

Original Article

The microRNA-410 targets Fos-related antigen 1 and inhibits malignancy in breast cancer cells

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Abstract: MicroRNAs belong to non-coding RNAs and play important roles in tumor progression. However, the role of microRNA-410 (miR-410) in breast cancer is largely unknown. In current study, we investigated the potential mechanisms of in breast cancer. We showed that the expression of miR-410 was substantially decreased in well-established breast cancer cell lines as well as solid tumors. Meanwhile, miR-410 transfection in MCF-7 and MDA-MB-231 significantly reduced the malignancy such as proliferation and invasion of these tumor cells. By bioinformatics strategies, we identified Fos-related antigen 1 (Fra-1) as a direct and plausible target downstream of miR-410. We further confirmed by experimental studies Fra-1 overexpression in Fra-1 overexpression can reverse the effect of miR-410 in MCF-7 and MDA-MB-231 cells. Taken together, our results have unraveled novel functions of miR-410 in breast cancer and provided insight into therapeutic intervention by targeting microRNAs.

Keywords: miR-410, breast cancer, malignancy, Fra-1

Introduction

Breast cancer denotes a situation where complexity and heterogeneity exist [1]. Gene profiling study also contributed substantially to the heterogeneous nature of breast cancer cells [1]. Breast cancer has posed serious threat to women and caught attention worldwide [2]. The pathological features of breast cancer should be investigated in depth, however, much is unknown about the exact nature of breast cancer progression and effective pharmacological intervention.

The microRNAs (miRNAs) are a class of noncoding and short-length RNAs which can suppress gene expression by base paring to the 3'-untranslated regions (3'-UTR) of targets [3, 4]. The imperfect binding to 3'UTR can either lead to the degradation of target messenger RNAs or prevention of their translation [5]. It has been reported that the microRNAs can exist either individually or being as a cluster among the whole genome. Meanwhile, they can

be also located in introns or exons of genes [6]. To date, thousands of microRNAs have been identified and implicated in wide repertoire of biological regulations. The role of microRNAs in breast cancer has also been extensively demonstrated. For example, miR-31 has been shown to inhibit breast cancer metastasis by using a microRNA sponge strategy [7]. The microRNA-200 family was also implicated in inhibiting the proliferation and invasion of breast cancer cells via a ZEP-SIP1 loop [8, 9]. In addition, the miR-124 utilized a different way by targeting multiple oncogenic genes and suppressed breast cancer development [10]. MiR-340 and miR-196 have been shown elsewhere and also play tumor suppressive roles in breast cancer [11, 12]. Some other microRNAs instead may promote tumor progression. For instance, miR-10b expression is correlated malignant phenotype of breast cancer possibly by initiating the epithelial-to-mesenchymal transition [13]. Meanwhile, miR-121/122 has also been shown to favor the EMT and increase the incidence of breast cancer via targeting TRPS1

[14]. However, the role of miR-410 in breast cancer has caught little attention.

The Fos-related antigen 1 (Fra-1, also named Fos1) was previously identified as a gene in immediate early response [15]. Fra-1 presents a negative regulator for AP-1 transcription factor and has transforming activity [16]. ERK-dependent activation of Fra-1 was through indispensable phosphorylation of Fra-1 at Thr-231 in transactivation domain especially in ERK sufficient JB6 cells [17]. However, similar effect was not observed in Fra-2 fusions suggesting a more specific role for Fra-1 [18]. Therefore, Fra-1 might be an important target for tumor prevention.

In current study, we first demonstrate that decreasing expression patterns for miR-410 in breast cancer cell lines and human tumors. We then investigated the role of miR-410 in shaping malignant phenotypes of breast cancer such as proliferation and invasion. In addition, we explored the potential targeting effect of miR-410 on Fra-1 as well as the regulatory functions of Fra-1 in miR-410 induced breast cancer inhibition. Our current study may shed light on the tumor suppressive role of miR-410 and provide critical implications to microRNA mediated cancer intervention.

