

# MicroRNA-146a inhibits cell migration and invasion by targeting RhoA in breast cancer

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Received December 31, 2015; Accepted February 11, 2016

DOI: 10.3892/or.2016.4788

**Abstract.** MicroRNAs (miRNAs) function as genetic modulators that regulate gene expression and are involved in a wide range of biological roles, including tumor cell migration and invasion. In the present study, we demonstrated that the migration and invasion activity in MDA-MB-231 breast cancer cells could be directly influenced by altering miR-146a expression. The expression of RhoA and miR-146a in the breast cancer cells showed an inverse correlation. Upregulation of miR-146a in the MDA-MB-231 breast cancer cells by transfection of miR-146a mimics resulted in decreased RhoA protein levels. Conversely, downregulation of miR-146a by transfection of miR-146a inhibitor resulted in increased RhoA protein levels. To confirm the fact that RhoA is a potential target of miR-146a, luciferase reporter containing the RhoA 3' untranslated region (3'UTR) was constructed. The results demonstrated that the luciferase reporter activity was reduced after overexpression of miR-146a. Moreover, the luciferase reporter which was constructed with the RhoA 3'UTR mutant did not show significantly altered luciferase reporter activity. Furthermore, after treatment with the RhoA inhibitor exoenzyme C3 transferase protein, the migratory capacity of the MDA-MB-231 cells was not significantly altered even though the amount of miR-146a was changed. Our results indicate that miR-146a functions as a tumor suppressor in breast cancer cells. Downregulation of the expression of miR-146a increased the migration of MDA-MB-231 cells, due to the upregulation of RhoA expression.

## Introduction

Breast cancer is the most common cancer among women, and its mortality rate ranks second only after lung cancer (1). Metastasis is the main reason for the mortality of breast cancer

patients, and thus, it is crucial to understand the molecular and cellular mechanisms that cause primary tumors to metastasize.

RhoA is a prototypical member of the Rho GTPase family, and plays an important role in the regulation of cytoskeleton reorganization. RhoA regulates signal transduction from cell surface receptors to intracellular target molecules and is involved in a variety of biological processes, including cell motility (2), morphogenesis (3), cytokinesis (4,5) and tumor progression (6,7). Rho proteins bind to and activate a number of downstream effectors, leading to a cascade of biochemical responses. Previous studies have found that RhoA is upregulated in several types of human cancers, including colorectal (8,9) and breast cancer (10,11), hepatocellular carcinoma (12,13), gastric cancer (14,15), and alteration of the expression levels of intracellular RhoA can directly affect the process of invasion and metastasis in a variety of tumors (7,16,17).

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs with 20-25 nucleotides that regulate the expression of hundreds of target genes. miRNAs bind target mRNA sequences through canonical base pairing between the seed sequence, which includes nucleotides 2-8 from the 5'-end, and the complementary sequence found in the 3' untranslated region (3'UTR) of its target mRNA (18-20). Recently, numerous studies have demonstrated that miRNAs suppress the expression of cancer-related genes and reduce tumorigenesis and metastasis in breast and several other cancers (21-23).

miR-146a was found to act as a tumor suppressor by directly regulating NF- $\kappa$ B expression and affecting tumor cell signaling pathways (24). miR-146a is involved in cell proliferation and influences cancer metastasis in various types of tumors, including breast cancer (25-27). However, the regulation of breast cancer metastasis by miR-146a is a complex biological network. Therefore, the regulatory mechanisms of breast cancer metastasis by miR-146a warrant further in-depth study.

In the present study, among metastasis-related genes as potential targets of miR-146a, an inverse correlation was noted between RhoA and miR-146a expression in breast cancer cell lines. Furthermore, we found that miR-146a specifically caused the downregulation of RhoA expression. miR-146a was also found to directly target the RhoA 3'UTR, thereby downregulating breast cancer cell migration and invasion. The present study aimed to elucidate the interrelation between miR-146a and RhoA, searching for promising molecular targets to inhibit

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**Key words:** microRNA, miR-146a, breast cancer, migration, RhoA

metastasis and potential anti-metastatic agents for possible use in breast cancer therapy.

## Materials and methods

**Cell culture.** Human breast cancer cell lines, MCF-7 and MDA-MB-231, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Gibco, Carlsbad, CA, USA). The cells were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere until reaching 75% confluency. To block the function of RhoA, the RhoA inhibitor exoenzyme C3 transferase protein (C3) (Cytoskeleton, Denver, CO, USA), was added to the culture medium at a final concentration of 2.0 µg/ml.

