

# MiR-155 promotes the proliferation and migration of breast cancer cells via targeting SOCS1 and MMP16

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**Abstract.** – **OBJECTIVE:** The aim of this study was to investigate the effect of miR-155 on the proliferation and migration of breast cancer cells, and to explore the underlying mechanism.

**MATERIALS AND METHODS:** The breast cancer cell line MDA-MB-231 was transfected with miR-155 mimics, inhibitor or negative control, respectively. The expression level of miR-155 was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Subsequently, the proliferation of MDA-MB-231 cells was detected by multi-cellular tumor spheroid (MTS) and colony formation assay. Cell migration was examined by transwell assay and scratch test. In addition, qRT-PCR was performed to analyze the expression of matrix metalloproteinase 16 (MMP16) after miR-155 mimics or inhibitor transfection in MDA-MB-231 cells. Meanwhile, Western blot was used to evaluate the protein expression levels of suppressor of cytokine signaling 1 (SOCS1) and MMP16 after miR-155 mimics or inhibitor transfection.

**RESULTS:** QRT-PCR results showed that miR-155 mimics significantly increased the expression of miR-155 in MDA-MB-231 cells, whereas miR-155 inhibitor markedly decreased miR-155 expression ( $p < 0.05$ ). Meanwhile, MTS and colony formation assay indicated that the proliferation of MDA-MB-231 cells was remarkably increased after miR-155 mimics transfection. However, miR-155 inhibitor transfection exhibited the opposite result in cell proliferation ( $p < 0.05$ ). Moreover, miR-155 overexpression significantly increased the migration of MDA-MB-231 cells ( $p < 0.05$ ). Western blot further confirmed that miR-155 overexpression down-regulated the expression level of target protein SOCS1 and upregulated the expression level of MMP16.

**CONCLUSIONS:** We found that miR-155 significantly enhanced the proliferation and migration of MDA-MB-231 cells, which might serve as

an oncogene in breast cancer. Therefore, it is preliminarily believed that miR-155 plays an important role in the proliferation and migration of breast cancer cells via down-regulating the expression of SOCS1 and up-regulating the expression of MMP16.

*Key Words:*

MicroRNA-155 (MiR-155), Breast cancer, Cell proliferation, Cell migration, MMP16, SOCS1.

## Introduction

Breast cancer is the most common malignant tumor that seriously endangers female health. The incidence of breast cancer accounts for 7%-10% of all types of human malignant tumors, which has been rapidly increased in recent years. Nowadays, multiple researches have investigated the pathogenesis of breast cancer. However, the underlying mechanism is still not fully understood.

MicroRNA (MiRNA) is one of the most important tumorigenic factors discovered in recent years. MiRNA is a type of endogenous, single-stranded, non-coding RNA discovered in recent years, with 19-24 nucleotides in length<sup>1-3</sup>. It is reported that miRNA exhibits a regulatory role, and can bind to the 3'UTR region of target genes in an incompletely complementary manner. MiRNA can degrade target genes at the mRNA level or inhibit protein translation. Moreover, miRNA negatively regulates the target genes at the post-transcriptional level. Several studies<sup>4-9</sup> have discovered that miRNA is involved in the proliferation, migration, and

invasion of malignant tumors. Multiple studies<sup>10-16</sup> have shown that miR-155 is significantly up-regulated in breast cancer, which is closely related to clinic-pathological markers, tumor stage, radiotherapy and chemotherapy sensitivity, and low survival rate.

In the present study, we aimed to investigate the function of miR-155 in breast cancer cells and to explore its underlying mechanism, thereby serving as the basis for targeted therapy of breast cancer in the future.

## Materials and Methods

### Cell Lines and Reagents

The breast cancer cell line MBA-MD-231 was purchased from the Cell Bank of Chinese Academy of Sciences in Shanghai (Shanghai, China). Hank's balanced salt solution, trypsin, and Lipofectamine RNAiMAX reagent were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and L15 were obtained from Gibco (Rockville, MD, USA). Transfection sequences were designed and synthesized by GenePharma (Shanghai, China) (miR-155 mimics: UUA AUGCUAAUCGUGAUAGGGGU-CCCUAUCAGAUUAGCAUUAUU; mimics NC: UUCUCCGAACUUCACGUTTACGUGACAGUUCGGAGAATT; miR-155 inhibitor: ACCCUAUCACGAUUAGCAUUA; inhibitor NC: CAGUACUUUUGUGUAGUACAAAA; matrix metalloproteinase 16 (MMP16) primer sequence: Forward: ATCCCAAGCCAATCA-CAGTCT; Reverse: TGGTCCATCACAGC-CCATAA).