Materials and methods

Breast cancer cells and human samples

Totally 8 breast cancer cell lines SUM190, MCF-7, MDA-MB-453, ZR-75, MDA-MB-231, MDA-MB-468, SKBR3 and BT474 were used in the study which are all commercially purchased from the American Type Culture Collection (ATCC) (Shanghai, China). A control normal breast cell line HBL-100 was also obtained from ATCC. All these cell lines were ordinarily cultured in RPMI-1640 medium (TIANGEN, Shanghai, China) supplemented with 10% fetal bovine serum (TIANGEN, Shanghai, China), and 100 U/mL penicillin plus 100 µg/mL streptomycin (TIANGEN, Shanghai, China) in a culture chamber with 5% CO₂ at 37°C. Human breast cancer samples were surgically retrieved from patients registered at the First Affiliated Hospital, Sun Yat-sen University between September 2013 and May 2015. The tumorous (T) breast cancer tissues and corresponding adjacent non-tumorous (ANT) tissues were all

paired samples. All patients have signed consent forms. The whole sets of surgical and experimental procedures associated with human samples were formally approved by the Human Research Ethics Committee of the First Affiliated Hospital, Sun Yat-Sen University (No. 2013FH045).

Transfection of microRNA-410

We utilized lentiviral transfection system to overexpress miR-410 in breast cancer cell lines MCF-7 and MDA-MB-231. The lentivirus with miR-410 mimics (Lenti-miR-410) and the negative control (Lenti-C) were purchased from TIANGEN (TIANGEN, Shanghai, China). Lentivirus transfection into breast cancer cell lines MCF-7 and MDA-MB-231 were performed using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocols. After 24 hours' transfection, cultured medium was regularly replaced.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNAs were harvested from both breast cell lines and related human samples with Trizol reagent (TIANGEN, Shanghai, China). The reverse transcript cDNA with the SYBR Premix Taq™ Toolkit (Takara, Japan) was obtained according to the manufacturer's protocols. To detect miR-410 gene, a well-established TaqMan miRNA qRT-PCR Kit (Applied Biosystem, USA) was used. To quantify Fra-1 mRNA, a SYBR Green PCR Master kit (Takara, Japan) was used. Noticeably, *GAPDH* was used as controls. Reactions were carried out with the ABI PRISM® 7000 Sequence Detection System (Applied Biosystem, USA) according to the manufacturer's protocols. Relative expressions were shown which denote the fold change relative to those under control.

Proliferation assay

The formal 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (TIANGEN, Shanghai, China) was used to calculate the proliferation. After treatment for 24 h, MCF-7 and MDA-MB-231 cells were all suspended and placed into 96-well plates (10⁵ units per well) for 5 days. 10 µL MTT solution (15 mg/mL) was added into the culture every 24 h for 4 days. Crystalline formazan was dissolved in 200 µL SDS (15%) solution for 24 h.

miR-410 in breast cancer

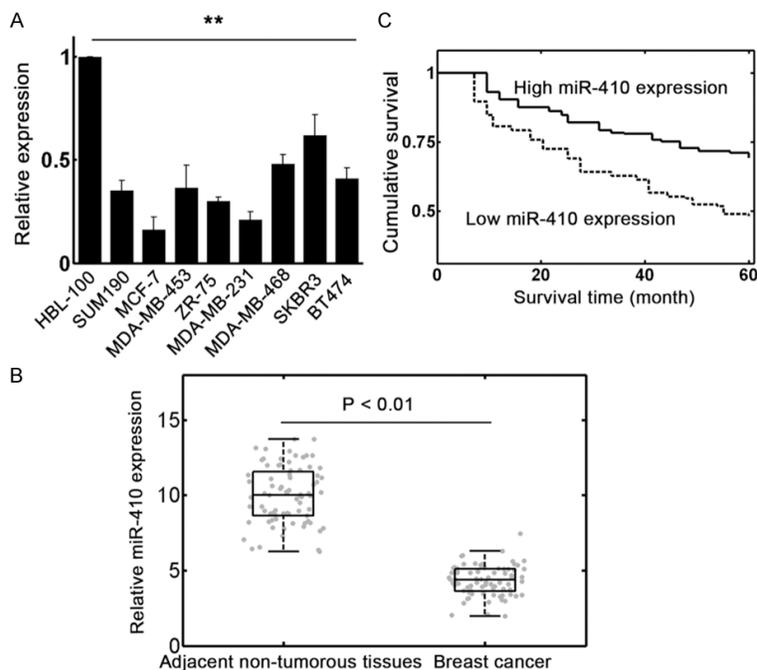


Figure 1. The miR-410 was decreased in breast cells. A. Quantitative RT-PCR results to quantify the expression of miR-410 in 8 breast cancer cell lines SUM190, MCF-7, MDA-MB-453, MDA-MB-231, ZR-75, MDA-MB-468, SKBR3 and BT474 normalized to HBL-100 (normal human epithelial breast cell line, **: $P < 0.01$). B. In 79 breast cancer patient samples, we used qRT-PCR to compare the expression of miR-410 between paired tumorous tissues and corresponding adjacent non-tumorous tissues (**: $P < 0.01$). Pair-wise *t* test was used. C. The survival curves for low and high miR-410 expression. The Kaplan-Meier χ^2 statistics was performed ($P = 0.002$).