**Transfection.** miR-146a mimics, miR-146a inhibitor, and control oligo were chemically synthesized by RiboBio (Guangzhou, Guangdong, China). All transfections were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The MDA-MB-231 cells were harvested 24 h after transfection, and various analyses were performed. Three independent experiments were carried out in duplicate.

**Plasmid constructs.** The 3'UTRs of the human RhoA gene (NM\_001664) were PCR amplified from human genomic DNA. The primers were digested and cloned into the pmiR-RB-REPORT™ vector (RiboBio) at the *Xho*I and *Not*I sites, and named pMIR-REPORT-RhoA-wt. The primer sequences were: h-RhoA-3UTR-forward (*Xho*I), 5'-CTTGAC TCGAGAACCTTGCTGCAAGCACAG-3' and h-RhoA-3UTR-reverse (*Not*I), 5'-ATTGCGGCCGCTGCCTTAT TCTATTAGTAG-3'. Site-directed mutagenesis with miR-146a target sites in the RhoA was carried out using a site-directed mutagenesis kit (Takara, Dalian, Liaoning, China), with pMIR-REPORT-RhoA-wt as a template, and named pMIR-REPORT-RhoA-mut, respectively. The primer sequences were: h-RhoA-3UTR-mut-forward, 5'-AGCCACGTCA AGAGATGGGACCCTCAGTCACAGAG-3' and h-RhoA-3UTR-mut-reverse, 5'-TCCCATCTCTTGACGTGGCTCCT CTGGGAGGGAAC-3'.

**Luciferase assay.** MDA-MB-231 cells were seeded into a 24-well plate 24 h before transfection. They were transfected with 0.4 µg of the pMIR-REPORT-RhoA-wt or pMIR-REPORT-RhoA-mut, 20 µM miR-146a mimics or control oligo using Lipofectamine 2000. Firefly and *Renilla* luciferase activities were measured consecutively using a Dual-Luciferase assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. In addition, 0.02 µg of the *Renilla* luciferase vector pRL-TK (Promega) was used for normalization. Three independent experiments were performed in triplicate.

**Quantitative real-time PCR analysis (qRT-PCR).** Total RNA was prepared using TRIzol, and cDNA was generated using the PrimerScript RT reagent kit and amplified using RhoA primers with SYBR Premix Ex-Taq™ kit (all from Takara). The primer sequences for RhoA were: 5'-TTTGGAGGTGGCATAGCCTT-3' (forward)

and 5'-ATGTTTAGTCAGCTGGAGAGAAGAG-3' (reverse), and the primer sequences for β-actin were: 5'-CCCTGGACTTCGAGCAAGAG-3' (forward) and 5'-AATGCCAGGGTACATGGTGG-3' (reverse). For analysis of miRNA expression by qRT-PCR, the miRcute miRNA isolation kit, miRcute miRNA First-Strand cDNA synthesis kit, miRcute miRNA qPCR detection kit and miR-146a detection primer were purchased from Tiangen Biotech (Beijing, China) to detect miR-146a and the control miRNA (U6 snRNA). Amplification and detection were performed using StepOne Plus QRT-PCR system (ABI, USA). β-actin was used to normalize the expression of RhoA, whereas U6 was used to normalize the expression of miR-146a; data were calculated based on the following equation:  $RQ = 2^{-\Delta\Delta Ct}$ .

**Western blot analysis.** Protein was extracted from breast cancer cell lines using RIPA buffer [150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA] including a protease inhibitor cocktail and phosphatase inhibitors (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Equal amounts of protein sample (50 µg) were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes using the Bio-Rad semi-dry transfer system. Western blotting was performed using anti-β-actin and anti-RhoA (CST, Denver, CO, USA). The signals were detected with an ECL kit (Beyotime, Nantong, Jiangsu, China) following the manufacturer's instructions.

**Cell migration assay.** Transwell invasion assays were performed using the MDA-MB-231 cells, which were transfected with the miR-146a mimics, miR-146a inhibitor and control oligo for 24 h. Then, the cells were resuspended in serum-free DMEM, and placed in the top chamber with a Matrigel-coated membrane. The lower portion of the chamber contained 20% FBS as a chemoattractant. After the cells were cultured for 24 h, we washed the chambers twice with phosphate-buffered saline (PBS), and stained the chambers using crystal violet. Five random fields were randomly selected, and images were captured from each chamber. Then, the chambers were decolorized by glacial acetic acid, and quantitative analysis was carried out using the enzyme marker at 570 nm wavelength. Three independent experiments were carried out in duplicate.

