deregulation in breast cancer was first reported in 2005<sup>8</sup>; since then many studies have focused on the expression of various miRNAs and

their roles in breast cancer. Numerous miRNAs have been reported to be associated with breast cancer, such as miR-206, miR-17-5p, miR-200c, etc.<sup>9–11</sup>. A recent study reported that miR-3188 was associated with nasopharyngeal carcinoma<sup>12</sup>. Its biological role has not yet been reported in breast cancer.

In the present study, we detected the expression of miR-3188 on breast cancer tissues and cells and then investigated the effects of its expression on proliferation, apoptosis, and migration. Additionally, we investigated the signal pathway involved in the regulation of breast cancer. This study may provide an essential theoretical basis for the pathogenesis of breast cancer.

## MATERIALS AND METHODS

### Patients

From January 2013 to March 2016, 37 patients with breast cancer who received surgical excision of tumor tissues were enrolled in this study. The diagnosis of breast cancer was pathologically defined according to a previous study<sup>13</sup>. Additionally, the adjacent normal tissues were excised as control. The tissues were stored at –80°C for further study. All patients provided their informed

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consent before the study. All procedures in this study were approved by our hospital's protection of human ethics committee.

#### *Cell Culture and Transfection*

Human breast cancer cells MCF-7 and normal breast epithelial cells MCF-10 (all purchased from the American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and incubated at 37°C in 5% CO<sub>2</sub>.

The vectors including miR-3188 inhibitor, miR-3188 mimic, and miR-3188 scramble were purchased from Sangon Biotech (Shanghai, P.R. China). Cell transfections were conducted with Lipofectamine 2000 reagent based on the manufacturer's protocol. Cells transfected with miR-3188 scramble and without vector transfection were considered as controls.

#### *Cell Viability Assay*

Cell viability was assessed based on the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] assay as previously described<sup>14</sup>. After 48 h of transfection, cells were adjusted to 5 × 10<sup>3</sup> cells/ml and injected into 96-well plates. Cells were centrifuged at 12,000 rpm, and supernatant was removed. Then 20 µl of MTT was added into each well and cultured for 4 h. Finally, 150 µl of dimethyl sulfoxide (DMSO) was added and reacted for 10 min. The absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Tokyo, Japan). All experiments were conducted three times independently.

#### *Colony Assay*

After transfection, cells were plated into 60-mm tissue culture dishes in triplicate at a cell density of 100 cells/dish. After growing in RPMI-1640 medium containing 10% FBS for 14 days, cells were fixed and stained with Diff-Quik and then dried. The colonies were counted under a microscope (IX83; Olympus). The cell number in each colony was at least 30 cells.

#### *Cell Apoptosis Assay*

Cell apoptosis was quantified using flow cytometry with Annexin-V-FITC Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h of transfection, cells were harvested and washed with PBS buffer (pH 7.4) three times and then resuspended in the staining buffer. Then 5 µl of annexin V-FITC and 5 µl of propidium iodide (PI) were mixed with the cells and incubated at room temperature for 10 min. The mixtures were analyzed using FACScan flow cytometry. Annexin V<sup>+</sup> and PI<sup>+</sup> cells were considered to be apoptotic cells.

#### *Cell Migration Assay*

Cell migration was evaluated using Transwell migration chambers (8-µm pore size; Millipore, Billerica, MA, USA). The membranes for the assay were coated with a diluted extracellular matrix (ECM) solution (Sigma-Aldrich, Shanghai, P.R. China). Cells (5 × 10<sup>4</sup>) were seeded into the upper portion of a chamber with serum-free medium after transfection. The medium contained 10% FBS and served as a chemoattractant in the lower chamber. After 24 or 48 h of incubation at 37°C, noninvaded cells on the upper membrane were removed by cotton swabs, and the invaded cells were fixed and stained with Diff-Quik staining and then counted under light microscopy.

#### *Luciferase Reporter Assay*

Dual-luciferase activity assay was performed according to a previous study<sup>15</sup>. The full-length 3'-UTR segments of tumor suppressor candidate 5 (TUSC5) mRNA that contained the miR-3188 binding site were amplified and inserted into the *Xba*I site of a pGL3 vector (Promega, Madison, WI, USA), which was named pGL3-TUSC5. The pGL3-TUSC5-mut reporter was synthesized using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Cells (1 × 10<sup>6</sup>) were then cotransfected with 1 µg of pGL3-TUSC5 (or pGL3-TUSC5-mut) plasmid, 50 pmol of miR-3188 inhibitor (or control miRNA), and 1 µg of a *Renilla* luciferase expression constructed pRL-TK (Promega) using Lipofectamine 2000. After 36 h, luciferase activity was measured using the dual-luciferase assay system (Promega) and normalized to *Renilla* luciferase activity.