The optical density (O.D.) at 490 nm was quantified with a Spectramax M5 microplate reader (Sigma, Shanghai, China) following the manufacturer's instruction.

Invasion assay

The 96-well transwell assay (QIANGEN, Shanghai, China) was performed to evaluate the invasion. The upper chamber was initially covered with Matrigel (Sigma, Shanghai, China) overnight. Lentivirus-treated MCF-7 and MDA-MB-231 cells were re-suspended and placed into the upper chamber (10^5 per well) in RPMI-1640 medium (Sigma, Shanghai, China). The lower chambers were replenished with RPMI-1640 medium plus additional 3% fetal calf serum (FBS). After 24-hour, upper chambers were all refreshed and migrating cells into the lower chambers were fixed with 3% paraformaldehyde (PFA) and stained with crystal violet. We monitored the transwell chamber with a Leica inverted microscope fluorescent microscope

(DM-IRB, Leica, Germany). Invasive capability was evaluated by dividing total invaded cells under each treatment by the number for control conditions.

Dual-luciferase reporter assay

Fra-1 gene was obtained from a human breast cDNA library and amplified with verification. The 3'-UTR of Fra-1 with predictive binding sites for has-miR-410 was cloned into the XbaI downstream of Renilla luciferase reporter plasmid pHRL-TK (Promega, LA, USA) to wild-type Fra-1 luciferase plasmids (Fra-1 3'UTR WT). Binding site for has-miR-410 upon Fra-1 3'UTR was also mutated by a Quik-Change™ Site-Directed Mutagenesis Kit (Stratagene, USA). The mutated Fra-1 3'UTR was accordingly incorporated into pHRL-TK to obtain the mutants (Fra-1 3'UTR MUT). In 293T cells, transfection was performed for 48 h.

The relative luciferase units (R.L.U) were followed by a dual-luciferase reporter assay (Promega, USA) according to the manufacturer's protocol.

In vivo implantation

MCF-7 cells were transduced with lentivirus for 12 h and then cultivated for another 24 hours. After that, all cells were suspended and 10^5 cells were implanted subcutaneously into nude mice. The *in vivo* volume of tumors was determined weekly (length multiply width multiply height). After 6 weeks, all mice were sacrificed for Ki-67 immunostaining (Sigma, Shanghai, China).

Fra-1 overexpression

Fra-1 sequence was cloned into a recombinant plasmid eukaryotic plasmid pcDNA3.1 (TIANGEN, Shanghai, China) to obtain the plasmid named pcDNA3.1/Fra-1. The transfection of pcDNA3.1/Fra-1 and the empty pcDNA3.1

