Abstract. MicroRNAs (miRNAs/miRs) are 19-25 nucleotide-long, non-coding RNAs that regulate the expression of target genes at the post-transcriptional level. In the present study, the role of miR-340 in breast cancer (BC) was investigated. The overexpression of miR-340 significantly inhibited the proliferation, migration and invasion of human breast MDA-MB-231 cancer cells in vitro. The Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) gene was identified as a target of miR-340; its expression was downregulated by overexpression of miR-340 by binding to its 3’-untranslated region. The short interfering RNA-mediated silencing of ROCK1 was also performed, which phenocopied the effects of miR-340 overexpression. An inhibitor of miR-340 was used to suppress miR-340 expression, which led to increased expression of ROCK1, thus improving the proliferation, migration and invasion of MDA-MB-231 cells. Data from the present study suggest that miR-340 inhibits MDA-MB-231 cell growth and its downregulation may lead to the progression and metastasis of BC. Thus, miR340 may act as a tumor-suppressor agent that could serve a key role in the diagnosis and therapy of BC.

Introduction

Breast cancer (BC) is the most common type of cancer in women worldwide and is the second-leading cause of mortality in women in the USA. In 2017, the projected number of new cases and number of mortalities of BC were 255,180 and 41,070, respectively in the USA (1). Although BC is detected at an earlier stage than it was in the past, the mortality rate of BC remains high; as many as 1 in 8 women may develop BC (2). Patients with triple-negative BC (TNBC) have the poorest outcome, owing to the high risk of metastatic progression and an absence of targeted treatments (3). The prognosis of BC can, therefore, be poor. The development of drug resistance and a poor understanding of the molecular mechanism by which BC progresses are two primary reasons behind the poor prognosis of BC (4-6). This frequent poor prognosis has led to a substantial interest in the quest for a novel predictive marker for BC.

MicroRNAs (miRNAs/miRs) are 19-25 nucleotide-long, non-coding RNAs that function as negative regulators of gene expression at the post-transcriptional level. miRNAs are transcribed by RNA polymerases II and III, and, following a series of cleavage steps, form mature miRNA. The regulatory function of miRNAs is mediated by the RNA-induced silencing complex (7). Dysregulation of miRNAs can lead to progression or inhibition of normal cell growth patterns, thus leading to oncogenesis (8). miR-340 suppresses cell migration and invasion by targeting the Wnt pathway (9). The aforementioned studies suggest that miR-340 acts as a tumor suppressor.

ROCK1 belongs to the AGC family of serine/threonine kinases. The human ROCK1 gene is located on human chromosome 18, at position 18q11.1. ROCK1 normally regulates the actin cytoskeleton through the phosphorylation of substrates and modulation of actin-myosin contractility. It also acts as a key modulator in the formation of focal adhesions, cell motility and tumor cell invasion, thus contributing to the regulation of morphology, gene transcription, proliferation, differentiation, apoptosis and oncogenic transformation (14).

In the present study, ectopic expression of miR-340 was observed to inhibit cell proliferation, migration and invasion in vitro. ROCK1 was identified as a direct target of miR-340.

MicroRNA-340 inhibits invasion and metastasis by downregulating ROCK1 in breast cancer cells

NIRAJ MASKEY*, DENGFENG LI*, HUI XU, HONGMING SONG, CHENYANG WU, KAIYAO HUA, JIALU SONG and LIN FANG

Department of Thyroid and Breast Surgery, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P.R. China

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Correspondence to: Professor Lin Fang, Department of Thyroid and Breast Surgery, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, 399 Yanchang Road, Jing’an, Shanghai 200072, P.R. China
E-mail: fanglin_f@126.com

*Contributed equally

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and was shown to function as a tumor suppressor by downregulating ROCK1, suggesting that it has potential as a diagnostic and therapeutic marker in the treatment of BC.

Materials and methods

Cell lines and culture conditions. The human BC cell line MDA-MB-231 was purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were incubated at 37°C in a humidified chamber with 5% CO2.