#### *qRT-PCR Analysis*

Total RNA was extracted from the tissues or cells with TRIzol reagent (Takara, Dalian, P.R. China). The concentration and purity of the isolated RNA were detected using SMA 400 UV-VIS (Merinton, Shanghai, P.R. China). Then 0.5 µg/µl of purified RNA mixed with nuclease-free water was used to synthesize cDNA with the PrimeScript 1st Strand cDNA Synthesis Kit (Invitrogen). Expressions for targets in tissues or cells were measured in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY, USA) using the SYBR ExScript qRT-PCR Kit (Takara). The 2<sup>-ΔΔ</sup> method was used to determine the relative gene expression levels. Melting curve analysis of amplification products was performed at the end of each PCR analysis to confirm that only one product was amplified and detected. GAPDH and U6 were chosen as the internal controls for mRNA and miRNA, respectively. Primers used for target amplification are shown in Table 1.

#### *Western Blotting*

Cells were lysed with RIPA assay and extraction buffer (Sangon Biotech) and were centrifuged at 12,000 rpm

**Table 1.** The Primers Used in This Study

Name	Forward Primer (5'–3')	Reverse Primer (5'–3')
GAPDH	AACGTGTCAGTOGTGGACCTG	AGT GGGTGTGCTGTGFGAAGT
TUSC5	ATTAGTAAAGTTGTTT	CAAAAACTCTAAAAAAA
p27	ATGTCAAACGTGCGAGTGTCTAA	TTACGTTTGACGTCTTCTGAGG
p21	TGTCCGTCAGAACCCATG	TGGGAAGGTAGAGCTTGG
Bcl-2	GACAGAAGATCATGCCGTCC	GGTACCAATGGCACTTCAAG
Bax	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
Cleaved caspase 3	TGGAGGCTGACTTCTGTATGCTT	ACGGGATCTGTTTCTTTGCGTGG
Procaspase 3	TACTGTTTAAAAGGGCTCGTTAAGCGG	TTAGTGGTGGTGGTGGTGGTGAGGAGTGAAGTACATCTCTTTGGT
E-cadherin	AACGCATTGCCACATACAC	AACGCATTGCCACATACAC
N-cadherin	AACGCATTGCCACATACAC	AACGCATTGCCACATACAC
Snail	TTCAACTGCAAATACTGCAACAAG	CGTGTGGCTTCGGATGTG
Vimentin	TCCAAGTTGCTGACCTCTC	TCAACGGCAAAGTTCTCTTC
U6	TATGGAACGCTTCAC	
miR-3188	AGAGGCTTTGTGCGGATACGGG	

for 10 min at 4°C. Supernatant was collected for the measurement of protein concentration using the BCA Protein Assay Kit (Pierce, Appleton, WI, USA). Then 25 µg of protein per cell lysate was subjected to a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was blocked in Tris-buffered saline Tween (TBST) containing 5% nonfat milk for 1 h at room temperature. The membrane was incubated with rabbit anti-human antibodies (TUSC5, p27, p21, Bax, Bcl-2, caspase 3, E-cadherin, N-cadherin, Snail, vimentin, p38, and JAK2; 1:1,000; Invitrogen) overnight at 4°C. Subsequently, the membrane was incubated with horseradish peroxidase-labeled goat anti-rat secondary antibody (1:1,000) at room temperature for 1 h. After washing, the signals were detected using the enhanced chemiluminescence (ECL) method. GAPDH served as the internal control.

*Statistical Analysis*

All data were expressed as mean±standard error of the mean (SEM). Independent sample *t*-test was used to calculate the difference between two groups using GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA). Post hoc Tukey test was used to calculate the difference among groups. A value of *p* < 0.05 was considered to be statistically significant.

**RESULTS**

*Expressions of miR-3188 and TUSC5 in Breast Cancer Patients and Cells*

The results of qRT-PCR detection showed that, compared with adjacent normal tissues and normal breast epithelial cells MCF-10, the expression levels of miR-3188

were significantly higher in breast cancer tissues and cells (MCF-7) (Fig. 1A and B). Additionally, we examined the expression levels of TUSC5 mRNA and protein in breast cancer tissues and normal tissues. The results showed that decreased expression of TUSC5 mRNA and protein was found in breast cancer tissues (Fig. 1C and D). In addition, TUSC5 mRNA and protein were also downregulated in MCF-7 cells compared to MCF-10 cells (Fig. 1E and F).