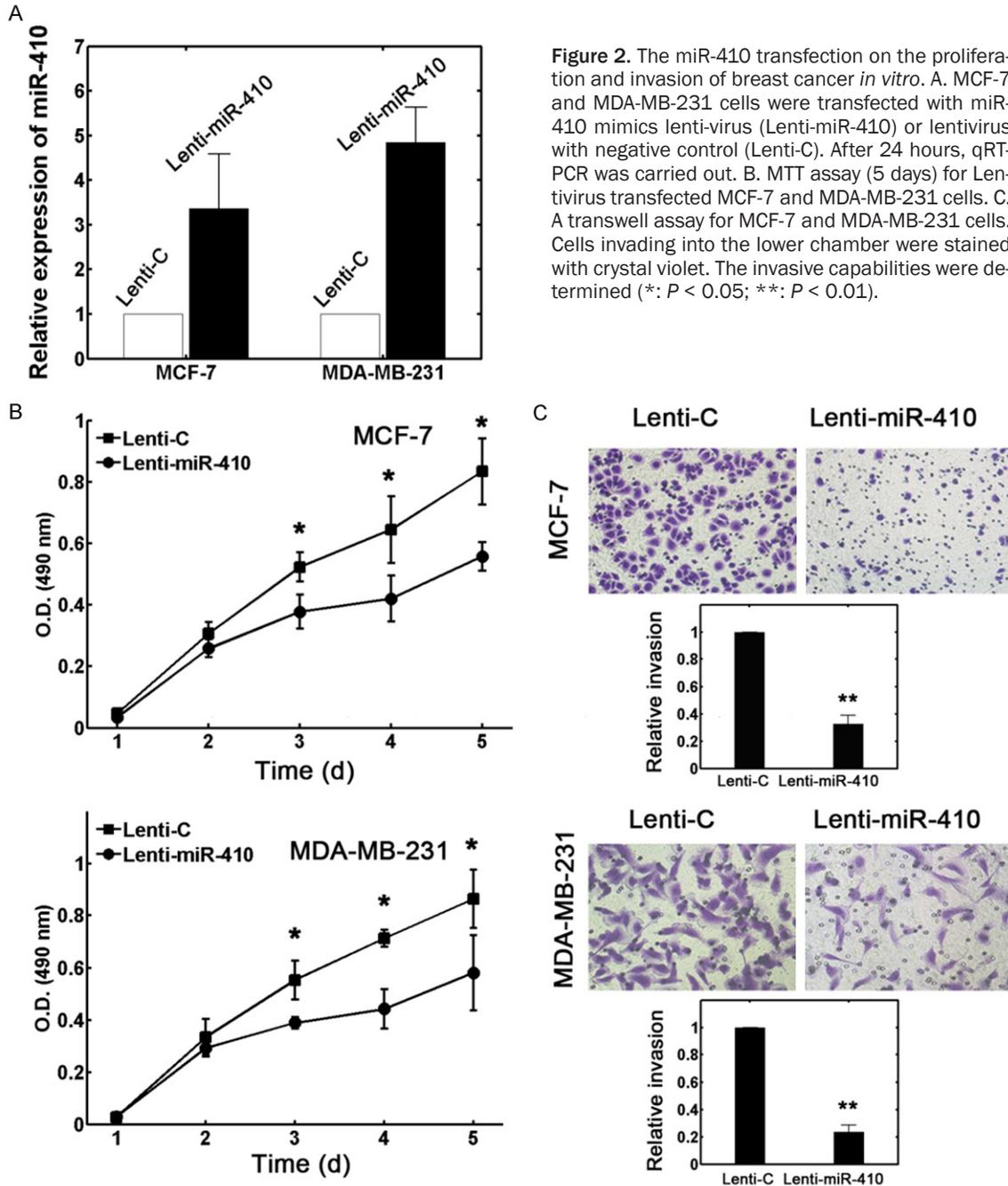


Figure 2. The miR-410 transfection on the proliferation and invasion of breast cancer *in vitro*. A. MCF-7 and MDA-MB-231 cells were transfected with miR-410 mimics lenti-virus (Lenti-miR-410) or lentivirus with negative control (Lenti-C). After 24 hours, qRT-PCR was carried out. B. MTT assay (5 days) for Lenti-virus transfected MCF-7 and MDA-MB-231 cells. C. A transwell assay for MCF-7 and MDA-MB-231 cells. Cells invading into the lower chamber were stained with crystal violet. The invasive capabilities were determined (*: $P < 0.05$; **: $P < 0.01$).

plasmid pcDNA3.1/+ into MCF-7 and MDA-MB-231 cells were performed with Lipofectamine 2000 (Invitrogen, Shanghai, China). After 24 h, cells were suspended and plated into 96-well plates.

Western blotting assay

MCF-7 and MDA-MB-231 cells were collected with lysis buffer containing 12% glycerol and

5% NP-40 (Sigma, Shanghai, China). The protein extracts (200 μg each) were dissolved in 15% SDS-PAGE and migrated to nitrocellulose membranes (Bio-Rad, USA). Antibodies against human Fra-1 (1:1000, Sigma, Shanghai, China) was carried out at 4°C overnight, and HRP-conjugated secondary antibodies (1:1000) at 20°C for 2 h. Immunoblots were shown with chemiluminescence film system (Amersham Pharmacia Biotechnology, Shanghai, China).