**Wound healing assay.** When cell confluency was <90% at 24 h after transfection, wounds were created in the confluent cells using a 200-µl pipette tip, and any free-floating cells and debris were removed by PBS. Medium was then added, and the culture plates were incubated at 37°C. Wound healing within the scraped wound line was observed at a 24-h time point, and representative scrape lines for each cell line were photographed. The optical distance of the wound was measured using ImageJ software. Three independent experiments were carried out in duplicate.

**Statistical analysis.** All data are presented as mean ± SD of three independent experiments. Assays for characterizing the phenotypes of the cells were analyzed by Student's t-test or

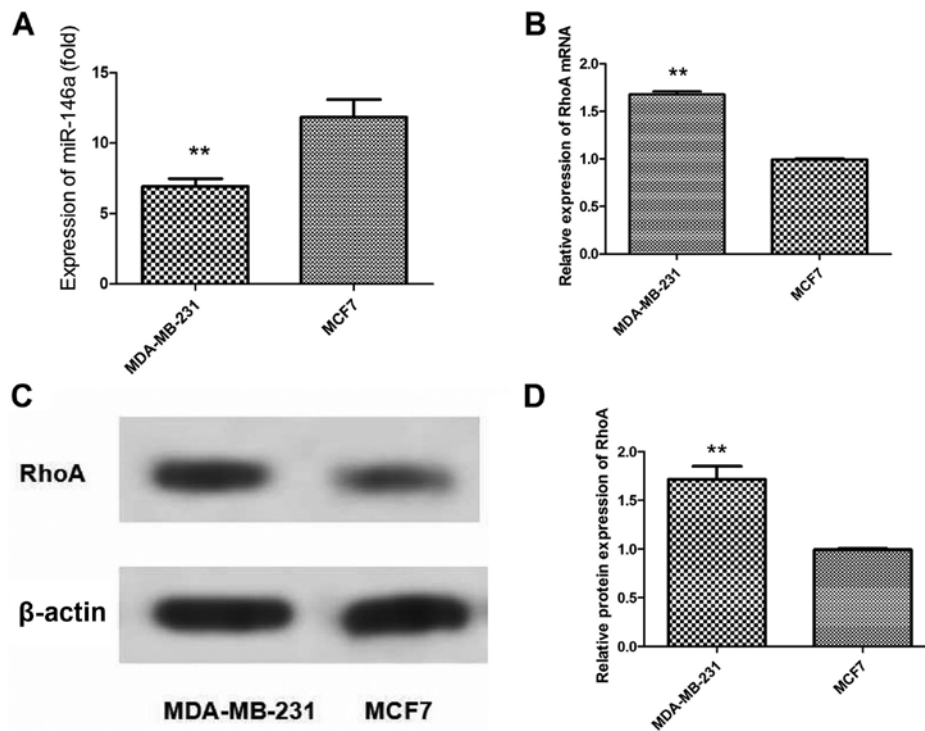


Figure 1. Differential expression of miR-146a and RhoA in breast cancer cell lines with different metastatic activity. (A) Expression of miR-146a in MDA-MB-231 and MCF-7 cells was assessed by qRT-PCR. The miR-146a expression level was normalized to U6 snRNA. We used  $RQ = 2^{-\Delta\Delta Ct}$  to represent the change in expression:  $-\Delta\Delta Ct = -[(Ct_{146aX} - Ct_{U6X}) - (Ct_{146aMCF7} - Ct_{U6MCF7})]$ . (B) Expression of RhoA mRNA in the MDA-MB-231 and MCF-7 cells was also assessed by qRT-PCR. In addition, the gene expression level was normalized to  $\beta$ -actin. (C and D) The relative protein expression of RhoA was measured by western blot analysis and normalized to  $\beta$ -actin. The data are presented as the mean  $\pm$  SD. n=3; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. the MCF-7 cells.

one-way ANOVA. P-value of <0.05 was considered to indicate a statistically significant result. All statistical analyses were performed using the SPSS 22.0 software package (SPSS, Inc., Chicago, IL, USA).

## Results

**Differential expression of miR-146a and RhoA in breast cancer cells with different metastatic activity.** To assess whether miR-146a levels were altered in breast cancer cell lines with different metastatic potential, two breast-derived cell lines were used to assess the relative levels of miR-146a. The basal expression level of miR-146a was generally increased in the tumorigenic but weakly metastatic cell line MCF7, compared with this level in the highly metastatic cell line MDA-MB-231 (Fig. 1A). In contrast, the expression levels of RhoA mRNA and protein in MCF7 cells were obviously decreased when compared with these levels in the MDA-MB-231 cells (Fig. 1B-D).

