

MiR-214 promotes carcinogenesis in triple-negative breast cancer by inhibiting FRK expression

Jieun Lee

Division of Medical Oncology, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Sooeun Oh

Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Dong-Min Kim

Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Jun Kang

Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Ahwon Lee (✉ klee@catholic.ac.kr)

Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Research Article

Keywords: breast cancer, triple-negative breast cancer, miR-214, FRK

Posted Date: May 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-468759/v1>

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Abstract

Background Triple-negative breast cancer (TNBC) is known to have a poor prognosis with limited treatment options. In-house data revealed that miR-214 was up-regulated in breast cancer tissue, but there have been conflicting results about the role of miR-214 in oncogenesis in breast cancer. In this study, we aimed to investigate the potential role of miR-214 and its target in carcinogenesis.

Methods We used three breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) and control breast epithelial cell line (MCF-10A) to evaluate miR-214 expression and function. Target genes were predicted based on pre-formed miRNA databases. miR-214 was transfected into cell lines, and cell proliferation, invasion and migration assays, and RT-PCR analysis were performed.

Results MiR-214 was significantly overexpressed in MDA-MB-231 line, which represents TNBC. Fyn-related kinase (FRK) was selected as a potential target of miR-214. Endogenous FRK levels were down-regulated in MDA-MB-231 cells. miR-214 transfected MDA-MB-231 cell line resulted in increased cell proliferation, and silencing FRK using si-FRK also induced cell proliferation in MDA-MB-231 cells. When MDA-MB-231 cells were transfected with miR-214, cell invasion and migration were enhanced. Furthermore, si-FRK-transfected MDA-MB-231 cells showed increased cell invasion and migration compared to the control cells.

Conclusions Our results suggest that miR-214 might play a role in tumor proliferation and invasion to promote breast cancer oncogenesis by suppressing FRK activity, leading to cancer progression.

Introduction

Breast cancer (BC) is the most common cancer in women worldwide [1] and is the most common type of cancer among Korean women [2]. As active screening with mammography is increasing worldwide, most BC patients are diagnosed at an early stage [3,4]. However, approximately 1 to 2% of patients are diagnosed with advanced or metastatic BC annually [5,6]. Triple-negative BC (TNBC) is a subtype of BC that is negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor-2 by immunohistochemical stain. TNBC accounts for about 10–20% of BCs, and is characterized by a high metastatic activity and poor clinical outcomes [7]. Cell proliferation, invasion, and migration are known to be the mechanisms of BC progression and metastasis. Discovering the underlying etiology of cell proliferation, invasion, and migration in BC, especially in TNBC, may lead to the development of new treatment options that may influence the survival outcome in these patients.

MicroRNAs (miRNAs) are non-coding small RNAs composed of 20-24 nucleotides, and deregulation of these molecules is known to be associated with the pathogenesis in various human malignancies [8]. MiRNAs act as tumor suppressors or tumor promoters by binding to the 3'-untranslated region (3'-UTR) of target genes [9]. Various miRNAs have been reported to be associated with BC development and progression [10]; however, no specific key miRNA has been validated as a major factor contributing to oncogenesis and progression of BC. We have previously analyzed whole miRNA expression profiles in

ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) [11]. During the examination of the data, we have discovered that miR-214-3p was up-regulated 2.2 fold and 18.3 fold each in recurrent DCIS and DCIS tissues adjacent to IDC respectively, compared to pure DCIS tissues. Based on these preliminary data, we suspected that miR-214-3p may play an important role in the oncogenesis and invasion of BC.

miR-214-3p functions as a major modulator of cancer cell growth, proliferation, migration, and metastasis in various malignancies, including breast, cervical, ovarian, and stomach cancers [12,13]. In BC, miR-214 targets multiple crucial genes, including phosphatase and tensin homolog (PTEN), β -catenin, TNF receptor-associated factor 3 (TRAF3), and p53 [14-17]. Deregulated miR-214 affects tumorigenesis and progression, but there are conflicting reports about the possible function of miR-214 of either a tumor suppressor or promoter [12,14,15,18]. These inconsistent findings may be due to heterogeneous subtypes of BC, or evolving role of miR-214 during tumorigenesis, invasion and progression from normal cells to DCIS, and to invasive carcinoma.

PTEN acts as a major tumor suppressor in various human cancers by regulating cell growth, proliferation, migration, DNA damage repair, and tumor metabolism [19]. PTEN is regulated by diverse interacting proteins, including Fyn-related kinases (FRKs). FRK is a non-receptor tyrosine kinase belonging to the BRK family kinases (BFKs) and is remotely related to Src family kinases (SFKs) [20]. FRK plays an important role during cancer formation and progression, and is regulated in a tissue-specific manner [20,21]. FRK functions as a tumor promoter in pancreatic and hepatocellular carcinoma [22,23], but there are conflicting reports suggesting that FRK may act as a tumor suppressor in BC, lung cancer, and glioma [20]. Previous studies have reported that FRK interacts with PTEN and promotes phosphorylation and stabilization of PTEN, thereby enhancing the tumor-suppressive effect in various cancers, including BC [20,24,25]. Expression of FRK is partially regulated by miR-214-3p in addition to various miRNA clusters [26]. The roles of miR-214-3p and FRK and its interaction are not well established in BC. Hierarchical control of FRK may control the PTEN-AKT pathway, thereby controlling cancer cell growth and progression in the cancer microenvironment [20].

Based on previous studies reporting the role of FRK and in-house data reporting up-regulated miR-214-3p in recurrent DCIS or DCIS specimens adjacent to IDC tissue, we hypothesized that miR-214-3p may regulate FRK expression and therefore influence oncogenesis and progression of BC. In this study, we aimed to analyze the role of miR-214-3p and its potential action on FRK using BC cell lines that represent the luminal and TNBC subtypes.