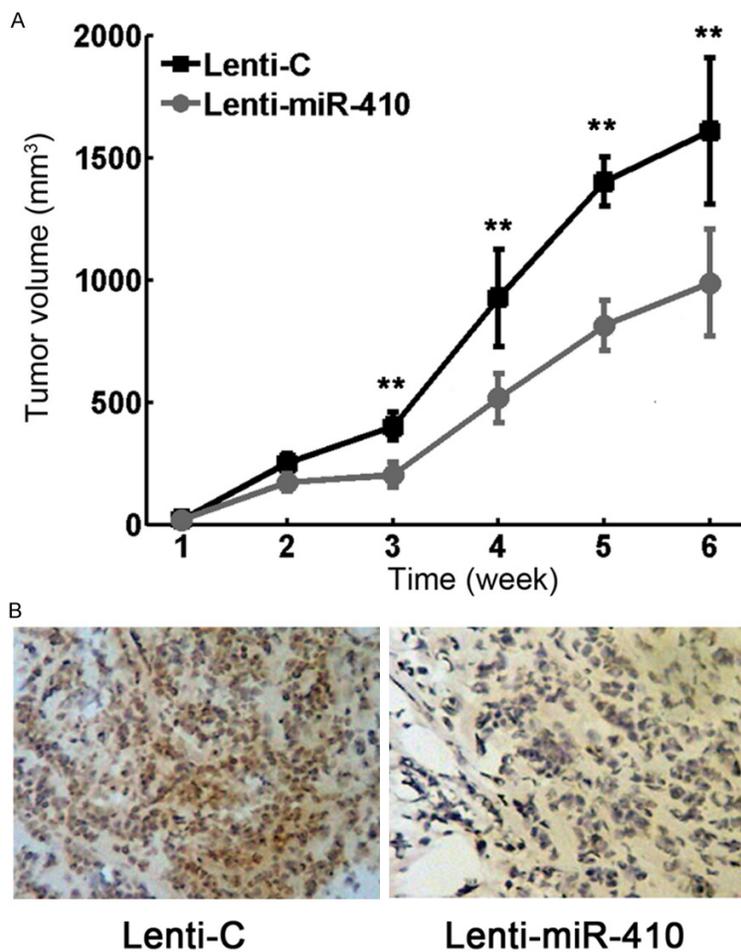


Figure 3. The expression of miR-410 on breast cancer implantation. A. 10^6 lentivirus transduced MCF-7 cells were subcutaneously injected into the flanking region of nude mice. The tumor volume *in vivo* was measured weekly (length \times width \times height) for a total duration of 6 weeks. B. By the end of the implantation, solid tumors were resected for Ki-67 staining on paraffin-embedded sections (*: $P < 0.05$; **: $P < 0.01$).

Statistical analysis

All experiments were performed with three replicates. Results were presented as mean \pm standard errors (SE). The significance for Kaplan-Meier curves was determined with a log-rank test. Statistical differences were quantified with student's *t*-test using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and the significance was evident if $P < 0.05$.

Results

Decreased expression of miR-410 in cancerous cells correlates with survival

We initially measured the expression of miR-410 in different breast cancer cell lines using

qRT-PCR. Human samples were also subject to PCR analysis. We found that the level of miR-410 was substantially decreased in all tested breast cancer cell lines compared with normal control (HBL-100) (Figure 1A, **: $P < 0.01$). We also compared miR-410 expression in tumor and associated adjacent non-tumorous tissues in 79 specimens (Figure 1B, **: $P < 0.01$). The results showed that miR-410 was also significantly downregulated in tumorous tissues (Figure 1B, **: $P < 0.01$). Additionally, patients with increased survival also significantly correlated with higher miR-410 expression ($P = 0.002$, Figure 1C). Taken together, these results suggested that miR-410 is decreased in breast cancer cells and correlated with survival.

Overexpression of miR-410 inhibits breast cancer malignancy

We then transfected the lentivirus vector expressing miR-410 into MCF-7 and MDA-MB-231 cells. After 24 hours' transfection, we used qRT-PCR to evaluate the efficiency. The results showed that miR-410 was elevated with lentivirus transfection (Lenti-miR-410) in both MCF-7 and MDA-MB-231 cells (Figure 2A, *: $P < 0.05$).

We accordingly transfected MCF-7 and MDA-MB-231 cells with lentivirus and then re-suspended these cells. After the treatment, cells were moved into 96-well plates for 5 days. The proliferation assay was performed every 24 h. The results showed that increased miR-410 expression strongly inhibited breast cancer proliferation in both MCF-7 and MDA-MB-231 cells (Figure 2B, *: $P < 0.05$). A subsequent transwell assay further confirmed that miR-410 elevation can decrease breast cancer invasion (Figure 2C). Quantification results showed that miR-410 upregulation reduced the invasion in

