

## Original Article

# MicroRNA-154 inhibits growth and invasion of breast cancer cells through targeting E2F5

Hui Xu<sup>1</sup>, Dan Fei<sup>2</sup>, Shan Zong<sup>1</sup>, Zhimin Fan<sup>1</sup>

<sup>1</sup>Department of Breast Surgery, The First Hospital of Jilin University, Changchun 130021, China; <sup>2</sup>Department of Ultrasonographic, China-Japan Union Hospital of Jilin University, Changchun 130033, China

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**Abstract:** Accumulating evidence suggested that microRNA-154 (miR-154) might play important roles in the development of various cancer types. However, the role of miR-154 in breast cancer progression remains largely unknown. Here, miR-154 expression level was measured via quantitative real-time RT-PCR (qRT-PCR) in 36 pairs of human breast cancer tissues and adjacent normal breast tissues and in a panel of human breast cancer cell lines. Cell proliferation, cycle, migration, and invasion were assessed by CCK8 assay, flow cytometer assay, wound healing assay and transwell invasion assay, respectively. Luciferase reporter assay and Western blot was used to verify E2F transcription factor 5 protein (E2F5) as a novel target gene of miR-154. Our results showed that miR-154 was frequently downregulated in breast cancer tissues and cell lines. Overexpression of miR-154 in MCF-7 cells significantly inhibited cell proliferation, migration, and invasion, and increased cell arrest at G0/G1 stage *in vitro*. E2F5 was identified as a target of miR-154, and its expression was inversely correlated with miR-154 expression in clinical breast cancer tissues. In addition, downregulation of E2F5 in MCF7 cells had similar effect on cell proliferation, cycle, migration and invasion by miR-154 induced. These findings indicate that miR-154 acts as a tumor suppressor by targeting E2F5, suggesting miR-154 as a potential therapeutic target for the treatment of breast cancer.

**Keywords:** Breast cancer, E2F5, proliferation, migration, invasion

## Introduction

Breast cancer is a malignant neoplasm originating from breast tissue and is the first leading cancer-related death in females worldwide in the last decade [1]. The main reason for the high mortality of breast cancer is tumor metastasis and proliferation [2, 3]. Therefore, it is urgent need to understand the molecular mechanism involved in breast cancer proliferation and metastasis for the development of novel diagnosis marker and therapeutic approaches in breast cancer.

MicroRNAs (miRNAs) are a new class of small non-coding RNAs that play crucial roles in the regulation of gene expression by triggering either mRNA degradation or translational repression through binding to a target site in the 3'-untranslated region (3'-UTR) messenger RNAs (mRNAs) [4, 5]. Accumulating evidence has shown that miRNAs involved in various biological procession, such as cell growth, differentiation, apoptosis and other metabolic

process [6, 7]. A large body of evidence has demonstrated that the abnormal expression of microRNAs is associated with various human tumors, and can function as tumor suppressors or oncogenes during the initiation and progression of cancer [8-10]. In human breast cancer, a largely number of miRNAs with aberrant expression has been identified, such as miR-21, miR-34a, miR-183, and miR-141, miR-145, which play oncogenic or suppressive roles [11, 12].

The miR-154, locates at chromosome 14q32, was downregulated and exerted suppressive role in several types of cancers including hepatocellular carcinoma [13], prostate cancer [14], osteosarcoma [15], colorectal cancer [16], non-small lung cancer [17], and thyroid cancer [18]. However, the biological function and underlying molecular mechanism of miR-154 in breast cancer has not been defined. Therefore, the aims of this study were to investigate the clinical significance of miR-154 in human breast cancer tissues and to clarify biological roles and the underlying mechanisms by which it is

involved in tumorigenic processes of breast cancer.

## Materials and methods

### *Patients and tissue samples*

Breast cancer samples and the corresponding adjacent normal tissues were taken from the 36 patients suffering breast cancer who underwent surgery at the First Hospital of Jilin University (Changchun, China) from July 2013 to August 2015. All of the tissues samples and clinical information were harvested after obtaining prior written informed consent from the patients or patient's family. The samples were immediately snap-frozen following surgery in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Patients received chemotherapy or radiotherapy prior to surgery was excluded in this study. This study is approved by Institutional Ethics Committees of Jilin University (Changchun, China).

### *Cell lines and transfection*

The noncancerous human mammary epithelial cell line: MCF-10A, and four human breast cancer cell line (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) were brought from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA), 100 units/ml penicillin or 100 mg/ml streptomycin under a humidified incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

miRNA-154 mimics and corresponding negative control mimics (miR-Ctrl), Small inhibitory RNA (siRNA) for E2F5 (si-E2F5), and scramble control siRNA (si-Ctrl) were all purchased from GenePharma (Shanghai, China). miR-154 mimic, miR-Ctrl, si-E2F5 and si-Ctrl were transiently transfected with MCF7 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. At 48 h post-transfection, transfection efficiencies were determined by qRT-PCR or Western blot.