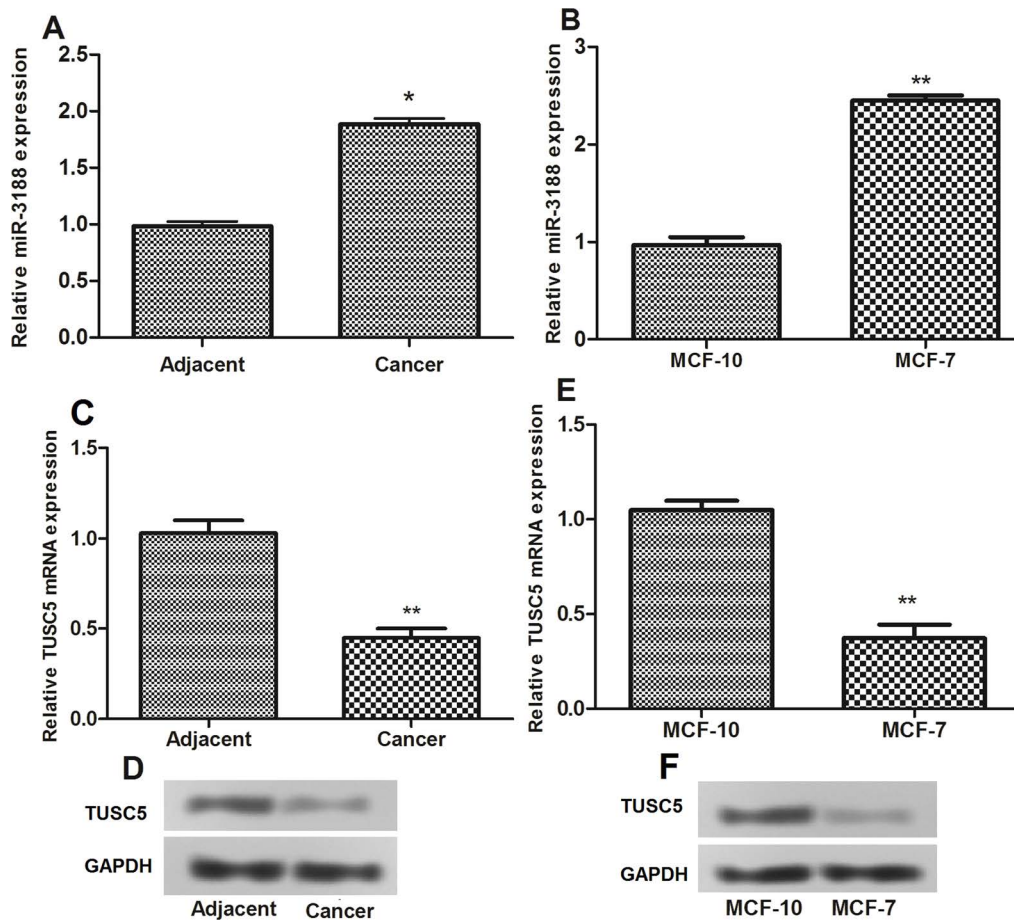
*miR-3188 Suppression Inhibited Breast Cancer Cell Proliferation*

After successful cell transfection (Fig. 2A), we investigated the effect of miR-3188 on the proliferation of MCF-7 breast cancer cells using MTT assay. As shown in Figure 2B, the viability of cells transfected with miR-3188 inhibitor was significantly lower compared with that of cells transfected with miR-3188 scramble or mimic. In addition, cells transfected with miR-3188 inhibitor had a significantly longer colony-forming efficiency than cells transfected with miR-3188 scramble (Fig. 2C and D). Moreover, miR-3188 suppression significantly increased the expression of p27 (Fig. 2E and F).

*miR-3188 Suppression Induced Cell Apoptosis and Inhibited Cell Migration*

We then investigated the effects of miR-3188 on the apoptosis and migration of MCF-7 cells. It showed that the percentage of apoptotic cells increased significantly when cells were transfected with miR-3188 inhibitor (Fig. 3A and B). Through detecting the expressions of apoptosis-associated proteins, we found that miR-3188 suppression induced apoptosis by regulating the expressions of Bax/Bcl-2 and caspase 3 (Fig. 3C and D).

Additionally, as shown in Figure 3E and F, the number of cells migrating across the membrane of cells transfected



**Figure 1.** (A, B) The relative expression levels of miR-3188 in breast cancer tissues and cells detected by RT-PCR). (C–F) The relative expression levels of tumor suppressor candidate 5 (TUSC5) in breast cancer tissues and cells detected by RT-PCR and Western blot. \* $p < 0.05$ , \*\* $p < 0.01$  compared with control.

with miR-3188 inhibitor was significantly less than that of cells transfected with scramble. Furthermore, we investigated the expressions of epithelial–mesenchymal transition (EMT) proteins, E-cadherin, N-cadherin, vimentin, and Snail, and found that miR-3188 suppression promoted E-cadherin expression and inhibited N-cadherin, Snail, and vimentin expressions (Fig. 3G and H).