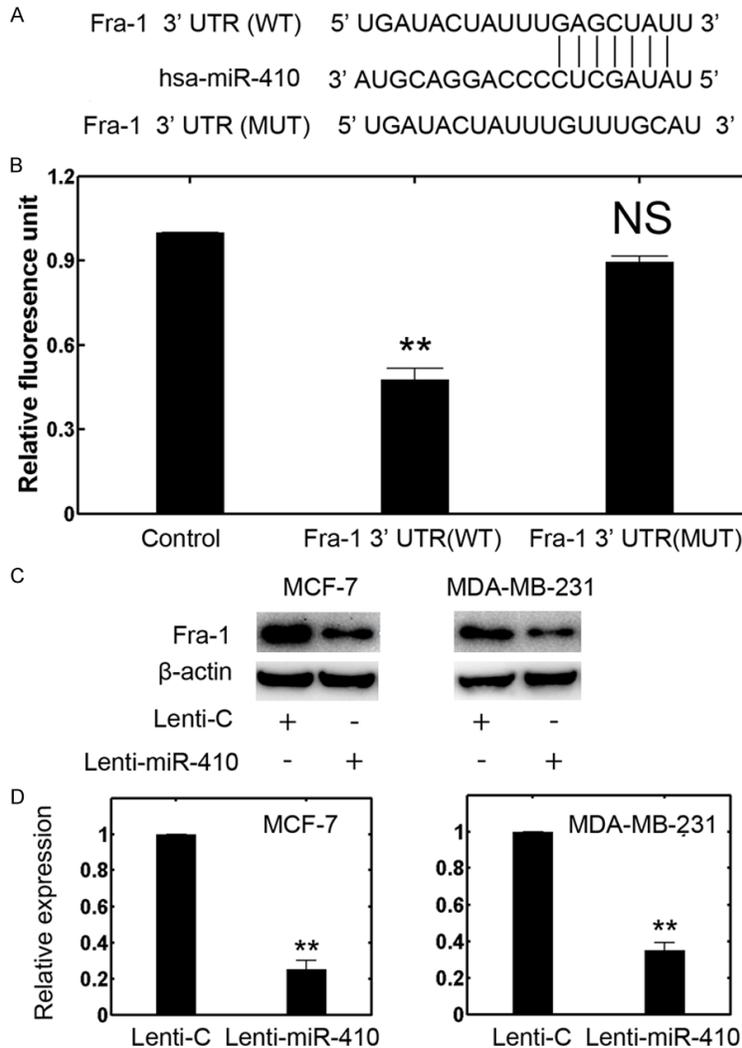


Figure 4. Fra-1 is the direct target of miR-410. A. DNA sequences showed the alignment of has-miR-410 on wild type (WT) Fra-1 3'UTR. Fra-1 3'UTR were mutated (MUT). B. 293T cells were transfected with Lenti-miR-410, wild-type Fra-1 plasmid (Fra-1 3'UTR (WT)), mutated Fra-1 plasmid with mutated miR-410 binding sites Fra-1-3'UTR (MUT) or an empty Renilla plasmid (control) for 24 h in a dual-luciferase reporter assay. Note that the luciferase activities were normalized. C. MCF-7 and MDA-MB-231 cells were transduced with lentivirus containing miR-410 mimics (Lenti-miR-410), or lentivirus containing negative control miRNA (Lenti-C) for 24 h. The protein levels of Fra-1 were shown by western blots. D. The expression of Fra-1 mRNAs were evaluated by qRT-PCR (*: $P < 0.05$; **: $P < 0.01$).

MCF-7 and MDA-MB-231 cells by over 50% (Figure 2C, *: $P < 0.05$). These results suggested that miR-410 can attenuate the malignancy of breast cancer cells.