Materials And Methods

Cells

Breast epithelial cell line MCF10A, breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-468 were purchased from the American Type Culture Collection (ATCC). Embryonic kidney cell line HEK-293 was also purchased from the ATCC. The non-tumorigenic epithelial cell line MCF10A was cultured in MEGM

medium (Lonza, Basel, Switzerland) supplemented with Bullet Kit™ (Lonza). The luminal breast cancer cell line MCF7 was cultured in RPMI1640 medium (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS, Corning, Corning, NY, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco BRL, Grand Island, NY, USA). HEK293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (Welgene, Gyeongsangbuk-do, South Korea) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco BRL). These cells were maintained at 37°C in the presence of 5% CO₂. Triple-negative breast cancer (TNBC) cell lines MDA-MB-231 and MDA-MB-468 were cultured in Leibovitz's L-15 medium (Welgene) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco BRL). MCF10A, MCF7, HEK293 cell lines were maintained at 37°C in 5% CO₂ and MDA-MB231, MDA-MB-468 cell lines were maintained at 37°C in air.

Selection of target gene

The target gene of miR-214-3p was selected based on the following miRNA databases: miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>), miRsearchV3.0 (<http://www.exiqon.com/mirsearch>), miRNAMap (<http://mirnamap.mbc.nctu.edu.tw/>) [26,27].

miRNA mimics, siRNA, and miRNA inhibitors

The miRNA mimics, FRK siRNA (siFRK1), and scrambled control miRNA that was used as a negative control were purchased from Genolution Pharmaceuticals (Seoul, South Korea). The sequences were as follows: 5'-ACAGCAGGCACAGACAGGCAGU-3' (miR-214-3p mimic), 5'-AGUCGAGGCACAGACAGGCAGU-3' (miR-214-3p mimic mutant; miR-214-3pm), 5'-GAUCAGAUGCAGAGAAACAUU-3' (siFRK), 5'-UUUUAACUCAGUAUUUUUA-3' (scrambled control). The miRNA inhibitors for miR-214-3p (LNA-miR-214-3p inhibitor) and negative control A were purchased from Qiagen (Hilden, Germany).

Luciferase reporter plasmid construction and site-directed mutagenesis

The 3'-UTR of *FRK* was amplified from the genomic DNA of MCF7 cells and cloned between the Renilla luciferase coding sequence and the poly(A) site of the *psiCHECK-2* plasmid (Promega, Madison, WI, USA) using XhoI/NotI sites to produce *psiC-FRK*. The primers used to amplify the *FRK* 3'-UTR were 5'-TCTAGGCGATCGCTCGAGCTTTTACAGTGTTCTTAACCTCCT-3' and 5'-TTATTGCGGCCAGCGGCCGCTAAGATTCAGTGGCTTGAAATC-3'. Mutations were introduced into the putative seed match sequences of *psiC-FRK* using the EZchange site-directed mutagenesis kit (Enzymomics, Daejeon, South Korea). The primer sequences were as follows: *psiC-FRK_m1*: 5'-CGTGGATCAAAGACCAAAGTAGATAAATCCAGG-3' and 5'-ACATGTTCCGGATGGGGTCTCTGAG-3'; *psiC-FRK_m2*: 5'-CGAGACTTAAATATCTCTGCCGGACAGC-3' and 5'-ACAAGCTGCTCCCATATCCACCC-3'.

Luciferase reporter assay

To test whether the miRNAs directly target the 3'-UTR of *FRK*, a luciferase reporter assay was performed. HEK293 human embryonic kidney cells, which are frequently used for luciferase reporter assays, and MCF7, MDA-MB-231, and MDA-MB-468 cells were seeded in 96-well white culture plates (5×10^3 cells/well). After 24 h, cells were co-transfected with 10 nM of each miRNA mimic and 20 ng psiC-FRK or a mutant reporter vector (*psiC-FRK_m1*, *psiC-FRK_m2*, or *psiC-FRK_m1m2*). Luciferase activity was measured 48 h post-transfection using the Dual-Glo™ Luciferase Assay System (Promega). Renilla luciferase activity was normalized to firefly luciferase activity for each sample.

Cell transfection

Cells were seeded 24 h prior to transfection in 60- or 100 mm diameter dishes containing 5 or 10 mL of culture medium, respectively. Transfection was performed with 50 nM of each miRNA mimic, siRNA, or miRNA inhibitor using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. Cells were harvested for RNA and protein extraction 48 h post-transfection.

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

Cells were harvested, and total RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 1 µg total RNA, oligo(dT) (Macrogen, Seoul, South Korea), and M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) for *FRK* was carried out using the TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX; Enzynomics, Daejeon, Korea) with the LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). Primer sequences were as follows: *FRK*, 5'-CACGAAATAAAGCTGCCGGT-3' and 5'-GGCACCTGTCATACCACTGT-3'; glyceraldehyde phosphate dehydrogenase (*GAPDH*), 5'-ATGGGGAAGGTGAAGGTCG-3' and 5'-GGGGTCATTGATGGCAACAATA-3'. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s for *FRK/GAPDH* detection. To confirm the specific amplification of the PCR products, dissociation curves were checked routinely. PCR products were ramped up from 55°C to 95°C at a heating rate of 0.1°C/s, and fluorescence was measured continuously. Relative gene expression was calculated according to the comparative Ct method using *GAPDH* as an internal standard.

Cell proliferation assay

Cells were seeded 24 h prior to transfection in 96 well culture plates containing 200 µL of culture medium. Transfection was performed with 50 nM of each miRNA mimic, siRNA, or miRNA inhibitor using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. Cell proliferation was assessed using the MTS cell proliferation colorimetric assay kit (BioVision, CA, USA). MTS-1 reagents (20 µL) were then added to the cells, which were incubated for a further 4 h at 37°C and 5% CO₂. The optical density was then assessed at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell migration and invasion assays

Cells were seeded 24 h prior to transfection in 6 well culture plates containing 1 mL of culture medium. Transfection was performed with 50 nM of each miRNA mimic, siRNA, or miRNA inhibitor using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. Transwell migration and invasion assays were performed using 24-well Transwell chambers with 8 µm polyethylene membranes (Corning, Corning, New York, USA). MCF-10A, MCF-7, and MDA-MB-231 cells were starved for 24 h, and 1×10^5 cells were seeded in the top chamber. The bottom chambers were filled with 20% FBS in RPMI. For the cell migration assay, cells were placed in the upper chamber with 100 µL serum-free medium. For the invasion assay, cells were placed in the upper chamber with 100 µL prechilled Matrigel (Corning). After 24 h, the migration and invasion activities of MCF-10A, MCF-7, and MDA-MB-231 cells were determined by counting cells positive for crystal violet staining for 20 min after fixation with 3.7% formaldehyde.