MDA-MB-231 cells were cultured in 6-well plates. When the logarithmic cell growth reached 50-70% confluence, cells were transfected with miR-340 mimics, miR-340 inhibitor or short interfering RNA (siRNA) of ROCK1 (Shanghai Integrated Biotech Co., Ltd., Shanghai, China) at a concentration of 100 nM using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All transfections were performed in triplicate. The sequences were as follows: miR-340 mimics sense, 5'-UUAAAGCAUGGAGACAU GAAU-3' and antisense, 5'-UCAGUCUCAUGCUUUAU AAU; NC mimics sense, 5'-UCACACCUCUCAAGGAAG AGUAGA-3' and antisense, 5'-UACUCUUUCAUGGAGGU UGGAU; miR-340 inhibitor, 5'-AAUCAGUCUCAUGC UUUAUA-3'; inhibitor NC, 5'-AAUCAGUCUCAUGC UUUAUA-3'; si-ROCK1 sense, 5'-UGAUGCAAGAUGUG ACUCTT and antisense, 5'-GAGUACAAUCUUUGCAUC ATT-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression level of ROCK1 was analyzed by RT-qPCR. RNA was extracted from mimic-transfected cells using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized by RT using a PrimeScript RT-PCR kit, according to the manufacturer's protocol (Takara Bio, Inc., Otsu, Japan). qPCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT-qPCR was performed using KAPA SYBR® FAST Universal qPCR kit (cat. no. kk 4601; Kapa Biosystems, Inc., Wilmington, MA, USA). The qPCR steps were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. GAPDH was used as the reference. For quantitative analysis, relative gene expression levels were calculated using the 2^ΔΔCq method (10). Expression of messenger RNA (mRNA) was assessed by evaluating quantification cycle values. The primer sequences were: ROCK1 forward, 5'-AAC ATGCTGTCGATAAATCTGG-3' and reverse, 5'-TGTATC ACATGTTACATGCC-3'; and GAPDH forward, 5'-ACA CTTTGTTATCGGAAGG-3' and reverse, 5'-GCCATC AGGCGACAGTTTC-3'. The experiment was performed in triplicate.

Cell proliferation assay. The effect of miR-340 and siRNA of ROCK1 on cell viability was measured using an MTT assay kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. In brief, cells (2x10^5 cells/well) transfected with miR-340 mimics, miR-340 inhibitors or siRNA of ROCK1 and their corresponding negative mimics were seeded into 96-well culture plates (BD Biosciences, Franklin Lakes, NJ, USA) and incubated overnight at 37°C in 5% CO2. Cell proliferation was assessed at 24, 48, 72, 96 and 120 h, following the addition of 5 mg/ml MTT solution. After 4 h of incubation, the medium was replaced with 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) and vortexed for 10 min. The absorbance was measured using a microplate reader at 490 nm. Each experiment was performed in triplicate.

Colony formation assay. MDA-MB-231 cells (2x10^3 cells/well) were transfected with miR-340 mimics, miR-340 inhibitors, a siRNA targeting ROCK1 and their corresponding negative mimics. At 24 h post-transfection, the cells were digested with trypsin and resuspended into single-cell status (determined by microscopy). A total of 500 cells from each group were cultured in a 6-well plate for 14 days. The cells were then fixed with 4% paraformaldehyde and stained with freshly prepared 0.1% crystal violet stain for 10 min. Following rinsing with distilled water, the colonies that had formed in each well were counted under microscopy (inverted microscope; Olympus CKX41, Shibuya, Tokyo, Japan) using x40 magnification. Each experiment was performed in triplicate.

Transwell invasion and migration assays. A Transwell invasion assay was performed to evaluate cell invasion ability. The filters (Corning Incorporated, Corning, NY, USA) were washed with serum-free DMEM and placed into the wells of a 24-well plate. The lower chamber contained DMEM with 10% FBS. For the upper chambers, 5x10^4 cells resuspended in 200 μl DMEM with 0.1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) were plated in the top chamber of the Transwell chamber (Corning Incorporated) with a 2 mg/ml Matrigel-coated membrane containing 8-mm diameter pores. Plates were then incubated at 37°C in 5% CO2. After 18 h, cells remaining on the upper membrane surface were removed by scrubbing with a cotton swab, while cells on the lower surface of the membrane were fixed in 10% formalin at room temperature for 30 min and stained with 0.5% crystal violet. Images of six randomly selected fields of view were captured by inverted microscopy (LI-COR Biosciences, Lincoln, NE, USA) and the cells were counted at a magnification of x200. For the migration assay, the transfected cells (2x10^4 cells per Transwell chamber) were placed in the top chamber without Matrigel. After 18 h, the migrated cells were lysed in glacial acetic acid and the solutions were transferred to a 96-well culture plate for the colorimetric reading of optical density at 560 nm. Each experiment was performed in triplicate.