**miR-146a suppresses metastatic activity in the MDA-MB-231 cells.** To confirm whether change in the expression of miR-146a can change the invasion and metastasis capabilities of the MDA-MB-231 cells, we upregulated miR-146a by transfecting the MDA-MB-231 cells with miR-146a mimics, and downregulated miR-146a by transfecting the MDA-MB-231 cells with the miR-146a inhibitor. The results of the Transwell experiments showed that the number of migrating cells was decreased after transfection with the miR-146a mimics. In

contrast, the number of migrating cells was increased after transfection with the miR-146a inhibitor (Fig. 2A). A similar trend was observed in the wound healing assays (Fig. 2B).

**Expression of RhoA varies with the amount of miR-146a expression in MDA-MB-231 cells.** To confirm whether suppression of migration by miR-146a is associated with changes in RhoA expression, we transfected miR-146a mimics and the inhibitor into the MDA-MB-231 cells and detected the expression of RhoA at the mRNA and protein levels by qRT-PCR and western blotting, respectively. The qRT-PCR results showed that the expression of miR-146a was significantly altered after transfection with the miR-146a mimics and inhibitor in the MDA-MB-231 cells (Fig. 3A). In addition, the qRT-PCR results showed that the expression of RhoA mRNA was not significantly altered after transfection with the miR-146a mimics and inhibitor in the MDA-MB-231 cells (Fig. 3B). However, in the western blot analysis (Fig. 3C and D), the RhoA protein concentration was decreased (P<0.01), when miR-146a was upregulated. In contrast, the RhoA protein concentration was increased (P<0.05), while miR-146a was downregulated. These results indicated that miR-146a post-transcriptionally regulated RhoA expression.

**RhoA 3'UTR is a potential miR-146a target gene.** To study how miR-146a may regulate migration, we proceeded to identify potential targets known to play a role in cell mobility using miRBase databases. Among the candidates surveyed, we found that the 3'UTR of the RhoA gene, which plays an important role