### *Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

Total RNA was extracted from breast cancer cells and tissues using TRIzol reagent (Invi-

trogen) according to the manufacturer's instructions. For detection of miR-154, cDNA was synthesized using One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantitative PCR was performed using the miRNA-specific TaqMan<sup>®</sup> MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) with miR-154 specific primers (Applied Biosystems) under ABI 7900 Sequence Detection System (Life Technologies, NY, USA). For detection of E2F5, cDNA was synthesized using the Primer Script RT reagent Kit (TaKaRa, Dalian, China). Quantitative PCR was performed using the Fast SYBR Green Master Mix (Applied Biosystems) under ABI 7900 Sequence Detection System (Life Technologies, NY, USA). The primers of E2F5 and  $\beta$ -actin were used in this study were used as described previously [19]. U6 and  $\beta$ -actin were used as internal standard to normalize the miRNAs and mRNA expression level using  $2^{-\Delta\Delta\text{Ct}}$  method. All experiments were performed in triplicate.

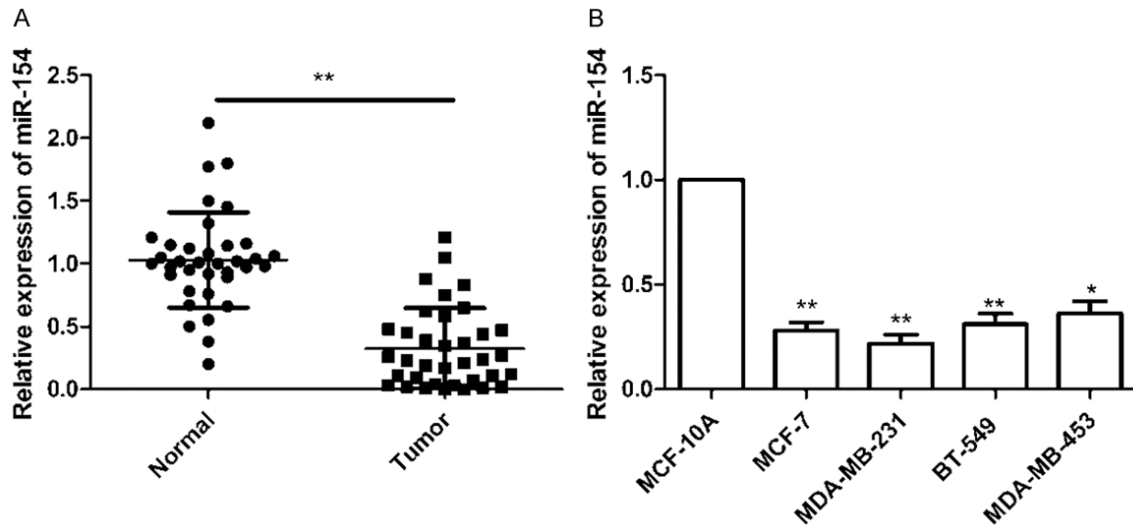
### *Cell proliferation and colony formation analysis*

Cell proliferation was determined using the CCK-8 Kit (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer protocols. Briefly, the transfected cells were seeded ( $5 \times 10^3$  cells/well) on 6-well plates and cultured for 24 h-72 h. In indicated time (24 h, 48 h and 72 h), 10  $\mu\text{l}$  of the CCK-8 solution was added to each well and cultured for 2 h, then the absorbance of the plate was measured at 450 nm under a microplate reader (Molecular Devices, Menlo Park, CA).

For colony forming assay, transfected cells (1,000 cells/well) were added to 6-well plates followed by incubation at the normal condition for two weeks. Colonies were fixed with 4% paraformaldehyde for 20 min and stained with 1% crystal violet. The clones were taken pictures and counted under a light microscope (Olympus, Tokyo, Japan).

### *Cell cycle assay*

Forty-eight hours after transfection, breast cancer cells were seeded in 6-well plates, and cultured for 48 h, thereafter, the cells were harvested and fixed in 70% ethanol, washed in PBS, re-suspended in 200  $\mu\text{l}$  of PBS containing 0.5 mg/ml RNase (Sigma), 0.05% Triton



**Figure 1.** miR-154 is downregulated in breast cancer tissues and cell lines. A. The expression of miR-154 was determined in 36 pairs of tumor tissues and the corresponding adjacent normal tissues by real time quantitative RT-PCR (qRT-PCR). \*\* $P < 0.01$  compared to normal tissues. B. The expression of miR-154 was determined in four breast cancer cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) and human mammary epithelial cell line (MCF-10A). \*\* $P < 0.01$  compared to MCF-10A.

X-100 (Sigma) and 10  $\mu\text{g/ml}$  propidium iodide (PI, Sigma), incubated for 1 hour at 37°C in the dark, and analyzed immediately using a FACS Calibur flow cytometer (BD Biosciences, Mansfield, MA, USA).

#### Wound healing assay

Transfected cells were seeded in 6-well culture plates at density of at  $2.0 \times 10^4$  cells/well. After cells had grown to confluence, the confluent monolayer in each well was created using a 100  $\mu\text{l}$  pipette tip and cultured for 24 h. Cells were photographed at 0 and 24 h under a light microscope (Olympus, Tokyo, Japan).

#### Transwell invasion assay

*In vitro* cell invasion assays were performed using 24-well transwell chambers (8- $\mu\text{m}$  pores, BD Biosciences, San Jose, CA). Briefly, The transfected cells ( $5 \times 10^4$  cells per well) were cultured in the top chamber coated with Matrigel with 100  $\mu\text{l}$  serum-free DMEM medium, 600  $\mu\text{l}$  complete media with 10% FBS was added into the lower chamber. After 24 h of cultivation, nonvading cells was gently wiped with a cotton swab, and the invaded cells were fixed in 4% paraformaldehyde, stained with 1% crystal violet solution (Sigma), and counted in selected randomly five fields under a light microscope (Olympus, Tokyo, Japan).