Results

Endogenous expression of miR-214-3p and FRK mRNA in various breast cancer cell lines

We analyzed endogenous miR-214-3p expression in three breast cancer cell lines (MCF7, MDA-MB-231, and MDA-MB-468). These cell lines represented hormone receptor-positive breast cancer and triple-negative breast cancer subtypes. To compare the expression levels of miR-214-3p in normal breast tissue, we used the MCF10A cell line. Among the four breast cancer cell lines, MDA-MB-231 showed marked expression of miR-214-3p compared to the other cell lines. Compared to MCF-10A cells, MCF-7 cells showed relatively higher expression of miR-214-3p. In contrast, *FRK* mRNA was up-regulated in MCF-7 and MDA-MB-468, and MDA-MB-231 showed low levels of *FRK* mRNA expression, which indicated a reverse correlation with miR-214-3p expression (Fig. 1). The MDA-MB-468 cell line lacks PTEN expression, which may result in the up-regulation of *FRK* [28], resulting in bias in the study. Considering intrinsic PTEN deficiency in the MDA-MB-468 cell line, we excluded this line from further analysis.

FRK as target gene of miR-214-3p

Based on miRNA database (miRTarBase, miRsearchV3.0, and miRNAMap) analysis [26, 27], *FRK* was selected as a potential target gene of miR-214-3p. A luciferase reporter assay was performed to demonstrate that miR-214-3p reduced the luciferase activity of *FRK* by binding to the following sites: 6086–6093, 6492–6498 of the *FRK* 3'-UTR. Scrambled control and mutated miR-214-3p (miR-214-3pm) were used as references. HEK293T cells were used as a control cell line to check the transfection ability of miRNA before the study. Transfection of miR-214-3p to MCF7 and MDA-MB-231 cells resulted in a significant reduction in *FRK* activity, compared to the scrambled control and miR-214-3pm transfected cell lines (Fig. 2). To confirm the functional main target of miR-214-3p in the *FRK* gene, two types of mutated miR-214-3p (m_1 , m_2) were constructed to target the mutant form of the *FRK* 3'-UTR seed match sequences (*psiC-FRK_m1*, *psiC-FRK_m2*, Fig. 3A,B). In HEK293T, MCF-7, and MDA-MB-231 cell lines, miR-214-3pm₂ transfected cell lines showed reduced luciferase activity, similar to wild-type miR-214-3p transfected cell lines (Fig. 3C-E). This result suggests that target site 1 is the main target of miR-214-3p.

MiR-214-3p suppress FRK and regulates breast cancer proliferation, migration and invasion

We hypothesized that miR-214-3p may suppress FRK expression, thereby promoting cell proliferation, migration, and invasion during carcinogenesis. The miR-214 mimic was transfected into MCF-10A, MCF-7, and MDA-MB-231 cells, and cell lines showed an increased rate of cell proliferation compared to the scramble control. (Fig. 4A-C). In contrast, silencing miR-214 resulted in decreased cell proliferation in all cell lines (Fig. 4D-F). We predicted that silencing FRK may lead to similar results compared to the miR-214 mimic-transfected MTS assay. In MDA-MB-231 cells, transfection with siFRK resulted in comparable results with the miR-214 transfected MTS assay (Fig. 4-C). In the MCF-7 cell line, siFRK-transfected cells showed increased proliferation compared to that in the cells transfected with the scramble control (Fig. 4-B). However, in the MCF-10A cell line, siFRK transfection did not influence the proliferation index compared to the scramble control (Fig. 4-A). Based on the MTS assay, we hypothesized that FRK acts as a tumor suppressor and miR-214 regulates FRK, resulting in tumorigenesis in breast cancer, especially in TNBC subtype represented by MDA-MB-231.

Similar results were observed in the cell migration and invasion assays. Transfection of miR-214 significantly enhanced cell migration in MCF-10A, MCF-7, and MDA-MB-231 cell lines (Fig. 5) and resulted in increased cell invasion in MCF-7 and MDA-MB-231 cells (Fig. 6). Inhibition of miR-214 resulted in decreased cell migration and invasion compared to the negative control (Figs. 5 and 6). Silencing FRK enhanced cell migration and invasion ability in cell lines, but to a lesser extent compared to miR-214 mimic transfection (Figs. 5 and 6).

Discussion

It is well known that miRNAs may act as oncomiRs or tumor suppressors during oncogenesis in various solid cancers [8,9]. We have shown previously that miR-214-3p was up-regulated in recurrent DCIS or DCIS tissues adjacent to IDC specimens, compared to pure DCIS tissue using miRNA hierarchical clustering assay [11]. MiR-214-3p is known to be one of the key regulators during tumor growth and progression, but there are conflicting results regarding whether miR-214-3p acts as a tumor promoter or tumor suppressor in breast cancer [12,14,15,18]. In this study, miR-214-3p was acting as a tumor promoter, enhancing tumor proliferation, invasion and migration, especially in TNBC cell line.

In breast cancer, miR-214-3p regulates the proliferation and apoptosis in malignant cells by controlling the levels of various tumor suppressors or promoters [15,18,29]. Multiple tumor promoters or suppressors, such as PTEN, WNT, TRAF3, and p53 [14-18] were reported to be regulated by miR-214-3p and influence tumor progression or suppression during tumorigenesis. In this study, we selected FRK as a potential target of miR-214-3p based on miRNA database analyses [26,27]. FRK is known to act both as a tumor suppressor or promoter, depending on organ-specific pathogenesis [20]. Previous studies have reported that FRK played a role as a tumor suppressor in breast cancer [24,30], but there are relatively few reports about the role of FRK in breast cancer; furthermore, the key regulator of FRK has not been reported.