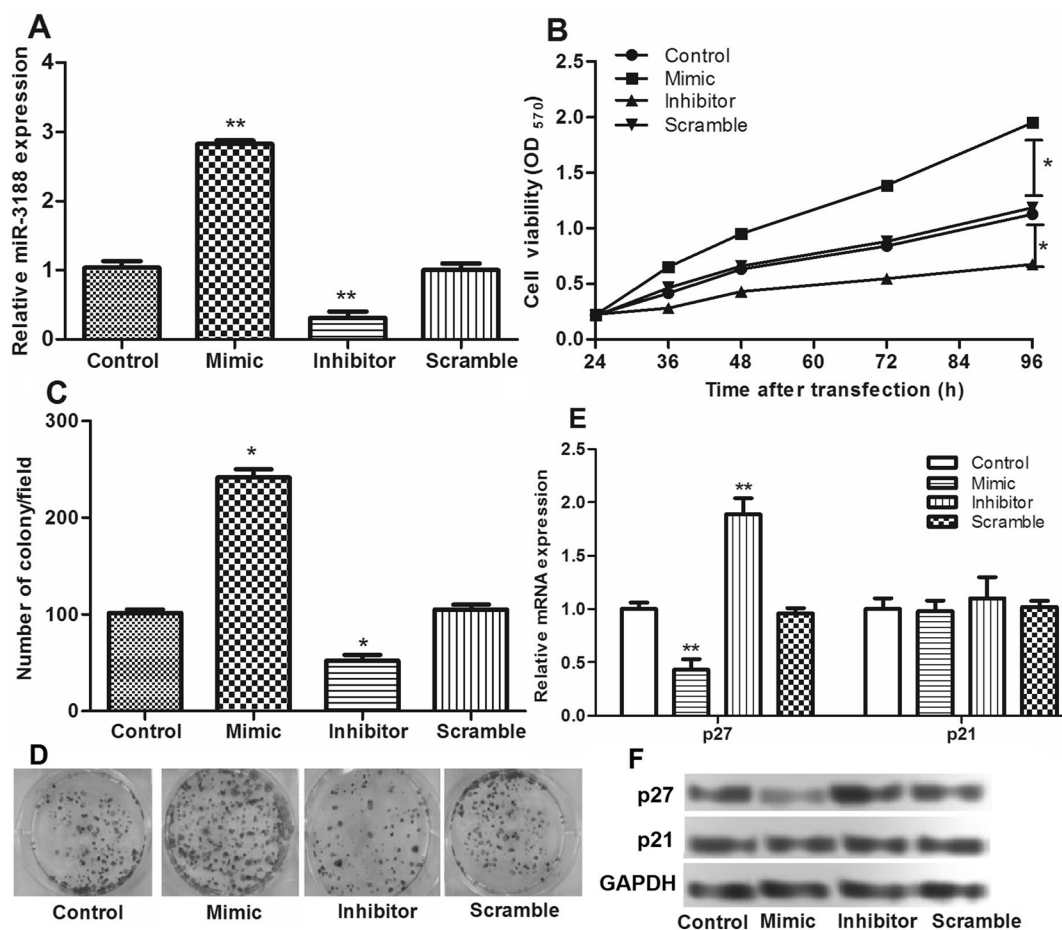
#### *miR-3188 Regulated Cell Apoptosis and Migration by Directly Targeting TUSC5*

We predicted that TUSC5 was a target gene of miR-3188 based on the TargetScan database (<http://www.targetscan.org/>) (Fig. 4A). To verify that TUSC5 was really a direct target of miR-3188, we employed a dual-luciferase reporter system. As shown in Figure 4B, miR-3188 inhibitor significantly downregulated the luciferase activity of the reporter. However, the luciferase expression of mutant TUSC5-3'-UTR was no longer regulated by miR-3188, which suggested that this site in the TUSC5-3'-UTR was the exact regulation site of miR-3188.

Subsequently, we detected the expression of TUSC5 in transfected cells. As shown in Figure 4C, there was a negative correlation between TUSC5 and miR-211-5p expressions. We then transfected si-TUSC5 into MCF-7 cells (Fig. 4D) and further investigated the effects of miR-3188 on cell apoptosis and migration. The results showed that, after TUSC5 suppression, the apoptosis-inducing effects of miR-3188 inhibitor disappeared (Fig. 4E and F). The inhibitory effects of miR-3188 inhibitor on cell migration were also significantly attenuated by the presence of siRNA to TUSC5 in breast cancer cell lines (Fig. 4G and H).