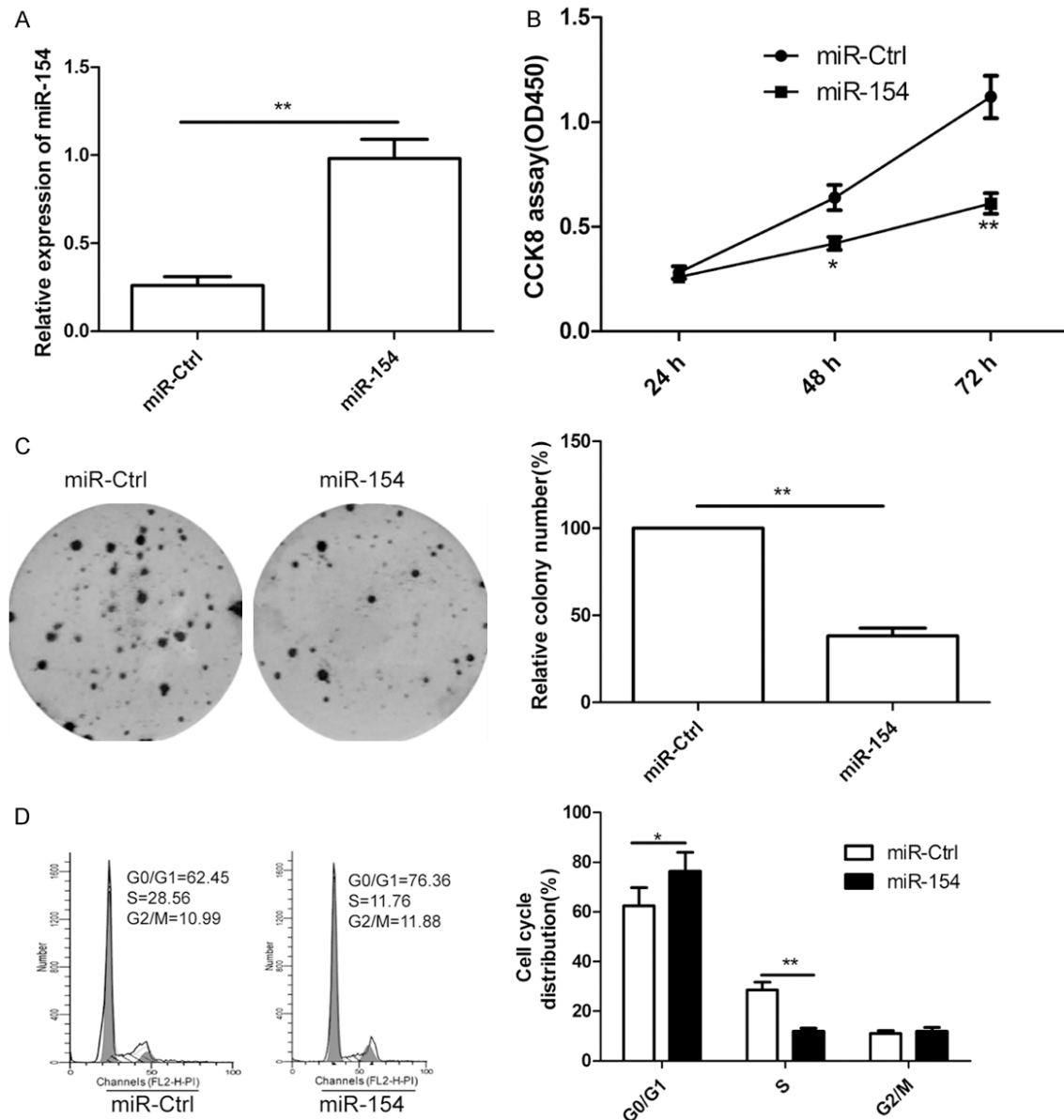
#### Luciferase reporter assays

A wild-type 3'-UTR and a mutant 3'-UTR of E2F5 were amplified from the Human cDNA of MCF-7 cells using PCR, and incorporated into the downstream of the firefly luciferase gene of a psiCHECK-2 vector (Promega, Madison, WI, USA). The inserts were confirmed by using DNA sequencing. For luciferase activity assay, MCF-7 cells were plated in 12-well plates in triplicate 24 h before transfection. miR-154 mimic, miR-NC and wide-type or mutant-type E2F5 reported plasmid were transiently co-transduced into MCF-7 cells by using Lipofectamine 2000 as recommended by the manufacturer's instructions. The luciferase activity was measured using a luciferase reporter assay system (Promega, WI, USA) after 48 h of incubation according to the manufacturer's protocol. Renilla luciferase activity was normalized to firefly luciferase activity.