### Cell Transfection

MDA-MB-231 cells were seeded into 6-well plates and cultured in a 37°C, 5% CO<sub>2</sub> incubator. After 24 hours of culture, the original culture medium was replaced. Transfection solution was prepared according to the instructions of Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, miR-155 mimics, miR-155 inhibitor or Lipofectamine RNAiMAX was diluted in serum-free medium, respectively. The OPTI-MEM I transfection solution was also diluted in the culture medium. Subsequently, diluted solution was incubated at room temperature for 5 min, followed by mixing together. After incubation at room temperature for 15 min, the mixture was added to each well for cell transfection.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of transfected cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The absorbance (A) at 260 nm and 280 nm was measured by an ultraviolet spectrophotometer. The concentration was calculated by A<sub>260</sub>, and the purity of was calculated by A<sub>260/280</sub>. TaqMan Real-time PCR was performed to detect the expression level of miR-155 in cells transfected with miR-155 mimics/NC and miR-155 inhibitor/NC. U6 was used as an internal reference. The reverse transcription system included: 0.15 μL dNTP (100 mM), 1 μL reverse transcriptase, 1.5 μL 10 × RT buffer, 0.188 μL RNase inhibitor, 1 μL RNA sample, 3 μL miR-155/U6 RT primer, and 8.162 μL NFH<sub>2</sub>O. Real-time PCR reaction system included: 10 μL 2 × PCR master mix, 1 μL 20 × Taqman assay mix, 1.33 μL miR-155/U6 RT product, and 7.67 μL H<sub>2</sub>O. QRT-PCR procedures were performed as follows: 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, for a total of 40 cycles. Primer sequences used in this study were as follows: microRNA-155, F: 5'-AACTTGTAACCTCCCTCGACTG-3', R: 5'-CCTTACGTGACCTGGAGTCG-3'; MMP16, F: 5'-TCACAGCGGATTTGGACAA-3', R: 5'-AATGCGAATAGCGACGTTCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### Multicellular Tumor Spheroid (MTS) Assay

MDA-MB-231 cells were seeded into 96-well plates at a density of 1 × 10<sup>3</sup> cells/well, with 6 replicates in each group. The cells were incubated in a 37°C, 5% CO<sub>2</sub> incubator for 24 h. Subsequently, the cells were transfected with mimics NC or miR-155 mimics, and inhibitor NC or miR-155 inhibitor according to standard instruction, respectively. 24, 48, and 72 h after transfection, the original culture medium was discarded. Next, 100 μL serum-free F12 culture medium and 14.5 μL MTS mixture (PMS: MTS = 1:19) were added to each well (Abcam, Cambridge, MA, USA), followed by incubation for 1-3 h. The optical density (OD) value at the wavelength of 490 nm was measured by a microplate reader, and the growth curve was plotted.

### Colony Formation Assay

MDA-MB-231 cells were seeded into 24-well plates at a density of 3-4 × 10<sup>3</sup> cells/well. After culturing for 24 h, the cells were transfected

with mimics NC/miR-155 mimics, and inhibitor NC/miR-155 inhibitor, respectively. 24 h after transfection, the cells were digested with trypsin, centrifuged at 1000 rpm/min for 3 min, and then resuspended in 1 mL complete culture medium for cell counting. Then, the cells were seeded into 6-well plates at a density of 800-900 cells/well, and the inoculated cells were evenly distributed, followed by incubation for 7-8 days. Subsequently, the culture medium was discarded and the cells were washed with phosphate-buffered saline (PBS) once. The cells were fixed with 10% formaldehyde and stained with crystal violet for 10-15 min. After washing with PBS and air dry, the colonies were captured and the number was calculated.

### **Scratch Assay**

MDA-MB-231 cells were seeded into 12-well plates with 20% cell confluence. After culturing for 24 h, the cells were transfected with mimics NC/miR-155 mimics, and inhibitor NC/miR-155 inhibitor, respectively. When the cell confluence reached 80%, a sterile 10  $\mu$ L micropipette tip was used to scratch the cell plate vertically. After removing exfoliated cells with PBS, serum-free medium was added for 48 h-incubation. Cell migration was observed under an inverted microscope, and the width of the scratch was measured and photographed.

### **Transwell Assay**

MDA-MB-231 cells were seeded into 12-well plates at a density of  $1 \times 10^5$  cells/well, with 3 replicates in each group. After culturing for 24 h, the cells were transfected with mimics NC/miR-155 mimics, and inhibitor NC/miR-155 inhibitor, respectively. 8 h after transfection, the culture medium was replaced with serum-free medium for continuous culture for 18 h. Then, the medium was removed, and the cells were washed with Hank's salt solution once and then digested with trypsin. Subsequently, 200  $\mu$ L cell suspension and 500  $\mu$ L medium containing 10 ng/mL HGF (hepatocyte growth factor) were added into the lower and the upper chamber, respectively. After culturing for 48 h, the cells were fixed with 10% formaldehyde for 15-20 min and stained with crystal violet for 20-30 min. The cells on the upper surface of the membrane were gently wiped with cotton swabs, and the cells on the lower surface were retained. Totally 5 randomly selected fields were observed under a microscope, and the number of migrated cells was counted.