In this study, endogenous expression of miR-214-3p was inversely related to FRK expression in breast cancer cell lines (Figure 1). Transfection of miR-214-3p into breast cancer cell lines (MCF-7 and MDA-MB-231) resulted in decreased expression of FRK and increased tumor cell proliferation, migration, and invasion. In MDA-MB-231 cell line, which represents TNBC, silencing FRK showed an increase in tumor cell proliferation and a partial increase in tumor cell migration and invasion similar to that in the miR-214 mimic-transfected cells. In the case of MCF-7 cells, representing the luminal type, si-FRK-transfected cells showed a partial increase in cell proliferation, migration, and invasion. Based on this result, we validated that miR-214-3p may act as an oncomiR and suppress FRK expression, which may be related to oncogenesis. The role of miR-214-3p and FRK in tumor invasion and proliferation may be more dominant in TNBC.

We hypothesized that FRK may be a direct target of miR-214; and silencing FRK may enhance cell proliferation as transfection of miR-214 mimic increased proliferation, invasion, and migration in breast cancer cells. However, transfection of si-FRK into a breast cancer cell line showed a partial increase in cell proliferation, invasion, and migration compared to that in miR-214 mimic-transfected cell lines. These differences may be due to complex interactions between miR-214-3p and other oncogenes and tumor suppression genes, including FRK. MiR-214-3p is one of the major miRNAs regulating tumor growth and targeting diverse oncogenes and tumor suppressors such as the PTEN/AKT pathway, β -catenin, p53, and various tyrosine kinase receptor pathways [20]. Unlike our primary hypothesis that that was proposed in this study, miR-214-3p may act as an upper-regulatory oncomiR and regulate not only FRK but also other major oncogenes, and FRK acting as a tumor suppressor located at a sub-specific level and may also be regulated by various miRNAs other than miR-214-3p. Based on our study, miR-214-3p may be suggested as an oncomiR in breast cancer oncogenesis and FRK as one of its target genes influencing tumor invasion, progression, and migration in breast cancer, especially in TNBC.

This study has some limitations. We validated the role of miR-214-3p and FRK in certain breast cancer cell lines. Considering that these data are based on preclinical studies, careful interpretation of the results is needed, and further validation of study results using human tissues such as surgical specimens may be needed. At present, we are planning to analyze the expression patterns of miR-214-3p and FRK in human TNBC tissues stratified by tumor grade and stage.

In summary, we validated the potential role of miR-214-3p as an oncomiR in breast cancer, especially in TNBC, that acts by suppressing FRK during tumorigenesis. MiR-214-3p may function as a major regulator during oncogenesis in breast cancer, and inhibition of FRK may have a role in cancer cell invasion, proliferation, and invasion. Further validation of the role of miR-214-3p and FRK in human tissues is warranted, and targeting miR-214-3p and FRK may reduce breast cancer progression, leading to the development of a new treatment option in the near future.

Declarations

Ethics approval and consent to participate

This study was approved by the IRB of the Seoul St. Mary's Hospital of the Catholic University of Korea (KC17SNSI0384).

Consent for publication

Not applicable

Availability of data and materials

All data from this study are included within this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (NRF-2017R1D1A1B03034165). Proofreading of English manuscript was supported by the Catholic Medical Center Research Foundation made in the program year of 2020.

Authors' contributions

JL and AL conceived and designed the analysis. JL, SO, DMK, JK acquired, analyzed, and interpreted the data. JL and AL drafted the work and AL substantially revised it. All authors reviewed and confirmed the final manuscript.

Acknowledgements

None

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Figures

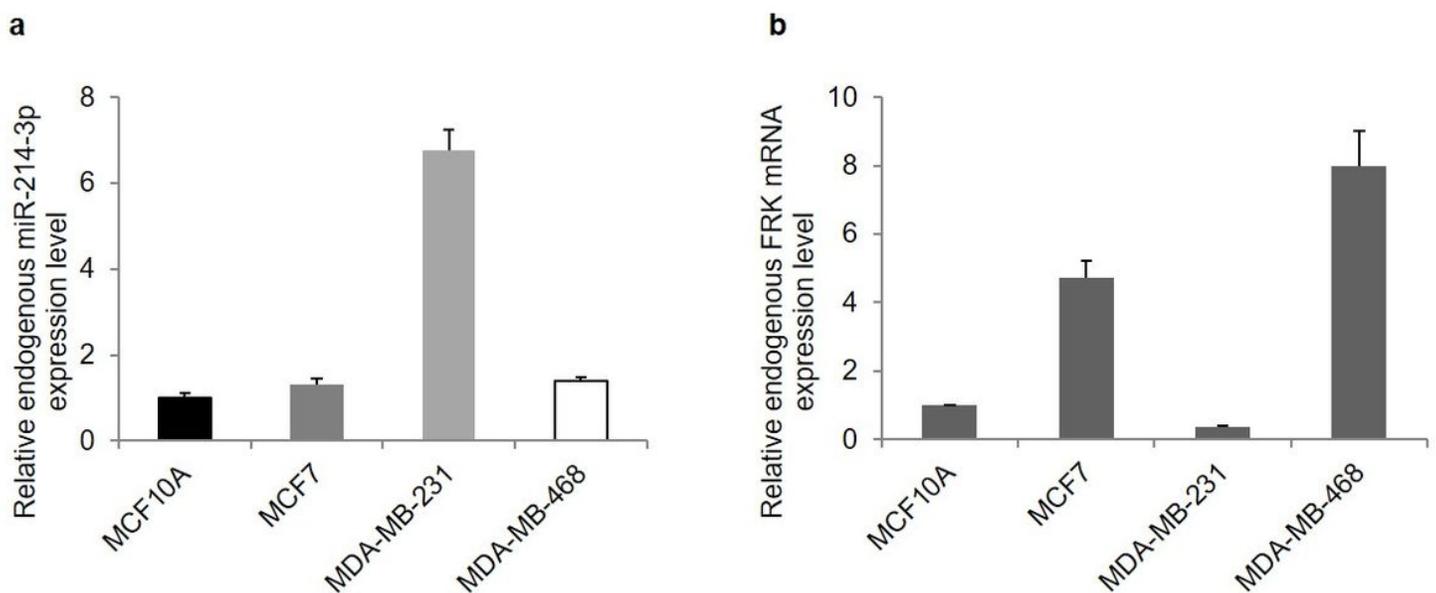


Figure 1

Expression level of endogenous miR-214-3p and FRK mRNA. Endogenous miR-214-3p (a) and FRK mRNA (b) expression level were measured by qRT-PCR. Relative gene expression was calculated according to the comparative Ct method, using GAPDH or U6 as an internal control (n=3). Error bars indicate standard deviation (SD).