#### Western blot analysis

Total proteins were extracted from breast cancer cell lines using RIPA buffer (Santa Cruz, Santa Cruz, CA, USA) containing protease inhibitors according to the manufacturer's protocol. The lysates were centrifuged at 25,000 g for 30 min at 4°C and the concentrations of total protein in supernatants were measured using a Bradford protein assay kit (Galen Bio-

## miR-154 inhibits breast cancer cell growth by targeting E2F5



**Figure 2.** miR-154 inhibits proliferation and colony formation of breast cancer cells. A. The expression of miR-154 was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl by qRT-PCR. B. Cell proliferation was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl by CCK8 assay. C. Cell colony formation was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl. D. Cell cycle arrest was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl by flow cytometer assay. \* $P < 0.05$ , \*\* $P < 0.01$  compared to miR-Ctrl.

pharm International Co., China) according to the manufacturer's protocol. The total extracts were separated using 10% SDS polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA). The membranes were probed with a primary antibody against human E2F5 (1:1000, Santa Cruz, CA, USA) or  $\beta$ -actin (1:5000, Santa Cruz) at 4°C overnight, followed incubation with HRP-labeled second-

ary antibody (1:10000, Santa Cruz) for 1 hour at room temperature.  $\beta$ -actin was used as internal control. Protein band were detected using the Supersignal West Pico ECL chemiluminescence kit (Thermo scientific, Rockford, IL).

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD) from at least three independent

experiments. All statistical analyses were performed using the SPSS 19.0 statistical software package (Chicago, IL, USA). Two-tailed Student's t test or ANOVA was used to determine the difference. The relationship between miR-154 and E2F5 was analyzed using Pearson's correlation assay. Differences were considered significant when  $P < 0.05$  (\*) and highly significant when  $P < 0.01$  (\*\*).

## Results

### *miR-154 is downregulated in breast cancer tissues and cell lines*

To determine the role of miR-154 in breast cancer progression, we investigated miR-154 expression in breast cancer samples and the corresponding adjacent normal tissues from 36 patients with breast cancer by real time quantitative RT-PCR (qRT-PCR). Our results showed that miR-154 expression was significantly lower in breast cancer tissues than in adjacent normal breast tissues (**Figure 1A**,  $P < 0.01$ ). We extended our investigations to four breast cancer cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) and found that their miR-154 expression levels were significantly lower than that of human mammary epithelial cell line (MCF-10A) (**Figure 1B**). These findings suggest that downregulation of miR-154 may affect breast cancer development.

### *miR-154 inhibits proliferation and colony formation of breast cancer cells*

To investigate the function of miR-154 in breast cancer tumorigenesis, we transfected miR-154 mimic or miR-Ctrl into MCF-7 cells and determined their miR-154 levels 48 hours after transfection. Results of qRT-PCR showed increased miR-154 levels in MCF-7 cells transfected with miR-154 mimic compared to miR-Ctrl transfected cells (**Figure 2A**). We then investigated the effect of miR-154 on MCF-7 cell proliferation and colony formation. As shown in **Figure 2B** and **2C**, restoration of miR-154 expression in MCF-7 cells significantly inhibited proliferation (**Figure 2B**) and colony formation (**Figure 2C**). It was well known that proliferation directly links to cell cycle distribution. We therefore tested cell cycle effect of miR-154 in breast cancer cells by flow cytometer assay. Our results showed that the percentage of G0/G1 phase cells increased, and the percentage of S phase cells decreased in miR-

154 transfected cells compared to miR-Ctrl transfected cells (**Figure 2D**).

### *miR-154 inhibits migration and invasion of breast cancer cells*

To investigate the function of miR-154 in breast cancer metastasis, the effect of miR-154 on cell migration and invasion were determined by wound healing and transwell invasion chamber, respectively. It was found that overexpression of miR-154 in MCF-7 cells led to significantly inhibited cell migration (**Figure 3A**) and cell invasion (**Figure 3B**) capability. Collectively, these results indicated that miR-154 suppressed breast cancer migration, and invasion.