#### *miR-3188 Regulated Breast Cancer Progression by Involving the p38 MAPK Signaling Pathway*

We then investigated the role of miR-3188 in the carcinogenesis of breast cancer at the pathway level. The expression of p38 MAPK in transfected cells was determined by Western blot. As shown in Figure 5A, when miR-3188 was overexpressed, the expression of p-p38



**Figure 2.** (A) The relative expression level of miR-3188 after cell transfection. (B) Cell viability assay after 24 to 96 h of cell transfection. (C, D) Clonogenic assay after cell transfection. (E, F) The relative expression level of p27 and p21 after cell transfection. \* $p < 0.05$ , \*\* $p < 0.01$  compared with control.

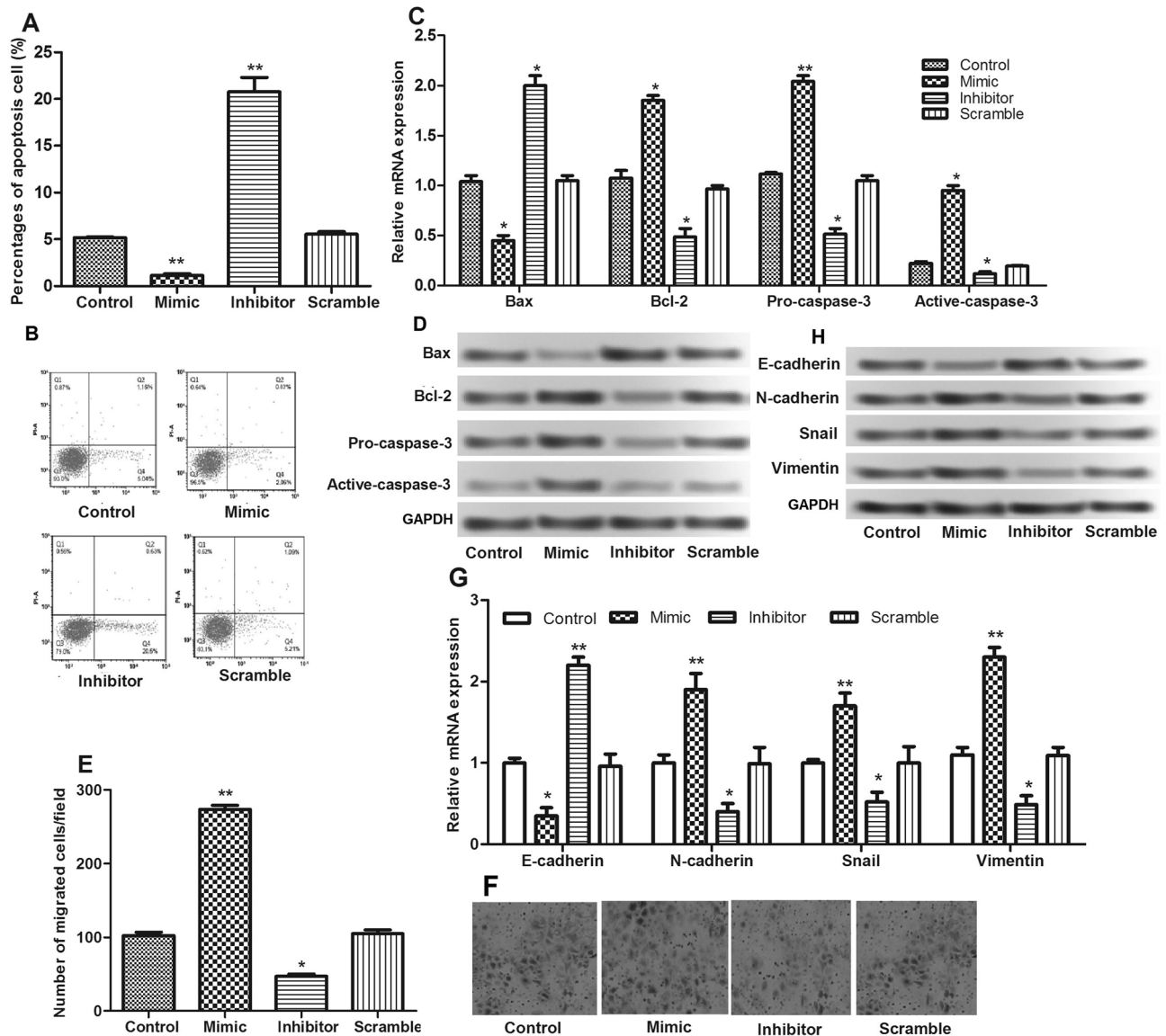
increased significantly. On the contrary, the expression of p-p38 significantly decreased when miR-3188 was suppressed. However, JAK2 expression was not affected by miR-3188. Afterward, we used SB203580, the inhibitor of p38 MAPK, to verify the results, and the p-p38 protein level in the miR-3188 mimic group decreased significantly after MCF-7 cells were treated with SB203580 (Fig. 5B). Further study found that the effects of miR-3188 mimic on apoptosis and migration were significantly reversed by SB203580 (Fig. 5C and D).