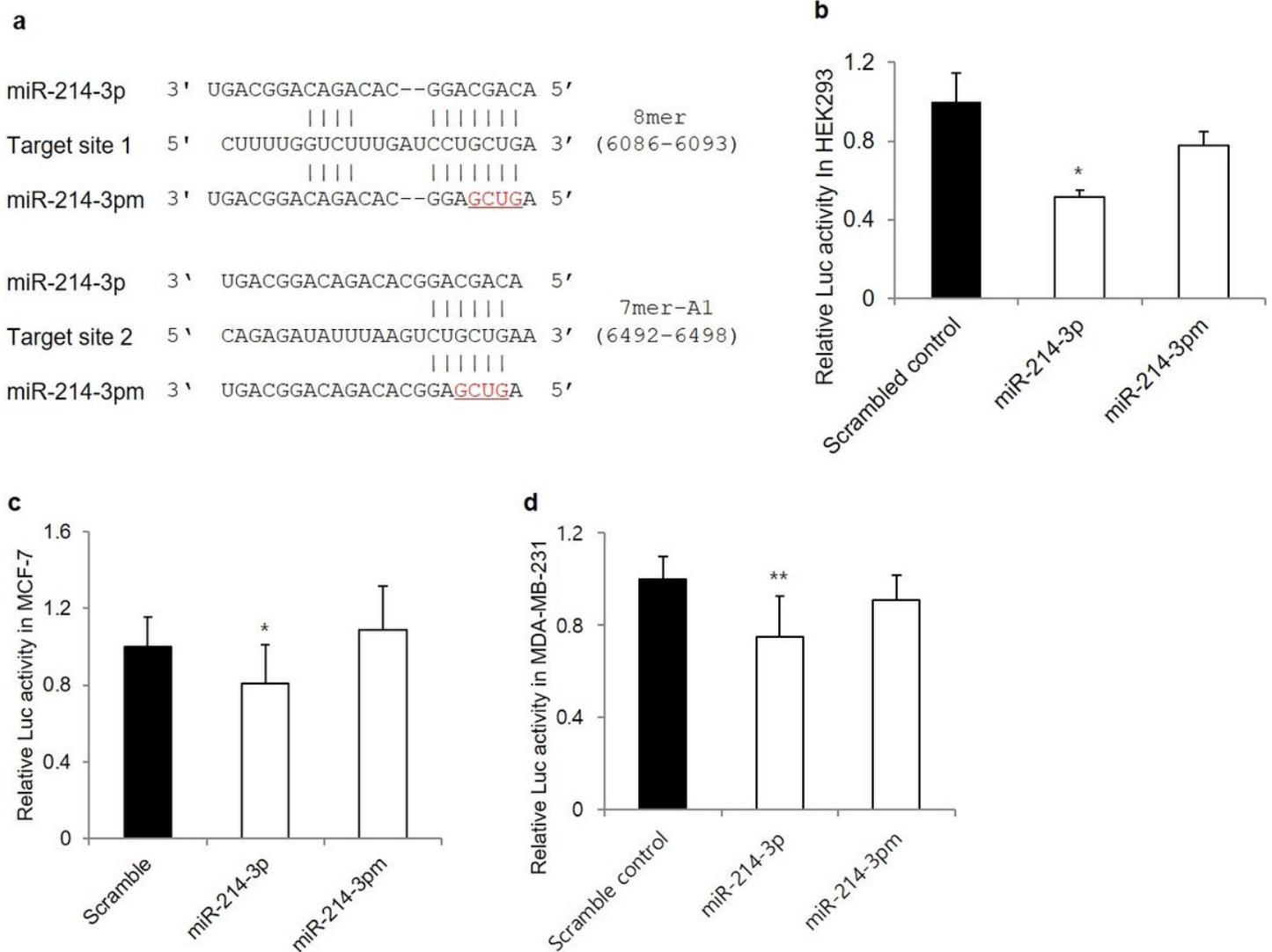


Figure 2

Luciferase reporter assay to identify miR-214-3p target sites in the FRK 3'-UTR. (a) Seed matches between miR-214-3p and 3'-UTR of FRK. Bottom bars indicate mutated nucleotides in miR-214-3pm. (b~d) Luciferase reporter assay testing the effect of miR-214-3p on FRK. A reporter construct containing (psiC-FRK) the FRK 3'-UTR and the Renilla luciferase gene was co-transfected into HEK293T cells, MCF7, and MDA-MB-231 with the miR-214-3p mimics. To confirm the sequence-specific function of miR-214-3p, the miR-214-3pm (mutant miRNA), which have nucleotide substitutions at two to four sites of miR-214-3p, were also used. Error bars indicate SD (n=3).