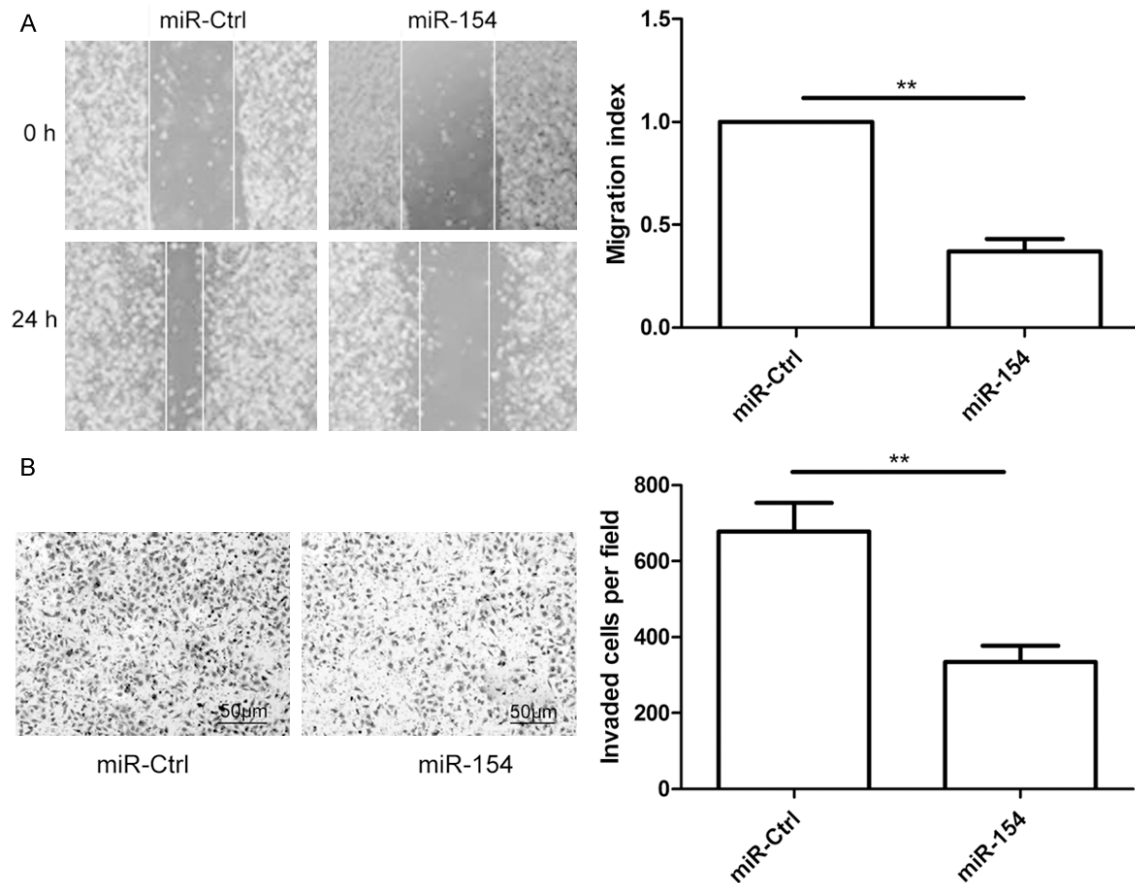
### *E2F5 is a direct target of miR-154*

To explore the underlying mechanism the growth inhibition by miR-154 in breast cancer cells, we used Targetscan6.2, microRNA.org, and miRWalk databases to predict potential miR-154 targets. Hundreds of potential targets were found, but we selected E2F5 for further analysis since it have previously been reported to involve in breast cancer procession [20]. We inserted wide-type or mutant-type 3'UTR into luciferase reporter vectors (**Figure 4A**) and cotransfected with miR-154 mimic or miR-Ctrl into MCF-7 cells. Forty-eight hours after transfection, luciferase activity were determined, and found that transfected with miR-154 mimic repressed wild-type 3'UTR-E2F5 reporter activity ( $P < 0.01$ ), while had no inhibition effect on the mutant 3'UTR-E2F5 reporter activity (**Figure 4B**), suggesting that E2F5 may be a target of miR-154 in breast cancer. We then sought to determine whether the overexpression of miR-154 in breast cancer cells can regulate E2F5 expression. Our results demonstrated that overexpression of miR-154 in MCF-7 cells obviously decrease E2F5 expression on mRNA level (**Figure 4C**) and protein level (**Figure 4D**).

### *E2F5 was up-regulated in breast cancer tissues and cell lines, and inversely correlated with miR-154 levels in breast cancer tissues*

As above results shows that E2F5 is a direct target of miR-154, we next detected expression in breast cancer samples and the corresponding adjacent normal tissues. qRT-PCR and Western blot assay showed that the expression





**Figure 3.** miR-154 inhibits migration and invasion of breast cancer cells. A. Cell migration was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl by wound healing assay. B. Cell invasion was determined in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl by transwell invasion assay. \*\* $P < 0.01$  compared to miR-Ctrl.

of E2F5 on mRNA and protein level was significantly higher in breast cancer tissues than those of adjacent normal tissues (**Figure 5A** and **5B**). In addition, Pearson's correlation assay showed that E2F5 mRNA expression level was inverse correlated with miR-154 expression in breast cancer tissue ( $r = -0.451$ ,  $P = 0.006$ , **Figure 5C**). We also investigated E2F5 protein expression in four breast cancer cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) by Western blot. As shown in **Figure 5D**, E2F5 protein expression level was obviously increased in four breast cancer cell lines compared with human mammary epithelial cell line (MCF-10A).