Dual-luciferase reporter assay. HEK 293T human embryonic kidney cells were provided by the Department of Central Laboratory, Shanghai Peoples Tenth Hospital (Shanghai, China). The cells were stored in liquid nitrogen at -191°C. These cells were seeded in 24-well plates (BD Biosciences) and cultured until the cells reached 80-90% confluence. The 3′-untranslated region (UTR) of ROCK1 containing the putative miR-340 binding site was amplified from genomic DNA via PCR in a total volume of 50 μl according to the manufacturer's protocol.
The reagents (Takara Bio, Inc., Otsu, Japan) used were 2X primer STAR GC buffer, dNTP Mixture, Primer1, Primer2, H2O and PimerSTAR HS DNA polymerase (Takara Bio, Inc.). The PCR steps were as follows: 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 12 sec (30 cycles). The ROCK1 primers used were: Forward, 5′-AACATGCTGCTGGATATCTGG-3′ and reverse, 5′-TGATACATCGTACCATGGCT-3′. The corresponding mutant constructs were created by mutating the seed regions of the miR-340 binding sites (5′-UUUAUA-3′ to 5′-AAAUAU-3′). Fragments were subcloned into the Xho site in the 3′-UTR of Renilla luciferase of the psiCHECK-2 reporter vector (Kapa Biosystems, Inc., Wilmington, MA, USA). HEK293T cells were transiently co-transfected with 0.2 μg psiCHECK-2/ROCK1 3′-UTR or psiCHECK-2/ROCK1 3′-UTR mutant receptor plasmids, together with 100 nmol/l miR-340 or negative control miRNA (miR-NC) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Luciferase activity was examined at 48 h after transfection using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA) and normalized to firefly luciferase activity. The ratio of Renilla:firefly luciferase was plotted. Three independent experiments were performed in triplicate.

Western blot analysis. Cells were lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) and the protein concentrations were quantified using a Pierce BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50 μg) were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane (Biozyme Institute of Biotechnology, Jiangsu, China). The membrane was blocked with 5% skimmed milk for 1 h. The membrane was incubated overnight at 4˚C with primary antibodies against ROCK1 (dilution, 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-tubulin (dilution, 1:1,000; cat. no. CW0098M; CWBio, Jiangsu, China) as a loading control. The membrane was treated with a horseradish peroxidase-conjugated secondary antibody (dilution, 1:2,000; cat. nos. 35571 and 35569; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Biosciences, Lincoln, NE, USA).

Clinical data analysis. The Cancer Genome Atlas (TCGA) provides a large amount of clinical data concerning different cancer types (15). Data on invasive BC were obtained from TCGA and analyzed using starBase v2.0 (16) and PROGeneV2 (17) to assess miRNA expression level and overall survival rate, respectively. miR-340 expression was compared against ROCK1 expression in 780 patient samples and miR-340 overall survival was assessed at median miRNA expression.

Statistical analysis. GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Pearson correlations and Kaplan-Meier analysis were used for all statistical analysis. Data were presented as the mean ± standard deviation from at least three separate experiments. A t-test (two-tailed) was used to compare between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Patients and BC cell MDA-MB-231 expression data support miR-340 and ROCK1 axis. Previous studies have analyzed the role of miR-340 in the development and progression of BC (5,9). A significant negative correlation was observed between miR-340 and ROCK1 expression using the TCGA BC clinical patient dataset (r=0.286; P<0.01; Fig. 1A). Analysis of the TCGA dataset also highlighted the prolonged overall patient survival that correlated with miR-340 expression in BC (P<0.05; Fig. 1B).

miR-340 suppresses MDA-MB-231 cell proliferation. The viability of cells transfected with miR-340 mimics and miR-340 inhibitors was compared with that of cells transfected with the corresponding miR-NCs at 24, 48, 72, 96 and 120 h post-transfection. Cells transfected with miR-340 mimics grew more slowly than control cells, whereas cells transfected with miR-340 inhibitors grew more rapidly than control cells at multiple time points (24, 48, 72, 96 and 120 h; Fig. 2A and B). Cells transfected with miR-340 mimics exhibited fewer colonies than NC groups, as determined by colony formation assays. miR-340 mimics decreased cell proliferation, whereas miR-340 inhibitors promoted it (Fig. 2C and D). These results suggest that the transient overexpression of miR-340 suppressed the proliferation and colony forming ability of MDA-MB-231 cells.

miR-340 suppresses MDA-MB-231 cell migration and invasion. Cells transfected with miR-340 mimics, miR-340 inhibitors and their corresponding NCs were tested for their migratory and invasive abilities. Images were obtained using an inverted microscope at x200 magnification. A total of three areas were randomly selected and the cells were counted (Fig. 3). The results demonstrated that miR-340 inhibited the migration and invasion of MDA-MB-231 cells whereas miR-340 inhibitor promoted the migration and invasion of MDA-MB-231 cells. These results indicate that the overexpression of miR-340 suppressed the migratory and invasive abilities of MDA-MB-231 cells.