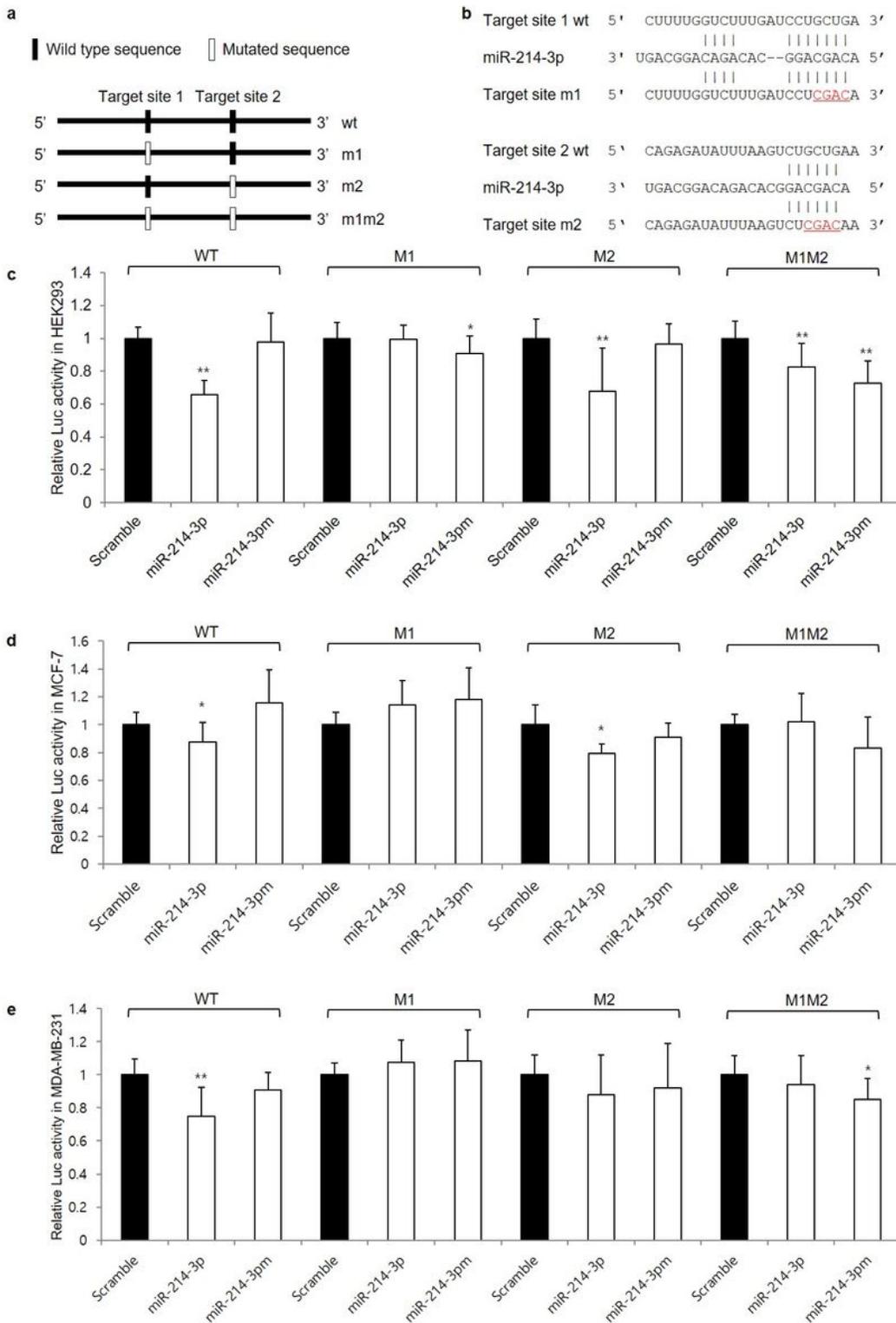


Figure 3

Luciferase reporter assay to identify miR-214-3p target sites in the FRK 3'-UTR. (a) Illustration showing the location of possible seed matched sites for miR-214-3p in the FRK 3'-UTR. Site-directed mutagenesis was performed to produce all possible mutant forms of the FRK 3'-UTR seed match sequences. Mutation signatures are as follows: m1 (psiC-FRK_m1), m2 (psiC-FRK_m2), m1m2 (psiC-FRK_m1m2). b Seed matches between miR-214-3p and the wild-type (wt) or mutated (m1 or m2) target sites in the 3'-UTR of

FRK. (c-e) Luciferase reporter assay testing the target sites of miR-214-3p on the 3'-UTR of FRK. HEK293T cells (c), MCF7 (d), and MDA-MB-231 (e) were co-transfected with miR-214-3p and luciferase reporter vectors containing the wt or mutated 3'-UTR of FRK. The scrambled control and miR-214-3pm (mutant miRNA) were used to confirm sequence-specific binding between miR-214-3p and the FRK 3'-UTR. Luciferase activity was normalized using firefly luciferase activity and is expressed as the ratio to the activity in scrambled control-transfected cells. Error bars indicate SD (n=3). *p<0.05, **p<0.01