**DISCUSSION**

Recent studies have found that dysregulation of miRNAs is linked with various types of human cancers by regulating multiple types of target gene expression<sup>16,17</sup>. In this study, we found that miR-3188 was overexpressed in breast cancer tissues and cells by targeting TUSC5. miR-3188 suppression could inhibit proliferation of cancer cells by promoting the expression of p27 and could

induce apoptosis by regulating the expressions of Bax/Bcl-2 and caspase 3. Additionally, miR-3188 suppression was found to inhibit cell migration by regulating EMT proteins. Further study found that miR-3188 regulated breast cancer progression through the p38 MAPK signaling pathway.

miR-3188 is a newly identified miRNA, which has been suggested to regulate nasopharyngeal carcinoma proliferation and chemosensitivity<sup>12</sup>. To the best of our knowledge, this is the first time it is suggested to be upregulated in breast cancer. We first investigated the effect of miR-3188 on the proliferation of breast cancer cells by transfecting MCF-7 cells with miR-3188 mimic or inhibitor. The results showed that miR-3188 suppression could inhibit proliferation of MCF-7 cells. It has been established that cell cycle progression is a predominant factor promoting tumor cell proliferation<sup>18</sup>. p27 belongs to the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitor proteins, which binds to cyclin D

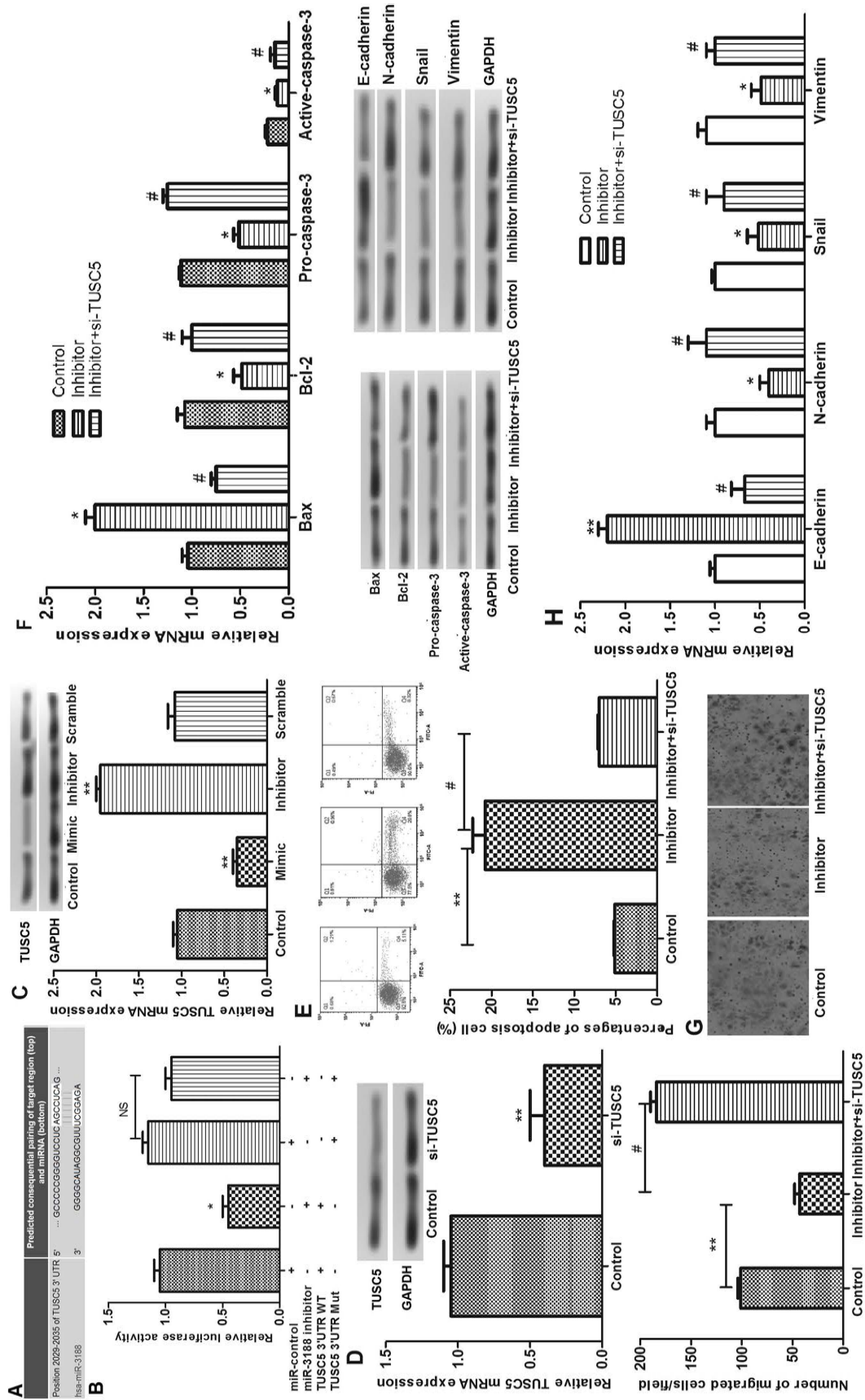


**Figure 3.** (A, B) Apoptosis assay after cell transfection. (C, D) The relative expression levels of apoptosis-related proteins (Bcl-2, Bax, and caspase 3) after cell transfection detected by qRT-PCR and Western blot. (E, F) Cell migration assay after cell transfection. (G, H) The relative expression levels of N-cadherin, E-cadherin, vimentin, and Snail after cell transfection detected by qRT-PCR and Western blot. \* $p < 0.05$ , \*\* $p < 0.01$  compared with control.

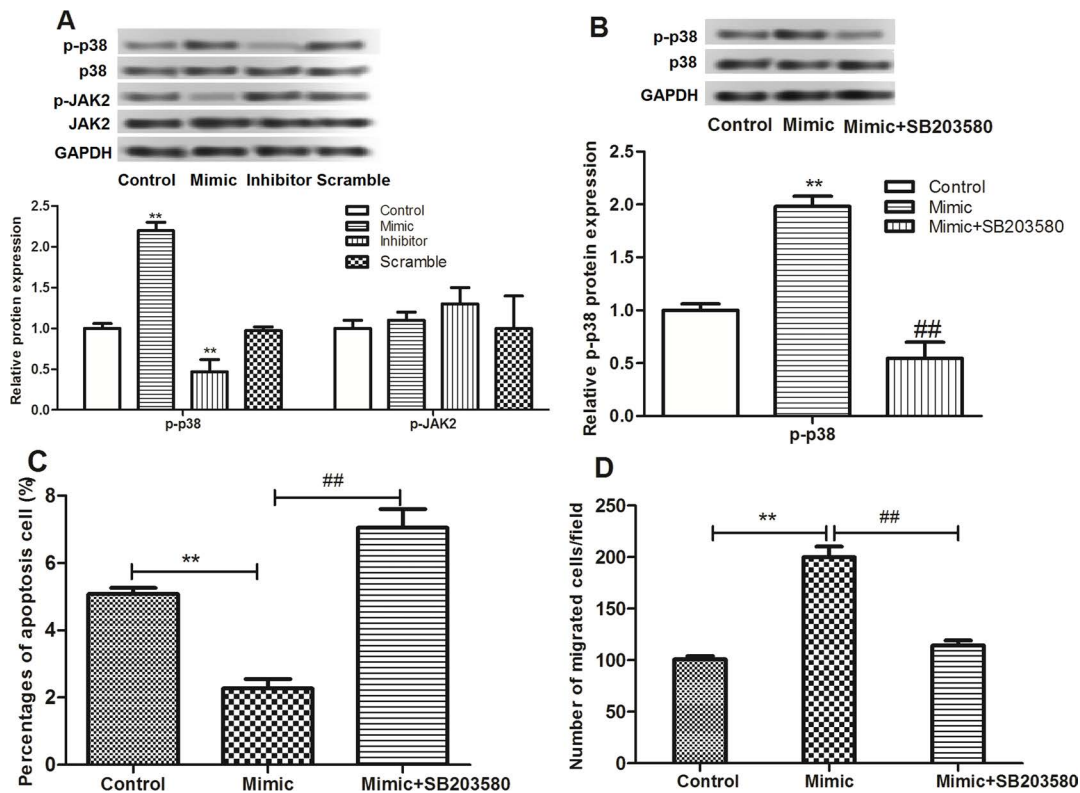
alone or to its catalytic subunit CDK4 to control the cell cycle progression at the  $G_1$  stage<sup>19</sup>. Additionally, it is also reported to regulate cell proliferation, motility, and apoptosis<sup>20</sup>. Low expression of p27 has been identified to be a predictor of poor prognosis in various human cancers<sup>21</sup>. The present result showed that miR-3188 mimic significantly decreased the expression of p27, suggesting that miR-3188 may promote breast cancer cell proliferation by inhibiting p27.