### **Prediction of Target Genes**

TargetScan online prediction website was applied to predict target genes of miR-155, and those with a high degree of match were selected.

### **Western Blot**

Transfected cells were lysed with cell lysis buffer. The concentration of extracted protein was measured by the bicinchoninic acid (BCA) reagent kit (Pierce Biotechnology, Rockford, IL, USA). Extracted proteins were separated on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with specific primary antibodies (dilution at 1:800) at 4°C overnight. Then, the membranes were washed with Phosphate Buffered Saline-Tween (PBST) (Beyotime, Shanghai, China) 3 times, followed by incubation with secondary antibodies at room temperature for 2 h. Subsequently, the membranes were washed with 3 times PBST, with 10 min each time. Finally, immunoreactive bands were exposed by the enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

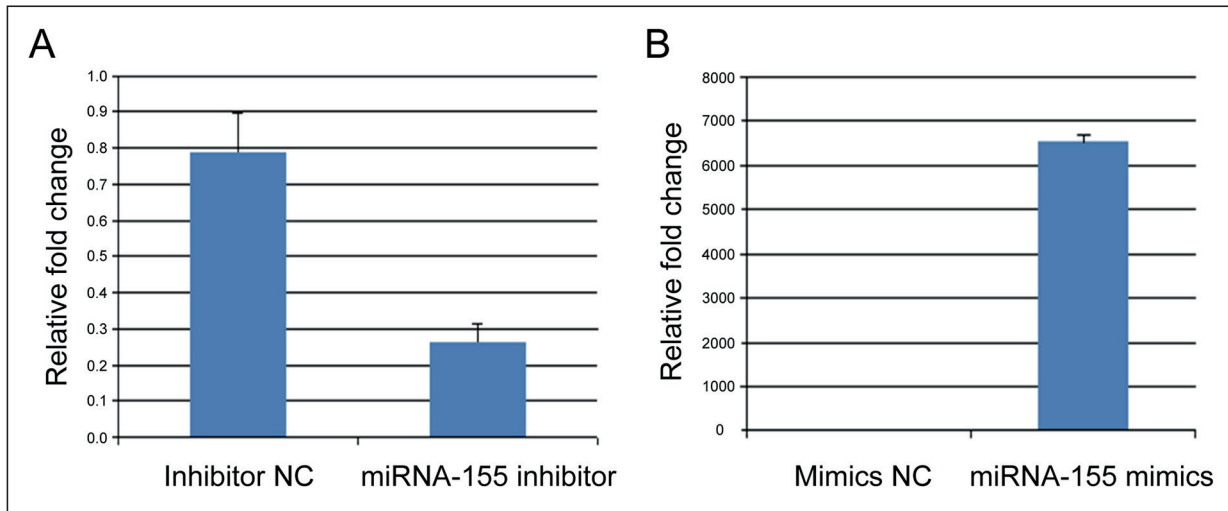
### **Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 17.0 Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean  $\pm$  standard error (mean  $\pm$  SEM). One-way ANOVA was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). Levene's test was used to measure the homogeneity of variance.  $p < 0.05$  was considered statistically significant.

## **Results**

### **Expression of MiR-155 After Mimics/Inhibitor Transfection**

MDA-MB-231 cells were transfected with mimics NC, inhibitor NC, miR-155 mimics, or miR-155 inhibitor, respectively. 48 h after transfection, we detected the expression level of miR-155 in MDA-MB-231 cells by qRT-PCR. Results showed that miR-155 mimics significantly up-regulated the expression level of miR-155 in MDA-MB-231 cells (mimics NC vs. miR-155 mimics:  $0.788 \pm 0.11$  vs.  $0.264 \pm 0.052$ ,



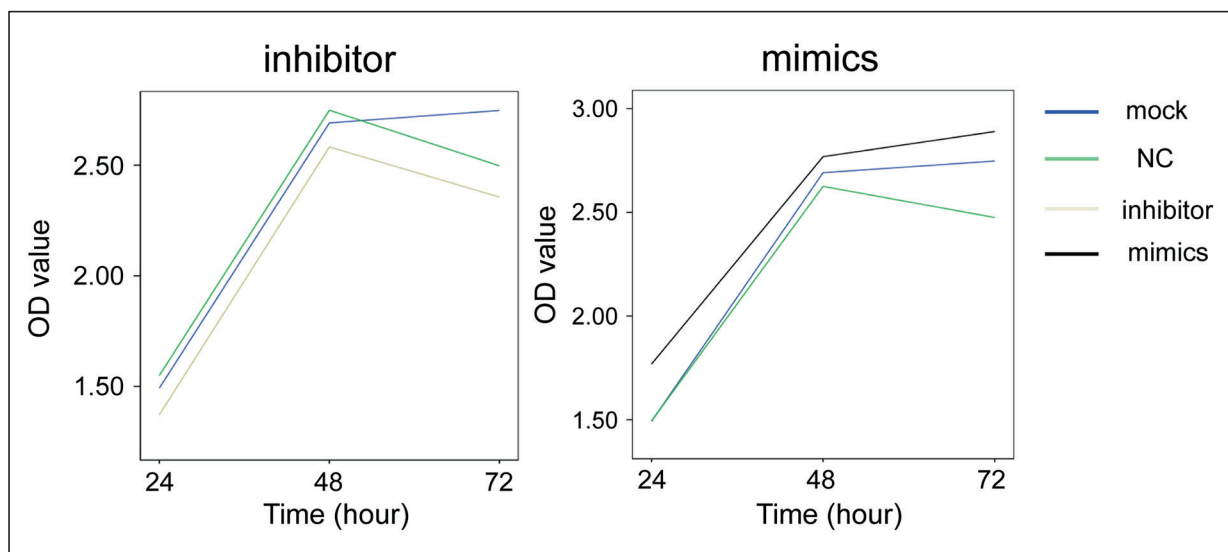
**Figure 1.** The expression level of miR-155 in MDA-MB-231 cells after transfection with (A) inhibitors or (B) mimics.