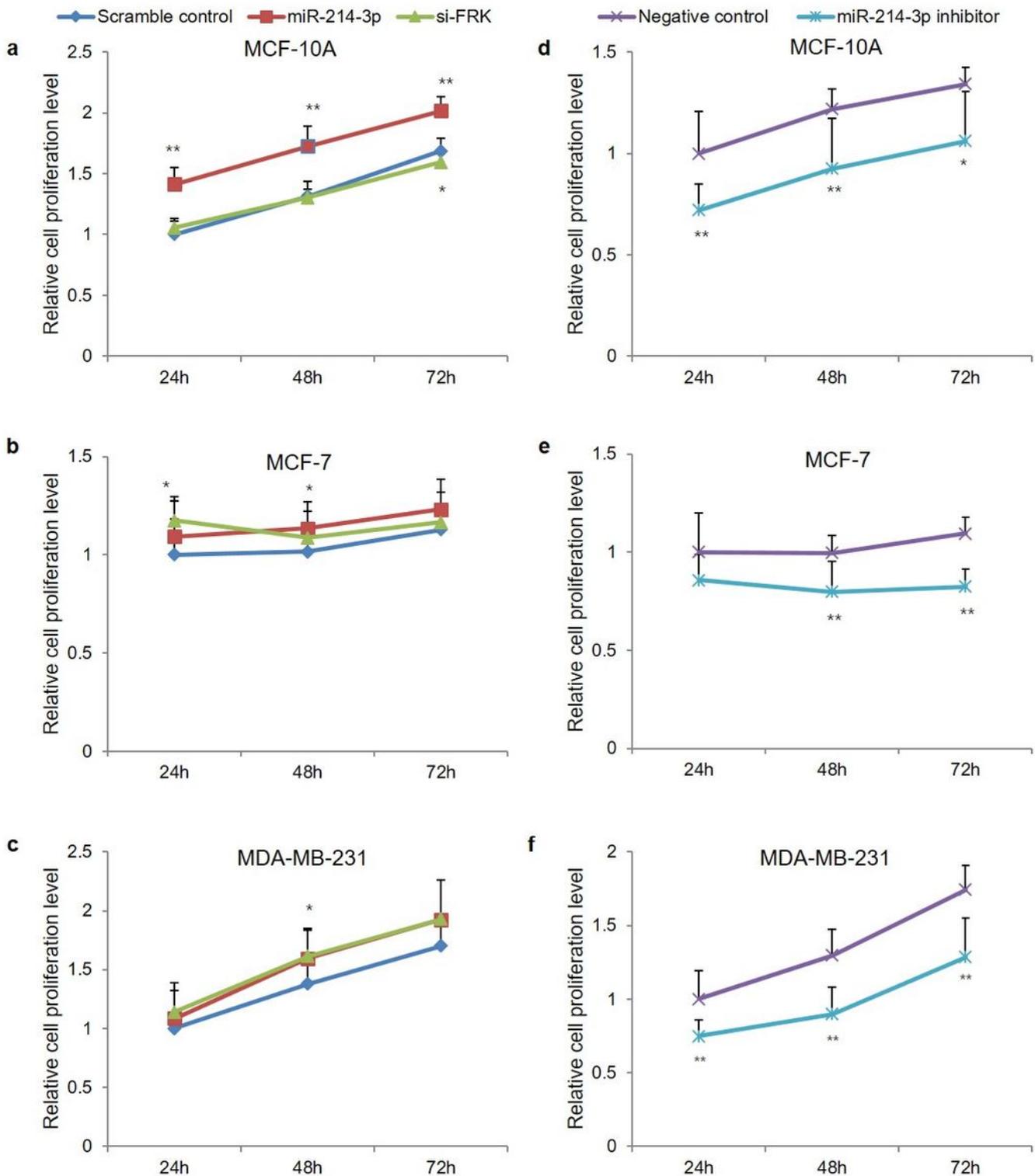


Figure 4

The effect of miR-214 or si-FRK on proliferation assay. (a-c) Overexpression of miR-214 or silencing of FRK promotes breast cell growth. The proliferation ability was determined by MTS assay at 24, 48 and 72h post-transfection with miR-214, si-FRK, scramble control in (a) MCF-10A, (b) MCF-7 and (c) MDA-MB-231. (d-f) The inhibition of miR-214 reduces to the proliferation of breast cells. The proliferation ability was determined by MTS assay at 24, 48 and 72h post-transfection with an inhibitor of miR-214 or control inhibitor in (d) MCF-10A, (e) MCF-7 and (f) MDA-MB-231. Error bars indicate SD (n=3). *p<0.05, **p<0.01