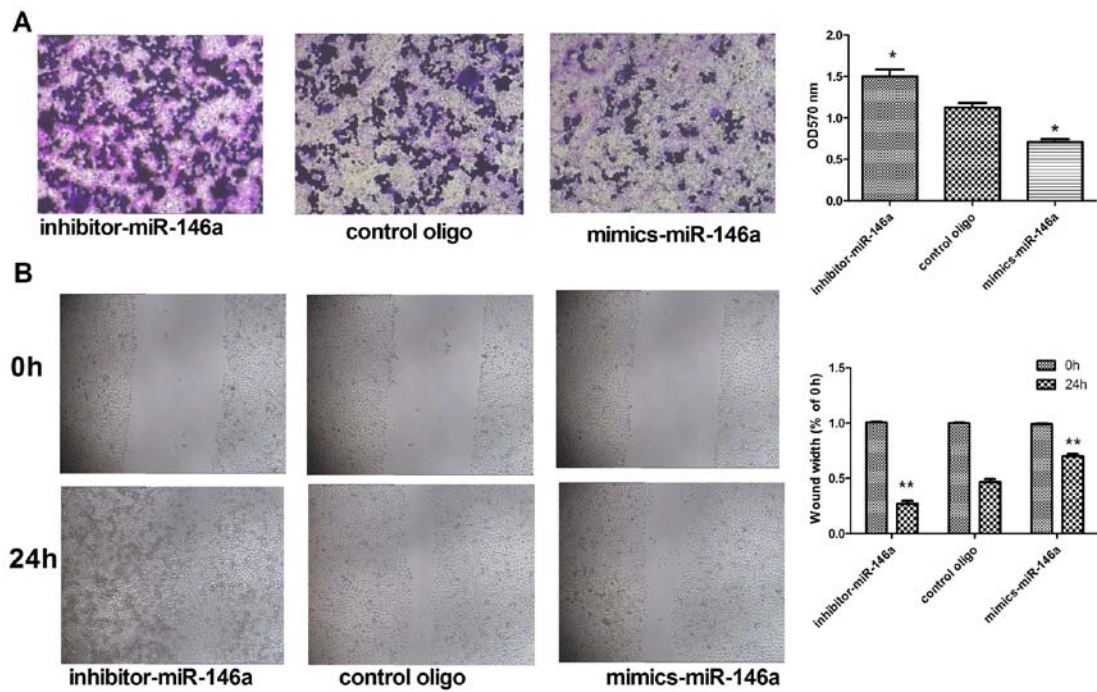


Figure 2. miR-146a suppresses the metastasis of MDA-MB-231 cells. (A) Effects of miR-146a on cell migration and invasion in the MDA-MB-231 cells was demonstrated by Transwell assay. The membrane of the upper chambers was coated with 1:7 Matrigel basement membrane matrix. Cells were harvested 24 h after transfection, then added ( $1.0 \times 10^5$  cells/well) to serum-free medium in the upper chambers, and incubated in complete medium containing 20% FBS for 24 h. Images of cell invasion through the polycarbonate membrane stained with crystal violet (magnification,  $\times 200$ ) are shown on the left. OD570 of crystal violet staining is shown on the right. (B) Effects of miR-146a on MDA-MB-231 cell migration by wound healing assay. MDA-MB-231 cells were plated in 6-well plates, and allowed to grow to 90% confluency in complete medium after transfection for 24 h. Cell monolayers were wounded with a sterile micropipette tip ( $200 \mu\text{l}$ ), and cells were further incubated for 24 h in serum-free medium and migrated into wound surface. Images were captured at 0 and 24 h after wounding, and the wound widths were quantified. Data are presented as the mean  $\pm$  SD.  $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs. the control group.

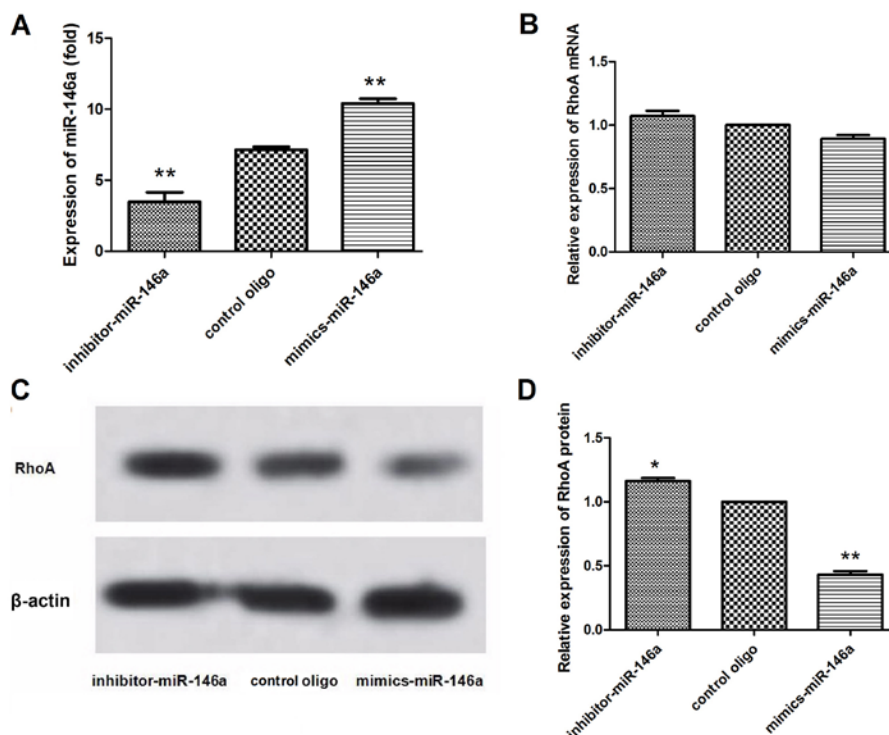


Figure 3. Expression of RhoA varies with the level of miR-146a expression in MDA-MB-231 cells. (A) To alter the amount of miR-146a expression, MDA-MB-231 cells were transfected with miR-146a mimics, miR-146a inhibitor and control oligo. Gene expression levels were assessed by qRT-PCR 48 h after transfection. The miR-146a expression level was normalized to U6 snRNA. We used  $RQ = 2^{-\Delta\Delta Ct}$  to represent the change in expression:  $-\Delta\Delta Ct = -[(Ct146ax - CtU6x) - (Ct146control - CtU6control)]$ . (B) Expression of RhoA mRNA in MDA-MB-231 cells with different levels of miR-146a expression were also measured by qRT-PCR. In addition, the gene expression level was normalized to  $\beta$ -actin. (C and D) The relative protein expression levels of RhoA were measured by western blot assay and normalized to  $\beta$ -actin. The data are presented as the mean  $\pm$  SD.  $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs. the control group.