$p < 0.05$ ) (Figure 1A). Meanwhile, miR-155 inhibitor significantly decreased the expression level of miR-155 (inhibitor NC vs. miR-155 inhibitor:  $1.092 \pm 0.099$  vs.  $6535.349 \pm 161.7$ ,  $p < 0.05$ ) (Figure 1B).

#### **MiR-155 Significantly Increased the Proliferation of Breast Cancer Cells**

MTS assay was performed to detect the proliferation of MDA-MB-231 cells at 24, 48, and 72 h after transfection, respectively. Results indicated that the proliferation of MDA-MB-231

cells transfected with miR-155 mimics was significantly increased when compared with those transfected with mimics NC ( $p < 0.05$ ). However, the growth of MDA-MB-231 cells transfected with miR-155 inhibitor was markedly decreased from the second day, which was significantly lower than that of the inhibitor NC group ( $p < 0.05$ ) (Figure 2). These results all indicated that miR-155 could remarkably enhance the proliferation of MDA-MB-231 cells and might play an important role in the regulation of breast cancer cell proliferation.



**Figure 2.** MiR-155 promoted the proliferation of MDA-MB-231 cells. MTS was used to detect the proliferation ability at 24, 48, and 72 h after transfection.

### **MiR-155 Significantly Increased the Colony Formation Ability of Breast Cancer Cells**

Colony formation assay was used to further confirm the effect of miR-155 on the growth of MDA-MB-231 cells. Our results showed that compared with the mimics NC group, the number of colonies in the miR-155 mimics group was significantly higher ( $p < 0.05$ ). However, the number of colonies in the miR-155 inhibitor group was significantly lower than that of the inhibitor NC group ( $p < 0.05$ ) (Figure 3). The above results suggested that miR-155 could enhance the colony formation ability of breast cancer cells.

### **MiR-155 Promoted Lateral Migration of Breast Cancer Cells**

As shown in Figure 4, after 2 days of scratch, the healing speed of the miR-155 mimics group was significantly faster than that of the mimics NC group. However, the healing speed of the miR-155 inhibitor group was markedly slower than that of the inhibitor NC group ( $p < 0.05$ ). These results demonstrated that miR-155 promoted lateral migration of breast cancer cells.

### **MiR-155 Enhanced Vertical Migration of Breast Cancer Cells**

Subsequently, the transwell assay was performed to further detect the effect of miR-155 on the vertical migration ability of breast cancer cells. Results showed that the number of migrated MDA-MB-231 cells transfected with miR-155 mimics was significantly higher than that of the mimics NC group (miR-155 mimics vs. mimics NC:  $376.7 \pm 25.2$  vs.  $166.7 \pm 15.3$ ,  $p < 0.01$ ). Conversely, the number of migrated cells in the miR-

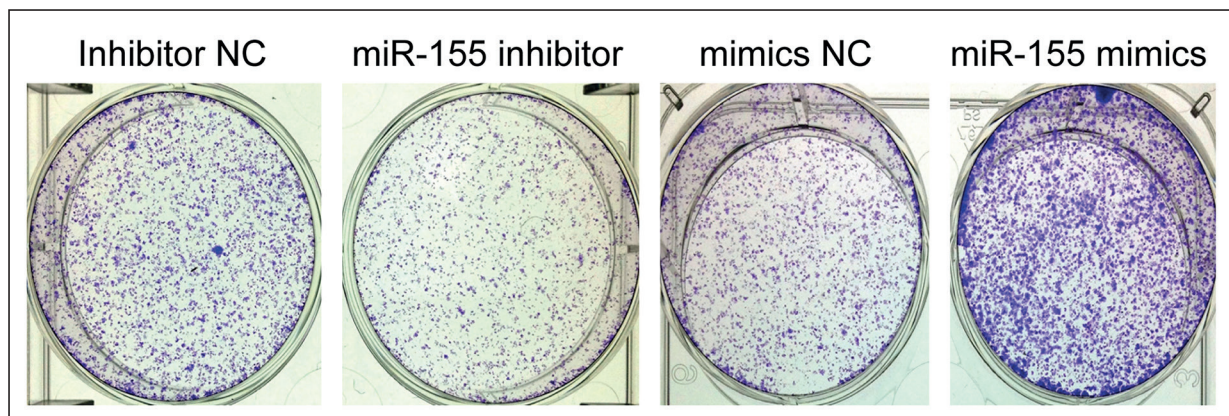
155 inhibitor group was markedly lower than the inhibitor NC group (miR-155 inhibitor vs. inhibitor NC:  $56.3 \pm 6.5$  vs.  $141.7 \pm 10.4$ ,  $p < 0.01$ ) (Figures 5 and 6). These results indicated that miR-155 enhanced the vertical migration ability of breast cancer cells.