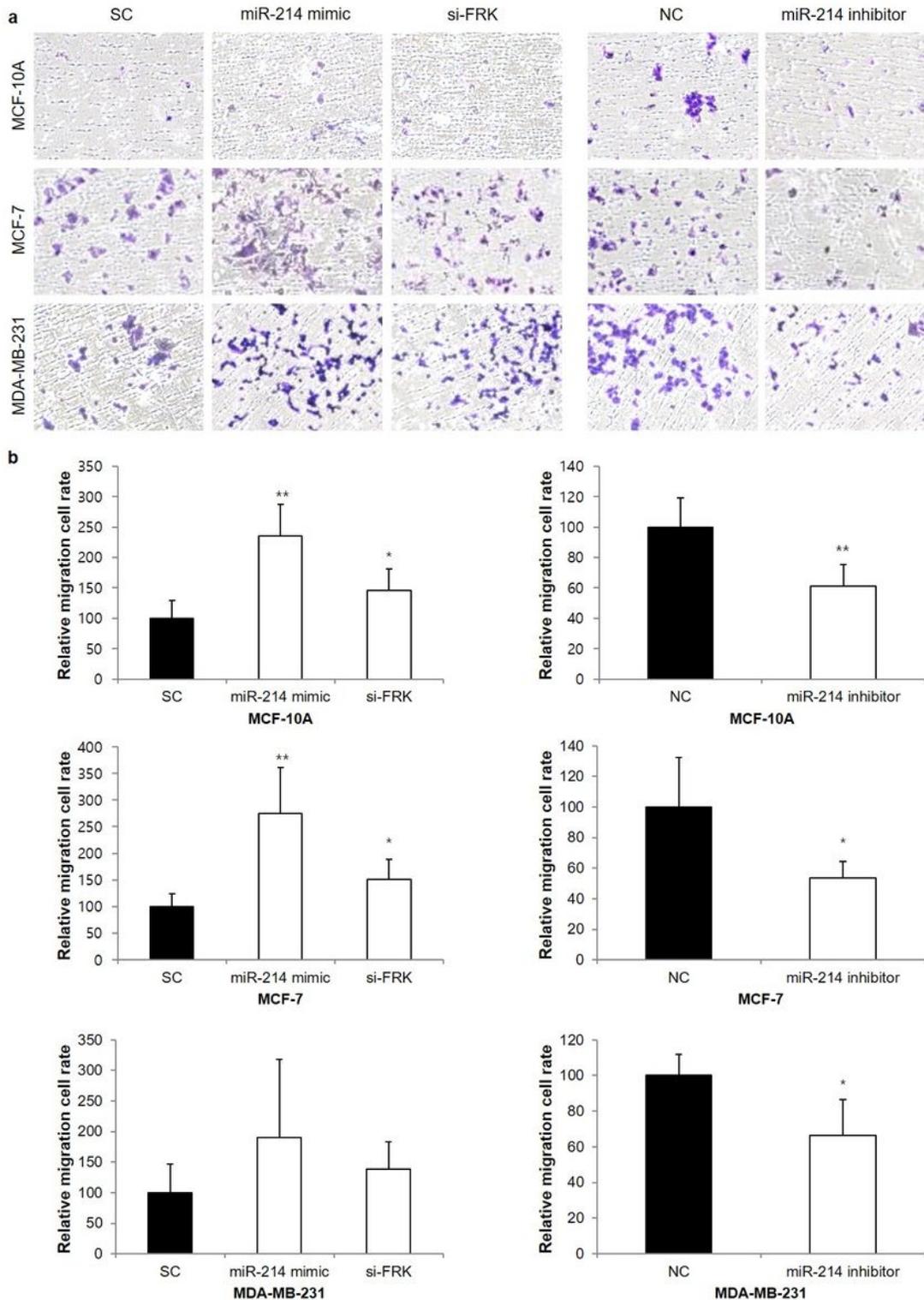


Figure 5

The effect of miR-214 or si-FRK on migration assay. (a-b) The migration ability was analyzed by transwell migration assay in MCF-10A, MCF-7 and MDA-MB-231. (A) The representative image of the transwell migration assay are presented (magnification x100); cells passing through the 8µm pore insert to the underneath well was stained. (B) The number of cells transfected with miR-214 mimic or si-FRK or miR-214 inhibitors passing through the underneath well shows a difference from the number of cells transfected with the control factor. Error bars indicate SD (n=3). *p<0.05, **p<0.01

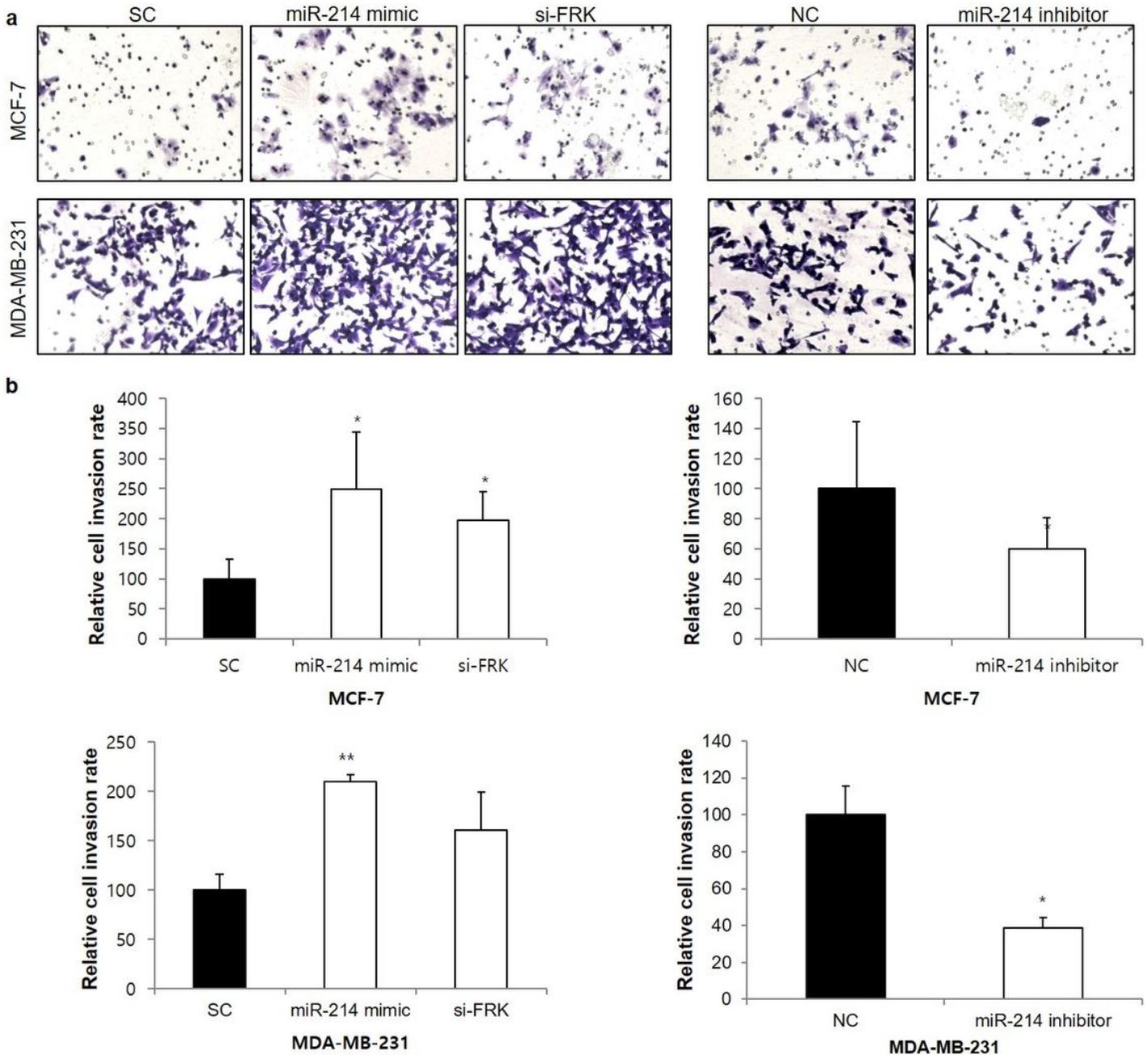


Figure 6

The effect of miR-214 or si-FRK on Invasion assay. (A-B) The migration ability was analyzed by transwell invasion assay in MCF-7 and MDA-MB-231. (A) The representative image of the transwell invasion assay are presented (magnification x200); cells passing through the 8µm pore insert to the underneath well was

stained. (B) The number of cells transfected with miR-214 mimic or si-FRK or miR-214 inhibitors passing through the underneath well shows a difference from the number of cells transfected with the control factor. Error bars indicate SD (n=3). *p<0.05, **p<0.01