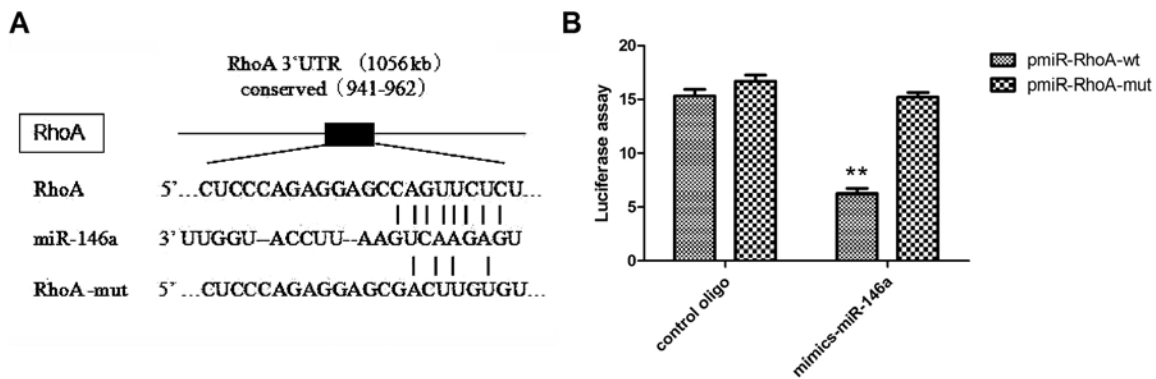


Figure 4. RhoA 3'UTR is a potential miR-146a target gene. (A) To predict and confirm that RhoA 3'UTR contains a conserved region that may serve as a binding site for miR-146a, the conserved region was determined by the miRanda algorithm. (B) A luciferase reporter of Rho A 3'UTR was constructed, which contained the binding sites of miR-146a by bioinformatics as predicted. Meanwhile the luciferase reporter of Rho A 3'UTR containing mutated binding sites of miR-146a was also constructed. miR-146a inhibits the luciferase activity of the reporter containing the 3'UTR of RhoA, when miR-146a mimics were transfected in the MDA-MB-231 cells. However, miR-146a did not inhibit the luciferase activity of the reporter containing a mutated sequence with the predicted target sites of the 3'UTR of the RhoA gene. Data are presented as the mean  $\pm$  SD. n=3; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. the control group.

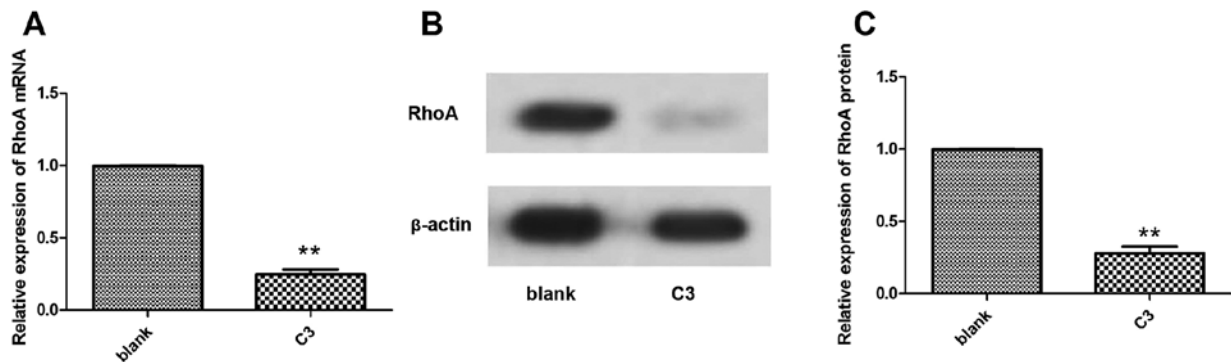


Figure 5. (A) Expression of RhoA mRNA in MDA-MB-231 cells treated with or without C3 was measured by real-time RT-PCR. In addition, the gene expression level was normalized to  $\beta$ -actin. (B and C) The relative protein expression levels of RhoA were measured by western blot assay, and normalized to  $\beta$ -actin. The data are presented as the mean  $\pm$  SD. n=3; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. the blank group.

in cell junction formation and stabilization (28,29), contains highly conserved regions that may serve as a binding site for miR-146a as determined at microrna.org (Fig. 4A).

To further demonstrate that RhoA is a potential target of miR-146a, we generated luciferase reporters that contained the 3'UTR of the RhoA gene. To determine whether the seeding site responds to miR-146a, the reporter plasmids were introduced into the MDA-MB-231 cells along with the miR-146a mimics or the control oligo. The results showed that the reporter activity was reduced by the ectopic expression of miR-146a (P<0.001; Fig. 4B). We also generated luciferase reporters that contained a mutated sequence within the predicted target sites of the 3'UTR of the RhoA gene to further demonstrate the interaction between miR-146a and the 3'UTR of RhoA. Data showed that the reporter activity was not reduced by the ectopic expression of miR-146a (Fig. 4B). Taken together, these results indicated that in MDA-MB-231 cells, the RhoA gene was a functional target of miR-146a.