### **Effect of MiR-155 on the mRNA Expression Level of MMP16**

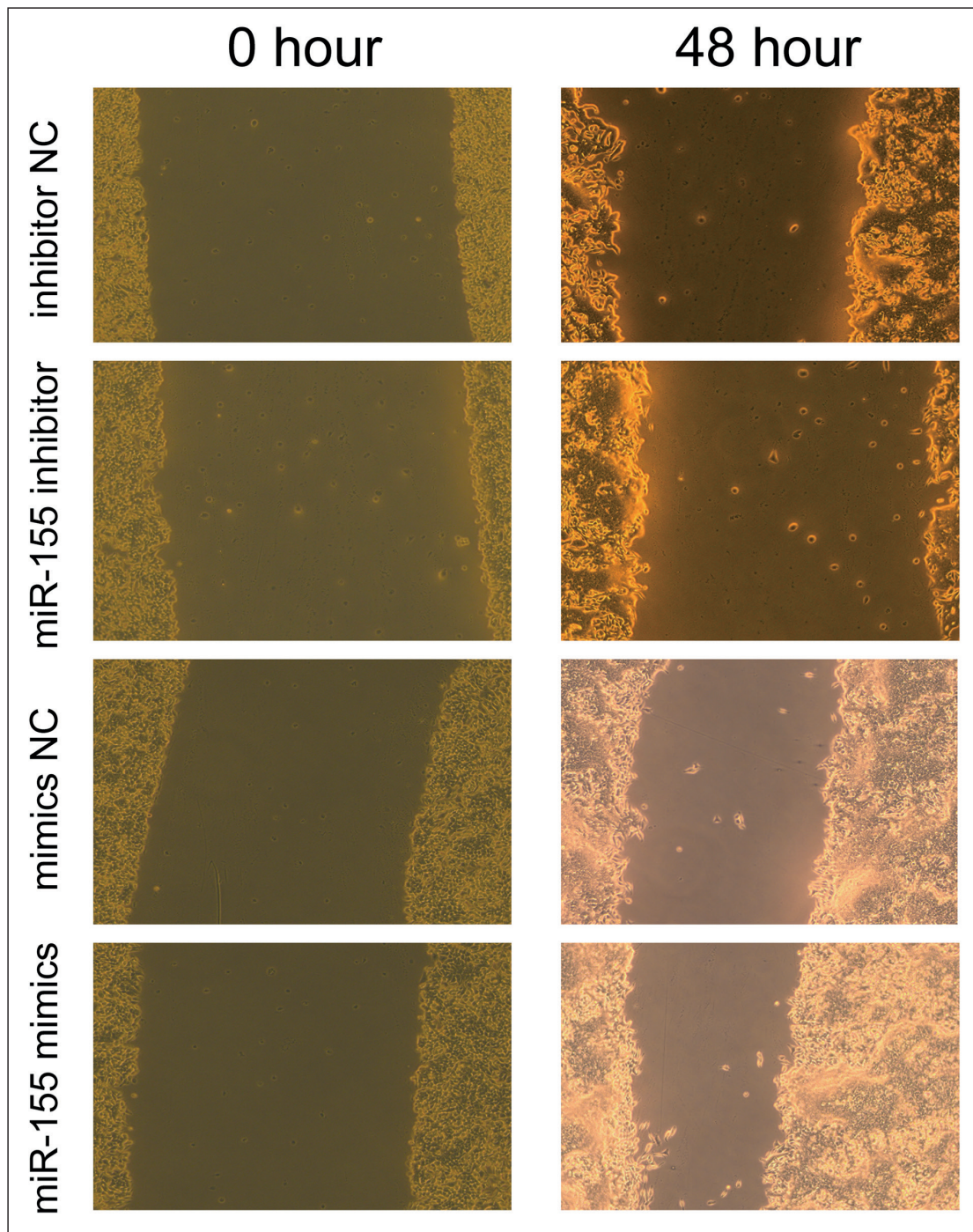
Subsequently, qRT-PCR was performed to detect the mRNA expression level of the target gene (MMP16) of miR-155. Results demonstrated that the expression level of MMP16 in MDA-MB-231 cells transfected with miR-155 inhibitor was significantly lower than that of those transfected with inhibitor NC. On the contrary, the expression level of MMP16 in the miR-155 mimics group was significantly higher than the mimics NC group ( $p < 0.05$ ) (Figure 7). Therefore, we concluded that miR-155 significantly enhanced the expression level of MMP16.