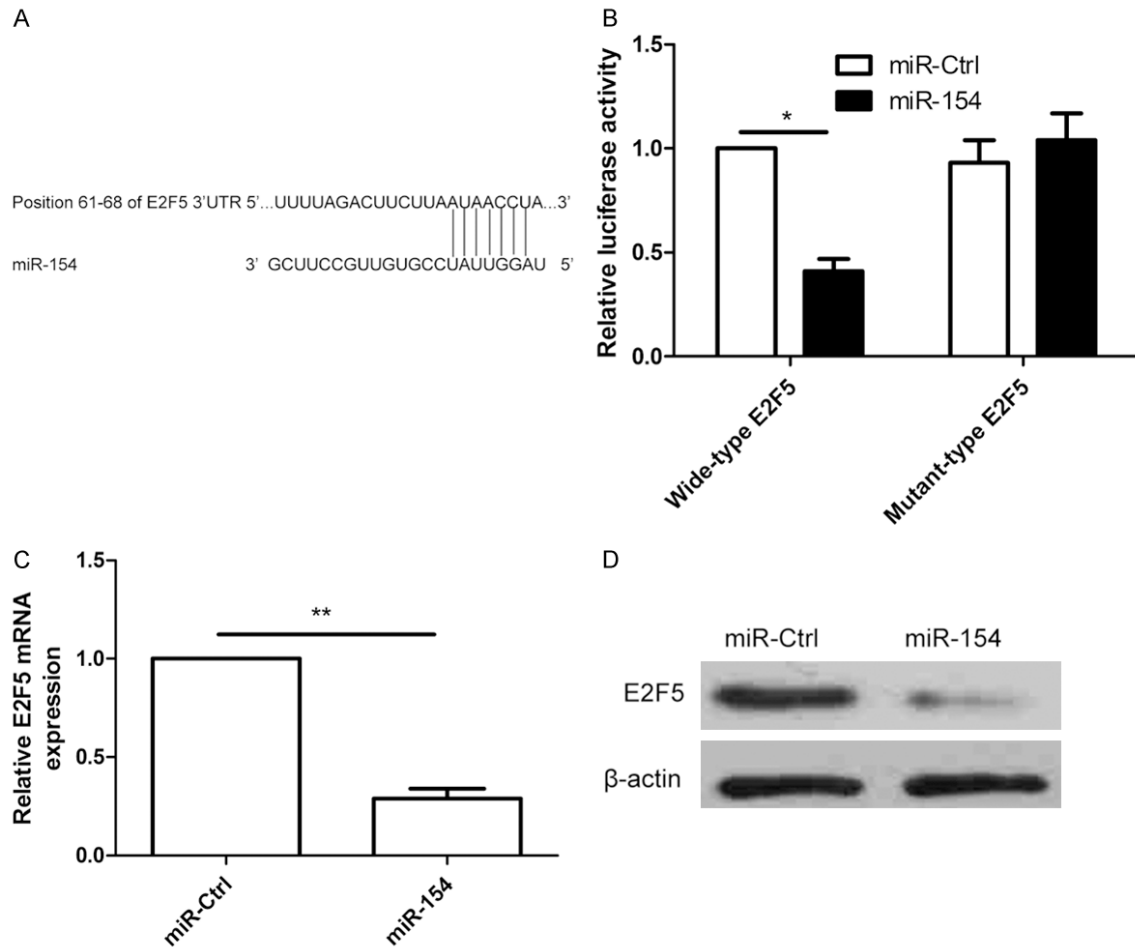
#### *Knockdown E2F5 inhibited cell proliferation, colony formation, migration, and invasion in breast cells*

To determine the biological role of E2F5 in breast cancer cells, MCF-7 cells were transfected

with si-E2F5 or the si-Ctrl, then E2F5 expression was determined by western blot 48 h after transfection. As shown in **Figure 6A**, the E2F5 protein expression levels was downregulated in si-E2F5 transfected cells compared with cells si-Ctrl transfected cells. Significantly, we also found that downregulation of E2F5 in MCF-7 cells significantly inhibited cell proliferation (**Figure 6B**) and colony formation (**Figure 6C**), increased cell arrest at G1/G0 stage (**Figure 6D**), suppressed cell migration (**Figure 6E**) and invasion (**Figure 6F**), which mimicked the effect of miR-154 overexpression on breast cancer cells.

#### **Discussion**

Dysregulation of miRNAs has been reported to be involved in tumorigenesis and progression in various types of cancer including breast cancer [11, 12]. In the present study, we found that miR-154 expression level was downregulated

