miR-410-3p suppresses breast cancer progression by targeting Snail

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Abstract. miR-410-3p acts as an oncogene or tumor-suppressor gene in various types of cancer. However, its role in breast cancer remains unknown. In the present study, expression of miR-410-3p in 30 breast cancer and paired adjacent normal tissues was detected by RT-qPCR. The expression of miR-410-3p was downregulated in 76.7% of the breast cancer samples. To further validate the expression of miR-410-3p in breast cancer, we analyzed miR-410-3p expression profiling data set from The Cancer Genome Atlas (TCGA) including 683 breast cancer and 87 normal breast tissues. We observed that the expression of miR-410-3p was downregulated in breast cancer tissues. Next, we investigated the influence of miR-410-3p on cell proliferation by transiently transfecting the miR-410-3p mimic or inhibitor, as well as their corresponding controls in the MDA-MB-231 and MCF7 cell lines. miR-410-3p overexpression reduced cell growth, colony formation and the number of EdU-positive cells in the MDA-MB-231 cells. In contrast, inhibition of miR-410-3p in the MCF7 cells resulted in a higher proliferation rate as assessed by MTT assay, plate colony formation and EdU assays. Furthermore, miR-410-3p inhibited epithelial-mesenchymal transition. In addition, Snail was found to be a direct target of miR-410-3p based on a luciferase assay. Overexpression of Snail was able to rescue the effect of miR-410-3p in breast cancer cells. Moreover, miR-410-3p was inversely expressed with Snail in breast cancer samples. Our data provide new knowledge regarding the role of miR-410-3p in breast cancer progression.

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Introduction

Breast cancer is one of the most commonly diagnosed cancers among female around the world (1). Clinically defined, patients do not die from the primary tumor, but from metastasis, which is resistant to systemic therapy. It is acknowledged that a series of genetic alterations promote normal epithelial cells to transform into malignant cancer cells, resulting in dysregulated cell growth and metastasis to distant sites. Therefore, a better understanding of the detailed mechanisms of breast cancer progression is urgently needed.

microRNAs (miRNAs), are a class of single-stranded small non-coding RNA molecules of ~22 nucleotides, and function as negative gene regulators to downregulate the expression of target genes. miRNAs play a vital role as both oncogenes and tumor suppressors, and are implicated in the hallmarks of breast cancer (2-5). Increased evidence indicates that aberrant expression of miRNAs plays important roles in diverse biological processes, including development, differentiation, growth and metabolism (6-8). miRNAs can function either as oncogenes or tumor suppressors during cancer development and progression. Deregulation of miRNAs is observed in many types of cancers, including gastric cancer, liver cancer, bladder cancer, prostate cancer, lung cancer, glioma and breast cancer (9-11).

Aberrant expression of miR-410-3p has been observed in various types of cancers, suggesting that miR-410-3p plays a significant role in cancer development and progression (12-19). Previous research has shown that miR-410-3p functions as a tumor suppressor by targeting MDM2 in gastric cancer or the angiotensin II type 1 receptor in pancreatic cancer, respectively (16,17,19). Furthermore, miR-410-3p regulates MET to influence the proliferation and invasion of glioma (14). However, several studies indicate that miR-410-3p functions as an oncogene to promote cancer proliferation (13,15,19), indicating that miR-410-3p plays dual roles in different types of cancers. The role of miR-410-3p in breast cancer development and progression remains unclear.

In the present study, we found that expression of miR-410-3p was downregulated in breast cancer tissues compared

with that noted in paired normal breast tissues. Moreover, overexpression of miR-410-3p promoted cell proliferation and invasion in breast cancer. Furthermore, Snail, an epithelial-mesenchymal transition (EMT)-related factor, was identified as a target of miR-410-3p. These results suggest that high expression of miR-410-3p may be involved in breast cancer progression.

Materials and methods

Cell culture and tissue specimens. The MCF7, T47D, BT474, BT549, MDA-MB-468 and MDA-MB-231 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured as previously described (20).

Breast cancer specimens were obtained from Tianjin Medical University Cancer Institute and Hospital. A total of 30 primary breast cancer tissues and paired adjacent normal breast tissue specimens were included in this study. All tumors were from patients with a newly diagnosed breast cancer who had received no therapy before sample collection. After mastectomy surgery, the primary breast cancer tissues and the adjacent normal tissues were flash-frozen in liquid nitrogen and stored at -80˚C. This study was approved by the Institutional Review Board of the Tianjin Medical University Cancer Institute and Hospital, and written consent was obtained from all participants.