### **Effect of MiR-155 on the Protein Expression of MMP16 and Suppressor of Cytokine Signaling 1 (SOCS1)**

Previous studies have shown that SOCS1 is a target gene of miR-155. Therefore, we further detected the protein expression of SOCS1 as well as migration related gene MMP16 in MDA-MB-231 cells by Western blot. Results illustrated that the protein expression level of SOCS1 in MDA-MB-231 cells transfected with miR-155 mimics was significantly lower than the mimics NC group. However, the protein expression level of MMP16 exhibited the opposite result ( $p < 0.05$ ). At the same time, when compared with



**Figure 3.** MiR-155 promoted the colony formation ability of MDA-MB-231 cells.

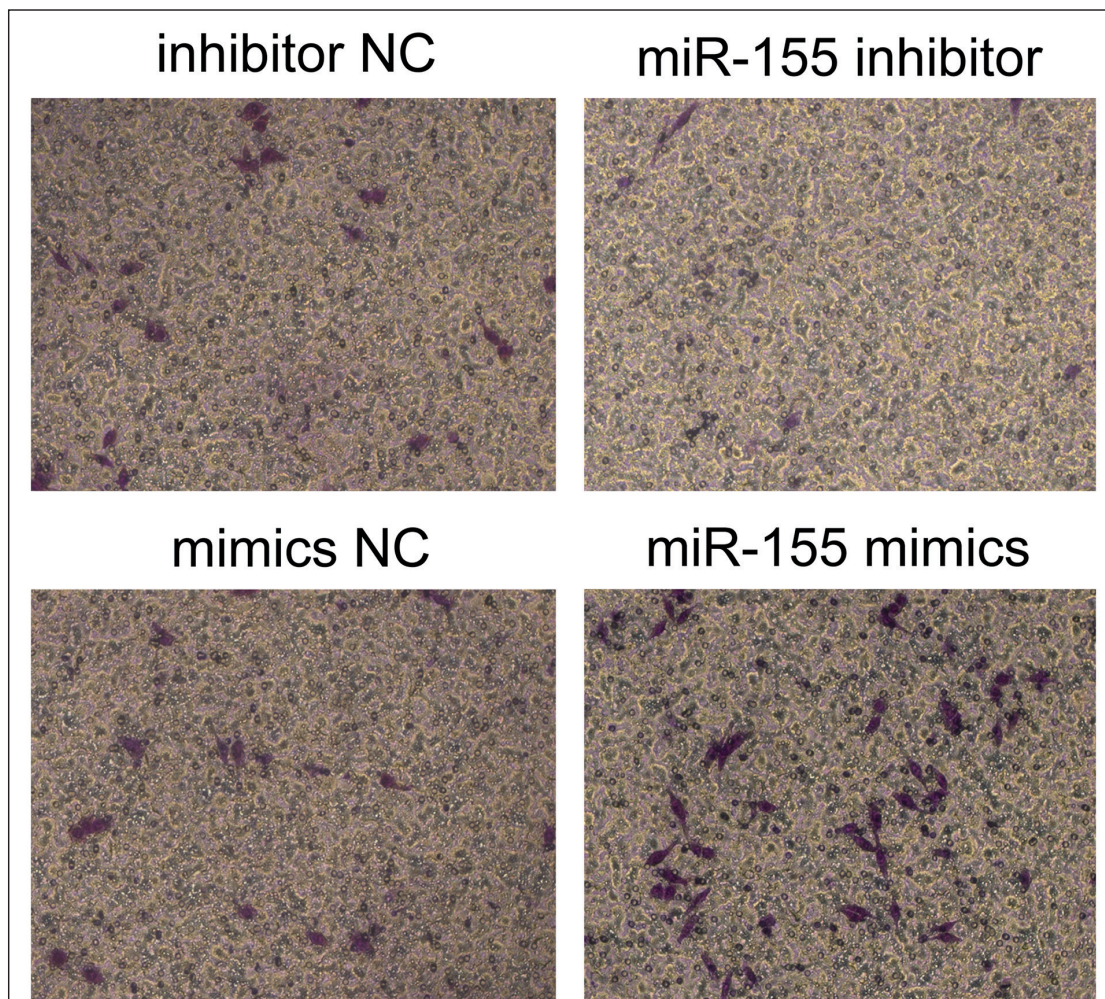


**Figure 4.** MiR-155 enhanced lateral migration of MDA-MB-231 cells (magnification: 100 ×).

the inhibitor NC group, the protein expression of SOCS1 increased significantly, and MMP16 was markedly decreased in the miR-155 inhibitor group. This demonstrated that miR-155 significantly decreased the protein expression of SOCS1 and increased the expression of MMP16 in breast cancer cells (Figure 8).