Increasing miR-410 inhibits breast cancer in vivo implantation

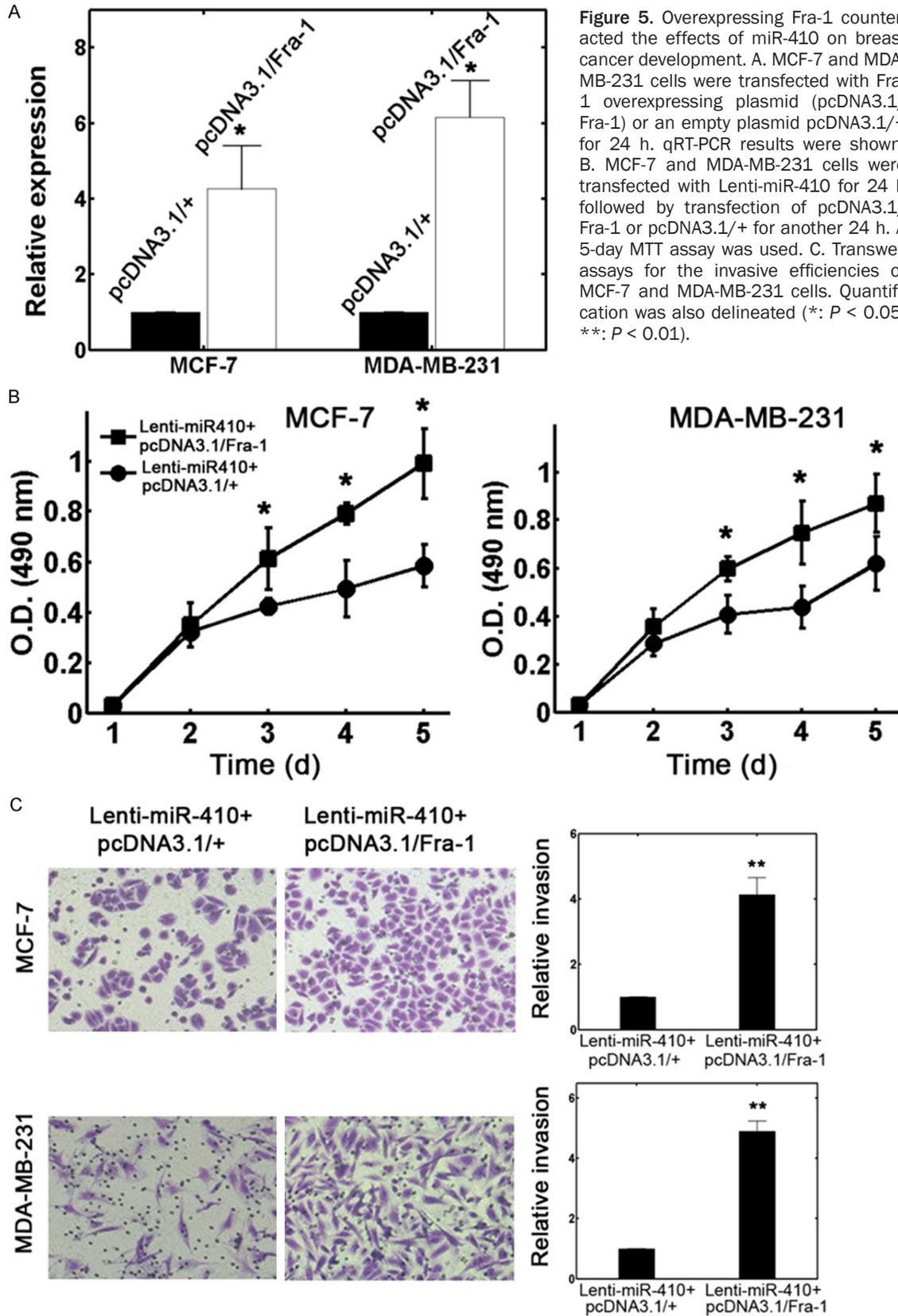
In addition to the effect of miR-410 *in vitro*, we also set out to determine whether miR-410

influenced tumor growth *in vivo*. As a result, we transduced MCF-7 cells with Lenti-miR-410 or controls for 24 h. Cells were then re-suspended (final loading is 10^6) were implanted subcutaneously into nude mice at rear flank. The volume of tumor was determined by length \times width \times height each week (with a total duration for 6 weeks). Results showed that higher miR-410 levels significantly correlated with lower tumor burden (Figure 3A). Ki-67 staining at 6 week after implantation was also performed. The results showed that Ki-67 staining also decreased with higher miR-410 expression (Figure 3B). These results further verified the tumor suppressive role of miR-410 *in vivo*.

MiR-410 may target Fra-1 in breast cancer

To identify plausible targets of miR-410, we used several miRNA targets prediction tools such as PicTar (pictar.mdc-berlin.de), TargetScan (www.targetscan.org) and miRDB (www.mirdb.org). Cross validation implied that Fra-1 might be the target (Figure 4A). Dual luciferase reporter assays were then used to experimentally confirm these predictions. We found that the plasmids with wild type (WT) Fra-1 3'UTR can be targeted by miR-410

to substantially reduce the luciferase activities (Figure 4B, *: $P < 0.05$). However, mutant luciferase plasmids (3'UTR MUT) were not affected by miR-410 transfection (Figure 4B, NS). Western blots were then performed and the results showed that lenti-miR-410 transfection can greatly decrease the expression of Fra-1 in both MCF-7 and MDA-MB-231 cells (Figure 4C). The results by qRT-PCR also showed consistent results (Figure 4D, *: P



< 0.05). These results suggested that Fra-1 might be a direct target of miR-410 in breast cancer cells.