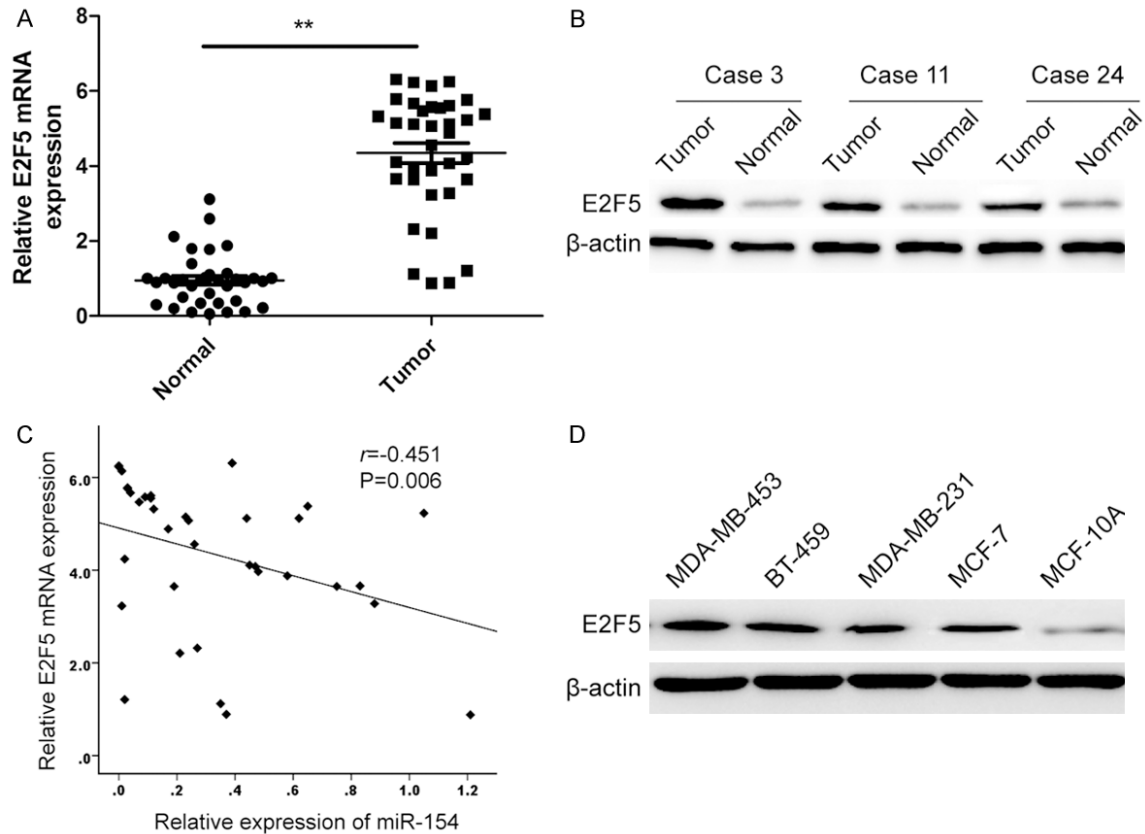


**Figure 4.** E2F5 is a direct target of miR-154. **A.** Schematic representation of the miR-154 targeting sequences within the 3'-UTR of E2F5. **B.** Luciferase activities were measured in MCF-7 cells 48 h after co-transfected with wide-type or mutant-type E2F5 3'-UTR luciferase plasmid and miR-154 mimic or miR-Ctrl. \* $P < 0.05$  compared to miR-Ctrl. **C.** Relative E2F5 mRNA expression was detected in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl. \*\* $P < 0.01$  compared to miR-Ctrl. **D.** E2F5 protein expression was detected in MCF-7 cells transfected with miR-154 mimic or miR-Ctrl. β-actin was used as internal control.

in human breast cancer tissues and cell lines compared with adjacent normal breast tissues and mammary epithelial cells, which was consistent with previous study [21]. In addition, we also found that restoration of miR-154 significantly suppressed breast cancer cell proliferation, migration, and invasion, and increased cell arrested at G1/G0 stage. E2F5, an important tumor promoter, is identified as a target of miR-154, and its expression was negative correlated with miR-154 expression. To the best of authors' knowledge, this is the first study to investigate miR-154 biological role and underlying mechanism in breast cancer.

miR-154 is located on human chromosome 14q32, which is frequently lost in human vari-

ous types of tumor, such as lung cancer [22], breast cancer [23] and multiple myeloma [24]. Recently accumulating evidence has demonstrated that miR-154 expression was downregulated and functioned as tumor suppressor miRNAs in several types of cancer [14-18]. For instance, Lin *et al* reported that miR-154 expression was downregulated in non-small lung cancer tissues and cell lines, and that restoration of miR-154 expression suppressed non-small lung cancer growth *in vitro* and *in vivo* [17]. Zhou *et al* showed that miR-154 exerted tumor suppressor role in osteosarcoma by targeting Wnt5a [15]. Pang *et al* found that overexpression of miR-154 in hepatocellular carcinoma cells can inhibit cell proliferation, colony formation, migration and invasion, and induced