## Discussion

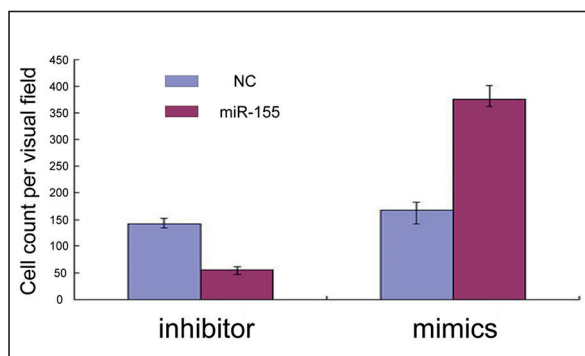
MiRNA is a type of non-coding small RNA widely distributed in eukaryotes. Several studies have indicated that miRNA mediates gene expression at the post-transcriptional level, and is closely related to the occurrence and develop-



**Figure 5.** MiR-155 promoted vertical migration of MDA-MB-231 cells (magnification: 200 ×).

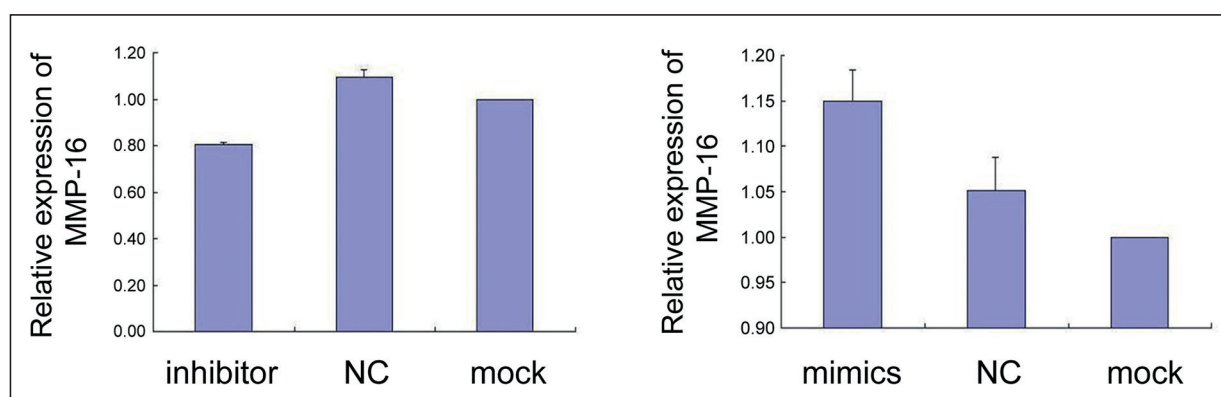
ment of human diseases, especially for cancer<sup>1,2</sup>. Differentially expressed miRNAs in tumor cells play an important role in the proliferation, apoptosis, migration, and invasion of tumor cells. As

reported previously, miR-155 acts as an oncogene in a variety of tumors, such as cervical cancer, breast cancer, lung cancer, gastric cancer, thyroid cancer, renal clear cell carcinoma, and B cell lymphoma<sup>3-11</sup>.



**Figure 6.** MiR-155 increased the number of perforated cells in MDA-MB-231 cells.

Breast cancer is one of the most common malignant tumors in women, whose incidence has increased rapidly in recent years. Nowadays, a large number of studies have shown that miR-155 is highly expressed in breast cancer, and is closely correlated with metastasis and infiltration, endocrine resistance, treatment, and prognosis of breast cancer. Moreover, the carcinogenic role of miR-155 has been widely investigated *in vitro* and *in vivo*<sup>12-30</sup>. Although these studies have preliminarily investigated the role of miR-155 in the diagnosis and prognosis of breast cancer, the specific underlying mechanism remains unclear. The regulatory



**Figure 7.** The expression level of MMP16 in MDA-MB-231 cells after transfection with inhibitors or mimics.

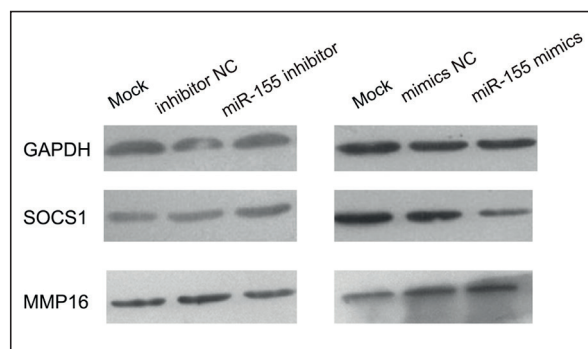
process of miR-155 on tumors is diverse and complex. Meanwhile, target genes in the downstream of miR-155 have not been well elucidated. Our study aimed to provide evidence for the development of novel targets for the treatment of breast cancer, eventually improving the diagnosis, treatment, and prognosis of breast cancer.