Apoptosis is an important biological process for the normal development and homeostasis of organisms<sup>22,23</sup>. Apoptosis failure is a crucial step in the initiation and

progression of cancer<sup>24</sup>. The present study found that miR-3188 overexpression could inhibit apoptosis of MCF-7 cells, and miR-3188 suppression could induce apoptosis, suggesting the carcinogenicity of this miRNA in breast cancer. Bcl-2 and Bax are members of the Bcl-2 protein family. Bcl-2 inhibits apoptosis, and Bax accelerates apoptosis<sup>25</sup>. Caspase 3 is a member of the caspase family, serving as an executor of programmed cell death<sup>26-28</sup>. Caspase 3 exists in cells as a low-activity zymogen, named procaspase 3, which can be activated by proteolysis into active caspase 3. The active caspase 3 is necessary for the initiation of apoptosis<sup>24,29</sup>. In this



**Figure 4.** (A) The gene sequences of suppressor of TUSC5 regulated by miR-3188. (B) The relative luciferase activities in the wild-type 3'-UTR of TUSC5 and mutant-type 3'-UTR of TUSC5 in transfected cells. (C) The relative expression level of TUSC5 detected by qRT-PCR and Western blot. (D) The relative expression level of TUSC5 after cells were transfected with si-TUSC5. (E) Apoptosis assay after cells were transfected with si-TUSC5. (F) The relative expression levels of apoptosis-related proteins (Bcl-2, Bax, and caspase 3) after cells were transfected with si-TUSC5. (G) Cell migration assay after cells were transfected with si-TUSC5. (H) The relative expression levels of N-cadherin, E-cadherin, vimentin, and Snail after cells were transfected with si-TUSC5. \* $p < 0.05$ , \*\* $p < 0.01$  compared with control; # $p < 0.05$  compared with the miR-3188 inhibitor group.



**Figure 5.** (A) The relative expression levels of p38 and JAK2 after cell transfection detected by Western blot. (B) The relative expression level of p38 after cells were treated with SB203580. (C) Apoptosis assay after cells were treated with SB203580. (D) Cell migration assay after cells were treated with SB203580. \*\* $p < 0.01$  compared with control; ## $p < 0.01$  compared with the miR-3188 mimic group.

study, miR-3188 overexpression could regulate the expressions of Bax, Bcl-2, and caspase 3, further indicating its role in inhibiting breast cancer cell apoptosis.

We then investigated the role of miR-3188 in cell migration and found that miR-3188 overexpression promoted the migration of breast cancer cells. Previous studies have reported that EMT plays a key role in the distant metastasis of tumors<sup>30-32</sup>. Yang et al. reported that cancer cells could reduce the expression of cellular adhesion protein E-cadherin and increase the expressions of mesenchymal markers including N-cadherin, vimentin, and Snail during the process of EMT<sup>33</sup>. In accordance with the findings above, we found that the E-cadherin expression significantly decreased, and the expressions of N-cadherin, vimentin, and Snail increased significantly when miR-3188 was overexpressed, which indicated the role of miR-3188 in promoting cell migration in breast cancer.

TUSC5 was originally identified as a gene locus disrupted in some lung cancers and was hypothesized to be involved in the attenuation of cancer cell proliferation<sup>34</sup>. A recent study reported that TUSC5 was downregulated in breast cancer<sup>35</sup>. Interestingly, our study found that TUSC5 was a target gene of miR-3188 and was also downregulated in breast cancer tissues and cells. Moreover,

TUSC5 suppression could attenuate the effects of miR-3188 inhibitor on cell apoptosis and migration. These findings suggested that miR-3188 is involved in the progression of breast cancer by targeting TUSC5.

Finally, we investigated the effect of miR-3188 on breast cancer at the pathway level. The p38 MAPK pathway is implicated in the regulation of various cellular processes, including cell proliferation, differentiation, survival, and migration<sup>36,37</sup>, which can be activated by various proinflammatory and stressful stimuli<sup>38</sup>. This study revealed that miR-3188 could activate this pathway. Specially, if we used the inhibitor of p38 MAPK (SB203580) to suppress this pathway, the effects of miR-3188 mimic on apoptosis and migration were significantly reversed, suggesting that miR-3188 regulated breast cancer progression via the p38 MAPK signaling pathway. In conclusion, our study revealed that miR-3188 was upregulated in breast cancer and had effects on cancer cell proliferation, apoptosis, and migration by targeting TUSC5 and activating the p38 MAPK signaling pathway. miR-3188 may serve as a potential therapeutic agent for the treatment of breast cancer.

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