ROCK1 is a target gene of miR-340. Bioinformatics analysis revealed that miR-340 binds to putative target sequences at position 1,317-1,723 of the ROCK1 3′-UTR (Fig. 4A). To confirm that ROCK1 was a direct target of miR-340, luciferase reporter constructs containing wild-type and mutant 3′-UTR of the ROCK1 gene were engineered. The luciferase reporter assay revealed that miR-340 significantly decreased the luciferase activity of ROCK1 3′-UTR wild-type but not that of the 3′-UTR mutant in HEK293T cells (Fig. 4B). RT-qPCR and western blot analyses revealed that the overexpression of miR-340 significantly downregulated the expression of ROCK1 at the mRNA and protein levels in MDA-MB-231 cells (Fig. 4C and D).

Expression of ROCK1 is downregulated by ROCK1-siRNA and the growth of MDA-MB-231 cells is inhibited by ROCK1-siRNA. The aforementioned results prompted an examination of whether the suppressive effect of miR-340 was mediated by repression of ROCK1 in MDA-MB-231 cells. The
knockdown efficiency of ROCK1 was verified by RT-qPCR and western blot analyses. The expression level of ROCK1 was significantly decreased in MDA-MB-231 cells transfected with siRNA of ROCK1, when compared with that of the siRNA NC group, at the gene and protein levels (Fig. 5A). The effect of ROCK1 silencing on cell viability was determined by MTT assay over a period of 5 days. The growth curve revealed a clear decrease in cell number in the MDA-MB-231 cells that had been transfected with siRNA targeting ROCK1 from day 2, compared with that in the control group (Fig. 5B). The total
number of colonies formed by MDA-MB-231 cells transfected with 100 nM siRNA of ROCK1 was visibly decreased compared with that in the control group (Fig. 5C). Further more, ROCK1 silencing significantly suppressed the migration and invasion of MDA-MB-231 cells (Fig. D and E), similar to the effect induced by miR-340. Taken together, these findings indicated that ROCK1 is a target of miR-340 that was involved in the suppression of the proliferation, migration and invasion of MDA-MB-231 cells.

Discussion
A number of studies have identified essential roles for miRNAs and genes in the cell growth and viability of BC (18-20). Normal tissue may have accumulated numerous detectable mutations, since breast tissue undergoes clonal expansion with more cell division compared with normal cells, which may lead to hypermutability (21). This clearly indicates the requirement for novel therapies to cure BC. With this hope, the
current study aimed to investigate the role of miR-340 in the tumor growth and metastasis of BC.

Although there are numerous reports of aberrant miRNA expression in cancer (22,23), data on the involvement of miR-340 in cancer are limited and few potential targets have been identified. A previous study reported that the target genes of miRNAs have similar functions; miR-340 was observed to mimic the effects of transforming growth factor-β activation, inhibiting cell proliferation by modulating cell scattering and cell-cycle arrest in lung cancer (24). The expression of miR-340 in glioblastoma is responsible for a strong tumor-suppressive effect in long-term survivors by downregulating the NRAS proto-oncogene (25). miR-340 inhibits prostate cancer cell proliferation and metastasis by targeting the mouse double minute 2 homolog-tumor protein 53 signaling pathway (26). miR-340 inhibits esophageal cancer cell growth and invasion by targeting phosphoserine aminotransferase (27). miR-340 inhibits the migration, invasion and metastasis of BC cells by targeting the Wnt pathway (13). These studies, together with the results of the present study, confirm that miR‑340 acts as a tumor-suppressor agent.

In conclusion, the present study demonstrated that increased expression of miR-340 inhibited the proliferation, migration and invasion of MDA-MB-231 cells. The tumor-suppressor function of miR-340 was mediated by the downregulation of its downstream target gene ROCK1. These results suggest that miR-340 may act as a tumor-suppressor agent whose downregulation may contribute to the progression and metastasis of TNBC.
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