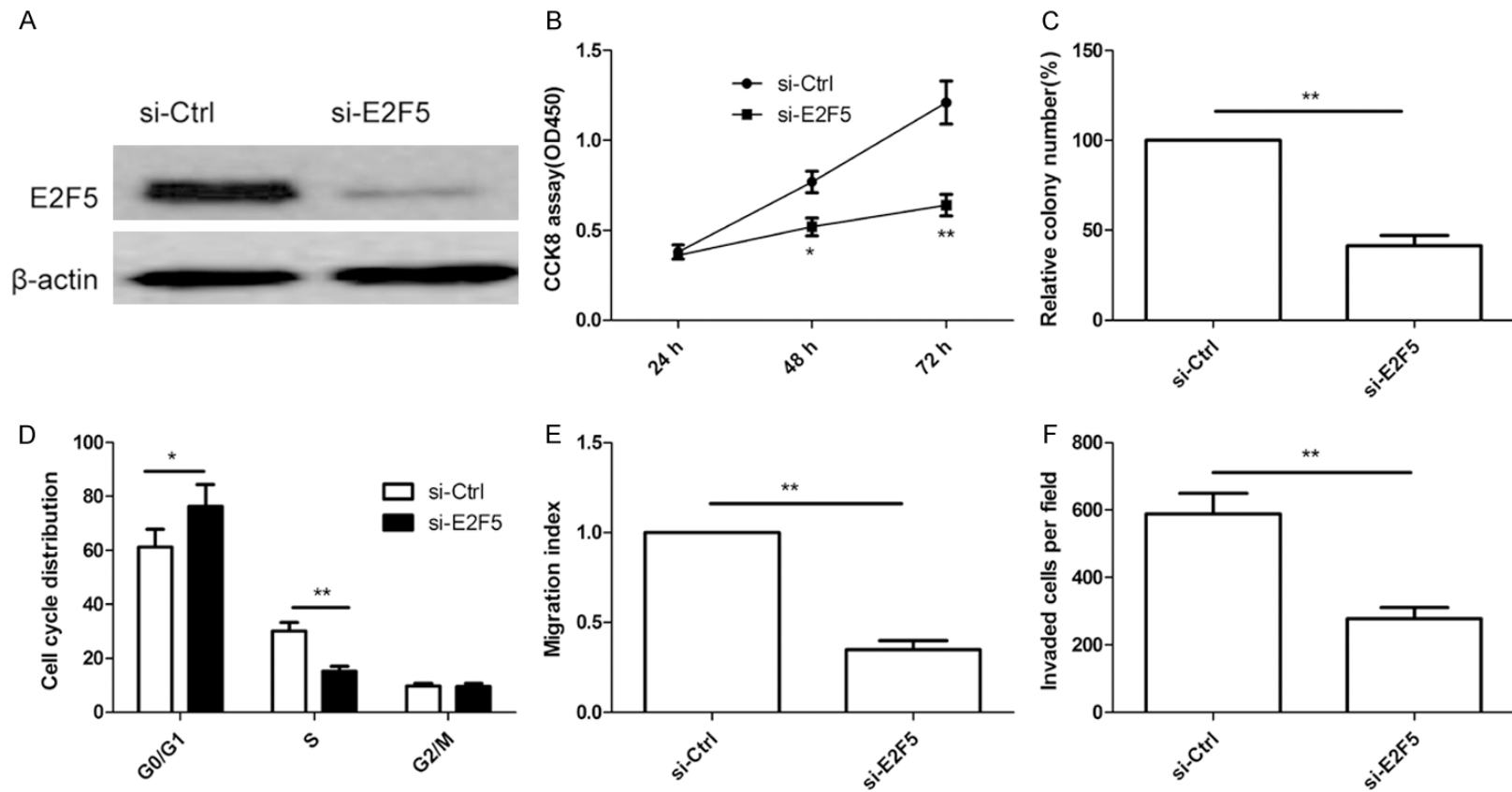
**Figure 5.** E2F5 was up-regulated in breast cancer tissues and cell lines, and inversely correlated with miR-154 levels in breast cancer tissues. A. Relative E2F5 mRNA expression was detected in 36 pairs of tumor tissues and the corresponding adjacent normal tissues by real time quantitative RT-PCR (qRT-PCR).  $**P < 0.01$  compared to Normal tissues. B. E2F5 protein expression was detected in breast cancer tissues and the corresponding adjacent normal tissues by western blot.  $\beta$ -actin was used as internal control. C. The correlation of the expression levels of E2F5 and miR-154 in 36 breast cancer tissue samples by Pearson's correlation assay. D. E2F5 protein expression was detected in four breast cancer cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) and human mammary epithelial cell line (MCF-10A) by western blot.  $\beta$ -actin was used as internal control.

cell apoptosis *in vitro*, as well as suppress tumor growth of PTC *in vivo* by targeting ZEB2 [13]. Here, our results showed that restoration of miR-154 significantly suppressed breast cancer cell proliferation, migration, and invasion, and increased cell arrested at G1/G0 stage by targeting E2F5, suggested that miR-154 function as tumor suppressor in breast cancer.

E2F transcription factor 5 protein (E2F5) belongs to the E2F transcription factor family, which involve in cell-cycle control, initiation of replication, DNA synthesis [25, 26]. E2F5 expression was upregulated in several types of cancer, including liver cancer [19], colorectal cancer [27], esophageal squamous cancer [28], prostate cancer [29], suggested that E2F5 function as oncogene. In breast cancer, a

report showed that the expression of E2F5 was upregulated in breast cancer, and its expression was associated with higher histological grade in human breast cancer and that the expression of E2F5 led to worse clinical outcomes with shorter disease-free survival [20]. Of note, recently several studies showed that E2F5 could be regulated by several miRNAs, including miR-34a [27], miR-181a [19] and miR-106 [30]. Here, we identified E2F5 as a target of miR-154 by luciferase reporter gene assays, qRT-PCR and Western blot. Our results also showed that E2F5 expression was upregulated in breast cancer tissues and cell lines, and was inversely correlated with miR-154 expression in breast cancer tissues. Knockdown E2F5 inhibited cell proliferation, colony formation, migration, and invasion in breast cells, which mimicked the effect of miR-154 overex-





**Figure 6.** Knockdown E2F5 inhibited cell proliferation, colony formation, migration, and invasion in breast cells. (A) E2F5 protein expression was detected in MCF-7 cells transfected with si-E2F5 or si-Ctrl by western blot. β-actin was used as internal control. (B-F) Cell proliferation (B), colony formation (C), cycle (D), migration (E) and invasion were determined in MCF-7 cells transfected with si-E2F5 or si-Ctrl. \* $P < 0.05$ , \*\* $P < 0.01$  compared to si-Ctrl.

pression on breast cancer cells. These finding suggested that miR-154 cell proliferation, colony formation, migration, and invasion in breast cells, at least in part, by repressing E2F5.

In conclusion, the present study demonstrates that miR-154 expression was downregulated in breast cancer tissues and cell lines, and that restoration of miR-154 expression inhibits breast cancer cell proliferation, migration, and invasion by targeting E2F5. These findings suggest that miR-154 and its target gene E2F5 might have a potential role in gene therapy and may be a promising target for the treatment of breast cancer in the future.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Zhimin Fan, Department of Breast Surgery, The First Hospital of Jilin University, Changchun 130021, China. E-mail: fanzhimin780213@126.com

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