*Migratory ability exhibited no change with the levels of miR-146a expression in RhoA inhibitor C3-treated MDA-MB-231 cells.* To further validate whether miR-146a inhibits migration in a Rho-dependent manner, we blocked RhoA

activity using the Rho inhibitor C3 at a final concentration of 2.0  $\mu$ g/ml. We found that the expression of RhoA mRNA and protein decreased after treatment with C3 compared with the untreated cells (Fig. 5).

Moreover, after treatment with C3, the migratory ability of the MDA-MB-231 cells transfected with the miR-146a mimics or the miR-146a inhibitor was neither decreased nor increased completely in the control oligo-transfected cells (P<0.05; Fig. 6A and B). These results suggest that miR-146a does not regulate RhoA when the RhoA pathway is blocked.

## Discussion

MicroRNAs (miRNAs) are a class of naturally existing small non-coding RNAs generated from large transcripts, termed pri-miRNAs and pre-miRNAs. In multicellular organisms, miRNA and the 3' untranslated region (3'UTR) of its target genes are incompletely complementary, regulating the gene expression at a post-transcriptional level and affecting almost all signaling pathways, which are involved in a variety of physiological and pathological processes and also play an important regulatory role in the occurrence and progression of tumors. Research shows that miRNAs have a role in the invasion and



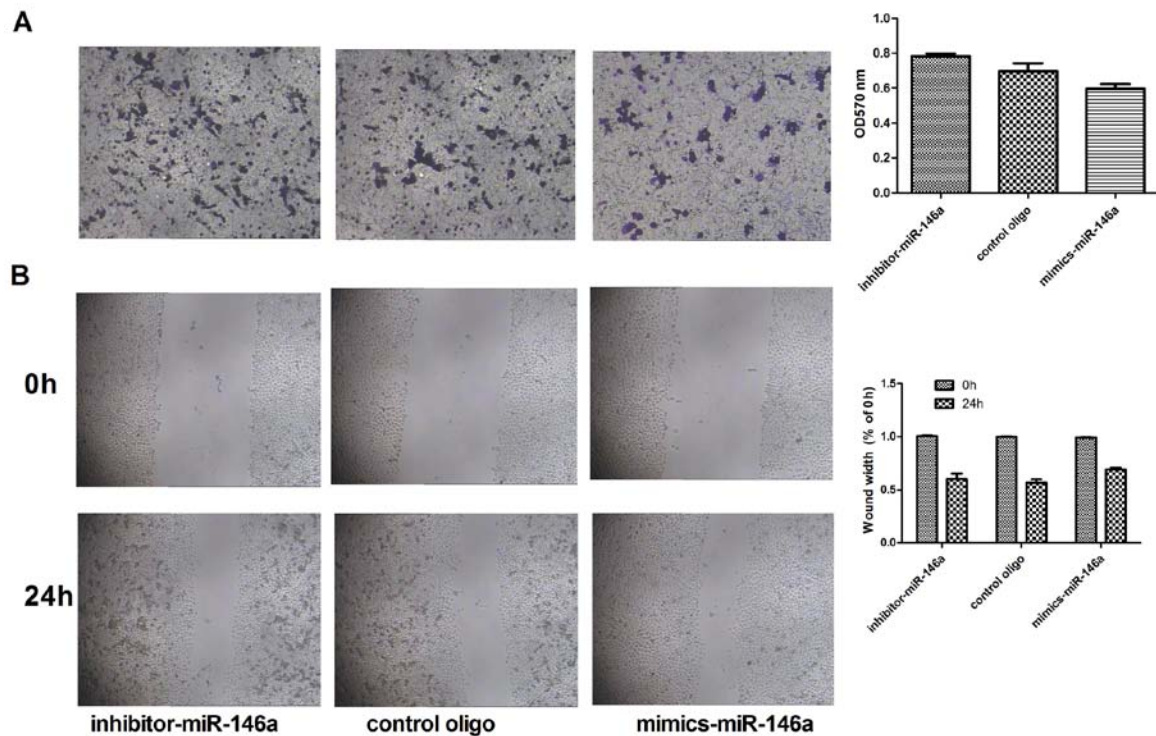


Figure 6. miR-146a suppresses the metastasis of MDA-MB-231 cells in a RhoA-dependent way. MDA-MB-231 cells were cultured in medium with 2.0  $\mu\text{g/ml}$  Rho inhibitor C3 for 24 h, before the expression of miR-146a was changed. (A) Effects of miR-146a on cell migration and invasion in the MDA-MB-231 cells were demonstrated by Transwell assay. Images of the cell invasion through the polycarbonate membrane stained with crystal violet (magnification, x200) are shown on the left. OD570 of crystal violet staining is shown on the right. (B) Effects of miR-146a on MDA-MB-231 cell migration by wound healing assay. Images were captured at 0 and 24 h after wounding and the wound widths were quantified. The data are presented as the mean  $\pm$  SD. n=3; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. the control group.

metastasis of tumor cells (21-23). miRNAs are primarily negative gene regulators of post-transcriptional repression (30), and their targets include tumor suppressors and oncogenes (18-20).