Malignant tumors are characterized by the uncontrolled proliferation of tumor cells. Therefore, anti-proliferation is one of the effective ways for tumor therapy. In this study, the breast cancer cell line MDA-MB-231 was transfected with miR-155 mimics, and the transfection efficiency was verified by qRT-PCR. Subsequently, MTS assay was performed to detect the proliferation of breast cancer cells. Results found that cell proliferation of the miR-155 mimics group was significantly higher than the mimics NC group. Meanwhile, colony formation assay obtained the similar results. However, the proliferation ability and colony formation ability of the miR-155

inhibitor were significantly lower than those of the inhibitor NC group, suggesting that miR-155 could act as an oncogene in breast cancer.

Migration and invasion abnormalities are also the main characteristics of malignancies. Tumor cell migration and invasion are mainly related to the adhesion between cells, as well as the interaction between extracellular matrix and cytoskeleton. It has been confirmed that miRNAs play an important role in regulating the migration and invasion of cancer cells. Distant metastasis is one of the leading causes of death in advanced breast cancer patients. In this study, we detected the lateral and vertical migration ability of MDA-MB-231 cells after transfection with miR-155 mimics/mimics NC and miR-155 inhibitor/inhibitor NC. Both scratch test and transwell assay suggested that miR-155 could promote the migration and invasion abilities of breast cancer cells.

According to the TargetScan prediction software, SOCS1, known as a tumor suppressor gene cytokine 1, is a target gene of miR-155. In 2010, Jiang et al<sup>24</sup> found that SOCS1 was a conserved target gene for miR-155 evolution. In breast cancer cells, the expression of SOCS1 is negatively correlated with miR-155. Meanwhile, it prevents the phosphorylation of STATs by binding and inhibiting JAKs or competing with STATs for the phosphorylation target of a cytokine receptor. Therefore, miR-155 promotes the proliferation of cancer cells by downregulating the expression of SOCS1 and activating the JAK-STAT signaling pathway. The JAK-STAT signaling pathway is one of the important inflammatory pathways. Meanwhile, JAK-STAT can enhance the formation of inflammatory-related tumors. Interleukin-6 (IL-6), inflammato-



**Figure 8.** The protein expression of SOCS1 and MMP16 in transfected MDA-MB-231 cells was detected by Western blot.



ry cytokine IFN- $\gamma$ , and lipopolysaccharide (LPS) can also stimulate the proliferation of breast cancer cells *via* the JAK-STAT signaling pathway, indicating that miR-155 may be a bridge between inflammation and breast cancer<sup>19-22</sup>. In this study, we observed that miR-155 could significantly reduce the protein expression of SOCS1.

MMP16, belongs to the matrix metalloproteinase family (MMPs), is an important cytokine regulating the migration and invasion of tumors. Researchers have found that MMP16 also participates in the tumorigenesis in addition to normal physiological processes, such as embryonic development and tissue remodeling. Lin et al<sup>31</sup> have indicated that miRNA-146-5p inhibits the migration and infiltration of pancreatic cancer through targeting MMP16. Other studies have demonstrated that miRNA-146a inhibits MMP16 in the colon cancer cell line HT-29, eventually suppressing its invasion ability<sup>32</sup>. Meanwhile, some scholars<sup>33</sup> have indicated that MMP16 is highly expressed in breast cancer. Here in our study, we found that miR-155 can positively regulate the expression of MMP16, thereby promoting the migration of breast cancer cells. However, the exact regulatory mechanism of miR-155 and MMP16 needs to be further investigated.

In 2013, Yan et al<sup>34</sup> found that liver cancer related mesenchymal stem cells promoted the progression of liver cancer through the S100A4-miR155-SOCS1-MMP9 axis. Other reports have indicated that MMP16 activates MMP2 and MMP9<sup>32</sup>. However, no researches have reported the relationship between MMP16, SOCS1 and miR-155. Our study suggested that there might be a miR155-SOCS1-MMP16-MMP9 pathway in breast cancer. We considered that miR-155 downregulated SOCS1, thereby promoting the phosphorylation of STAT3 and increasing the expression of MMP16. Subsequently, the expression of MMP9 could be activated, eventually promoting the proliferation and migration of breast cancer. However, our hypothesis still needs to be further investigated.

## Conclusions

We explored the role of miR-155 in breast cancer cells and investigated the possible underlying mechanism. We showed that miR-155 promoted the proliferation and migration of MDA-MB-231

cells by down-regulating the expression of SOCS1 and up-regulating the expression of MMP16. Therefore, miR-155 might be a molecular target in the diagnosis and treatment of breast cancer.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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