Restoring Fra-1 expression increases the malignancy of breast cancer cells

We next verified whether Fra-1 overexpression can reverse the effect of miR-410 transfection *in vitro*. We established a mammalian Fra-1 overexpressing plasmid (pcDNA3.1/Fra-1) in MCF-7 and MDA-MB-231 cells and the results showed that transfection of the plasmid can substantially upregulate Fra-1 expression (**Figure 5A**, *: $P < 0.05$). Dual luciferase reporter assay was then performed. MCF-7 and MDA-MB-231 cells were transfected with lenti-miR-410. After 36 hours, these cells were also transfected with either pcDNA3.1/Fra-1 or the empty control pcDNA3.1/+ for another 24 hours. The results showed that Fra-1 overexpression markedly relieved the inhibition of miR-410 co-transfection in the tested breast cancer cell lines (**Figure 5B**, *: $P < 0.05$). Transwell assay further confirmed that Fra-1 overexpression significantly increased the invasion of MCF-7 and MDA-MB-231 cells (**Figure 5C**). Furthermore, we noticed that the invasive capacity was enhanced by more than two-fold (**Figure 5C**). Overall, our results suggested that Fra-1 can be targeted by miR-410 and overexpressing Fra-1 can increase the adverse effect in breast cancer cells.

Discussion

The miRNAs denote short-length and noncoding RNAs which can regulate gene expression by directly targeting messenger RNAs for degradation or translational inhibition. Aberrant microRNAs are generally associated with malignant progression of many tumors [19]. These microRNAs may serve as important mediators in the progression of cancer cells [20, 21]. As recently implied, breast cancer patients ordinarily suffer from cancer cell metastasis before diagnosis which deteriorates current treatments such as radiotherapy and chemotherapy [22]. Therefore, in-depth investigation of the mechanisms in breast cancer is crucial for identifying novel therapeutics to increase overall survival.

Few studies reported the role of miR-410 especially in breast cancer. Recent microRNA profiling study suggested miR-410 was decreased in

bladder cancer cells [23]. The expression of miR-410 also correlates with the cisplatin sensitivity in more than half of the bladder tumor cell lines [23]. Another report instead showed that miR-410 can markedly regulate MET, the receptor for hepatocyte growth factor receptor (HGF) and influence AKT pathway activation in glioma cells [24]. Chen et al. therefore suggested that miR-410 may function as a tumor suppressor in glioma cells by reducing the proliferation and invasion [24]. Zhao et al. consistently demonstrated that miR-410 can repress vascular endothelial growth factor (VEGF) expression and inhibit osteosarcoma growth [25]. Their study showed a cooperative relation between VEGF and miR-410 in shaping osteosarcoma tumor development [25]. In addition to a tumor suppressive role, miR-410 has also been shown to promote tumor progression. Wang et al. recently found that miR-410 was frequently overexpressed in liver and colorectal tumors and correlated with poor survival [26]. They further confirmed that the tumor suppressor four-and-a-half LIM 1 (FHL1) as the potential target of miR-410 [26]. Therefore, the role of miR-410 was still contradictory and demands intensive investigation.

It was shown that Fra-1 belongs to AP-1 and is frequently upregulated in numerous cancers [27, 28]. High levels of Fra-1 are found in some samples of breast cancer [29]. Fra-1 is frequently elevated in breast cancer patients where Fra-1 knock-down can decrease the malignancy of tumors [30]. In addition, Fra-1 has multiple roles in cell invasion via its transcriptional activities and upregulates myriads of genes to promote metastasis [31]. Downregulation of miR-410 in cancer cells may therefore upregulate Fra-1 expression and favor tumor metastasis as stated in current research. Previous report showed that Fra-1 is primarily regulated by posttranslational modifications [32]. Our study therefore added another layer of complexity to the regulation of key oncogenic factor by showing that miR-410 directly targets Fra-1 through mRNA silencing.

In conclusion, we have shown for the first time that miR-410 expression is associated with breast cancer progression. We found that miR-410 can serve as a tumor suppressor in breast cancer. In addition, Fra-1 was confirmed by both bioinformatics and experiments as the direct downstream target of miR-410 in breast

cancer. The miR-410 can downregulate Fra-1 expression while Fra-1 transfection can partially reverse the adverse effect. Future studies are strongly needed to elaborate the roles of miR-410 in other tumors and this may lead to more sophisticated targets for pharmacological intervention.

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Disclosure of conflict of interest

None.

Authors' contribution

CQL, LLL and YBL conceived the study. CQL, MQM, YXZ performed the experiments. MQM and ZMN analyzed the data. CQL, LLL and YBL wrote the paper. All authors have read and approved the final version of the paper.

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