Present studies have shown that miR-146a can inhibit the occurrence, development and metastasis in gastric and pancreatic cancer, and other tumors, while its specific mechanism remains unclear (25-27,31-33). At present, scholars have already conduct research on the regulatory mechanism of miR-146a in breast cancer metastasis. miR-146a was found to act as a tumor suppressor directly regulating NF- $\kappa$ B expression, and affecting the tumor cell signaling pathway (24). miR-146a is involved in cell proliferation and influences cancer metastasis in various tumor types, including breast cancer (25-27). However, the regulation of breast cancer metastasis by miR-146a is a complex biological network, and the regulatory mechanism of breast cancer metastasis by miR-146a warrants further in-depth study. The results of the present study led us to make conclusions similar to these previous studies. We confirmed that the expression of miR-146a differs in human breast cancer cell lines with different metastatic potential, and miR-146a can reduce breast cancer cell migration and invasion.

Rho/ROCK signaling channel has been researched in regares to its involvement in the molecular mechanism of tumor metastasis (34). Rho-subfamily protein is one of the members of the Ras superfamily with GTP enzyme activity, and mediates a variety of cellular effects, such as cytoskeletal reorganization, membrane trafficking, proliferation, apoptosis/survival, cell polarity, cell adhesion, cell cycle and gene

transcription (35-38). As a key member of the Rho GTPase family, RhoA has been found to be upregulated in several types of human cancers, including colorectal (8,9) and breast cancer (10,11), hepatocellular carcinoma (12,13) and gastric cancer (14,15). Previous studies have found that alteration of the expression levels of intracellular RhoA can directly affect the process of invasion and metastasis in a variety of tumors (7,16,17,20). The results of our study suggest that miR-146a directly targets RhoA, and may be involved in the migration of MDA-MB-231 cells.

Based on our data, we demonstrated that in the breast cancer cell line MDA-MB-231, miR-146a directly regulates the target gene RhoA by incomplete complementarity with the 3'UTR, ultimately inhibiting the expression levels of the RhoA protein, and thereby controlling the migration of cells. We used bioinformatics to determine that RhoA is closely associated with cell migration and is a target gene regulated by miR-146a. In addition, miR-146a targeting of RhoA 3'UTR was confirmed using luciferase reporter gene containing RhoA 3'UTR or specific mutated sites RhoA 3'UTR in the MDA-MB-231 cells.

The results confirmed our hypothesized that upregulated expression of miR-146a negatively inhibited the expression of RhoA protein, due to incomplete complementarity to nucleotides within the 3'UTR of RhoA. Downregulation of miR-146a expression led to increased expression of RhoA protein. However, the level of RhoA mRNA had no significant changes. The inconsistency of the degree of RhoA transcription and levels of protein expression suggest that RhoA may be subject to post-transcriptional regulation by miR-146a. According to

the experimental results, we can infer that miR-146a may act as an anti-oncogene by suppressing MDA-MB-231 cell migration via the post-transcriptional repression of RhoA. After we blocked RhoA activity with the Rho inhibitor C3, we found that miR-146a was unable to regulate RhoA. These results further validated the possibility that miR-146a inhibited migration via a RhoA-dependent pathway.

Collectively, we demonstrated that miR-146a post-transcriptionally regulates the 3'UTR of RhoA mRNA and inhibits expression of RhoA protein, ultimately leading to suppress the migratory capacity of MDA-MB-231 cells. It is likely that miR-146a is involved in the RhoA-associated pathway, which affects the migration of the breast cancer cell line MDA-MB-231.

### Acknowledgements

The present study was supported by grants from the Zhejiang Provincial Natural Science Foundation (no. LQ13H160023), the Zhejiang Provincial Natural Science Foundation (no. Y12H290042) and the Population and Family Planning Scientific Research Project of Zhejiang Province (no. 2014KYA237). We are grateful to Dr Cai Zhijian (Immunology Research Institute of Zhejiang University, Hangzhou, China) for providing the MDA-MB-231 and MCF-7 cell lines. We are grateful to Dr Xiongfa Yang (Key Laboratory of Organosilicon Chemistry and Material Technology of Education Ministry, Hangzhou Normal University, Hangzhou, China) for the statistical analysis. We also thank the members of our laboratory for the technical assistance and useful suggestions.

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