To further validate the expression of miR-410-3p in breast cancer, we analyzed miR-410-3p expression profiling data set from The Cancer Genome Atlas (TCGA) including 683 cases of breast cancer tissues and 87 cases of normal breast tissues.

Plasmid, miRNA and antibodies. In pcDNA3.1-HA, annealed oligonucleotides encoding the HA tag were ligated into the *Hin*dIII and *Bam*HI sites of pcDNA3.1 (Invitrogen). The ORF of human Snail was generated from MDA-MB-231 cells, the resultant PCR product of which was connected together with pcDNA3.1 tagged HA (Snail-HA), the Snail 3'-UTR containing the miR-410-3p binding site or the miR-410-3p binding site mutated fragments were cloned into the pGL3-Control vector (Snail-3'UTR-wt and Snail-3'UTR-mu; Promega, Madison, WI, USA) and the resulting constructs were confirmed by DNA sequencing. miRNAs were purchased from RiboBio (Guangzhou, China). Antibodies against Snail (Abcam, Cambridge, MA, USA) and β-actin (Cell Signaling Technology, Beverly, MA, USA) were used. Recombinant human TGFβ1 was purchased from R&D Systems (Redmond, WA, USA).

RNA extraction and reverse transcription quantitative-PCR. Total RNA from the cultured cells and surgically resected fresh breast tissues was extracted using mirVana PARIS kit (Life Technologies) according to the manufacturer's instructions. For miRNA detection, miRNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit and real-time quantitative PCR was performed using TaqMan miR-410-3p and U6 RNA (used as a normalizer) assays (Life Technologies) following the manufacturer's instructions.

Western blot analysis. Cells were lysed in RIPA buffer protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Samples were denatured for 5 min at 95˚C and subjected to 10% SDS/PAGE. The separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 5% (w/v) skim milk-TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.3) solution, followed by incubation with the primary antibodies diluted in skim milk-TBST solution overnight at 4˚C. Then the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature, and the immunoreactive protein bands were visualized by enhanced chemiluminescence reagents (Millipore).

Transfection and luciferase assay. For transfection, the cells were plated at a density of $2x10^5$ cells/well in 6-well plates. When the cells reached 60% confluency, 50 nmol/l miRNA or 4 μg Snail-HA was transfected into the cells using Lipofectamine 3000 (Invitrogen) for 48 h, according to the manufacturer's recommendations. After transfection, the RNA and protein were extracted after 24 and 48 h, respectively.

Luciferase assay was carried out on extracts from the different breast cancer cells co-transfected for 24/48 h with the corresponding plasmids or miRNAs using a dual-luciferase assay kit (Promega) according to the manufacturer's recommendations. The results were normalized against *Renilla* luciferase activity. All transfections were performed in triplicate.

Cell proliferation assay. MTT, plate colony formation and EdU assays were used to evaluate the ability of cell proliferation.

For the MTT assay, the cells were seeded in 96-well plates ($5x10^3$/well). Cell viability was examined during the following 5 days. After incubation for the indicated time, the cells were incubated with 20 μl MTT (5 mg/ml in PBS; Sigma-Aldrich) at 37˚C for 4 h. Then, the medium was removed and the formazan was dissolved in 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich). The absorbance was measured at 570 nm using a microplate auto-reader (Bio-Rad).

For the colony formation assay, 24 h after transfection, the cells were seeded into 6-well plates at a density of 500 cells/well. After ~15 days, the cells grew to visible colonies and were stained with crystal violet. The colonies were counted and compared with the control cells.

The EdU assay was performed using the EdU labeling/detection kit (RiboBio) according to the manufacturer's protocol. Briefly, after transfection for 48 h, the cells were incubated with 25 μM EdU for 12 h. The cells were fixed with 4% formaldehyde for 30 min at room temperature and treated with 0.5% Triton X-100 for 15 min at room temperature for permeabilization. After washing with PBS, the cells were reacted with Apollo reaction cocktail for 30 min. Times before fixation, permeabilization, and EdU staining. Subsequently, cell nuclei were stained with Hoechst 33342 at a concentration of 5 μg/ml for 30 min. Then the cells were observed under a fluorescence microscope. The pecentage of EdU-positive cells was examined by fluorescence microscopy.

Invasion assay. The invasive ability of the breast cancer cells *in vitro* was evaluated using Matrigel-coated Transwell inserts (BD Biosciences, San Diego, CA, USA), respectively. Briefly, $5x10^4$ cells in 500 μl serum-free medium were added to the

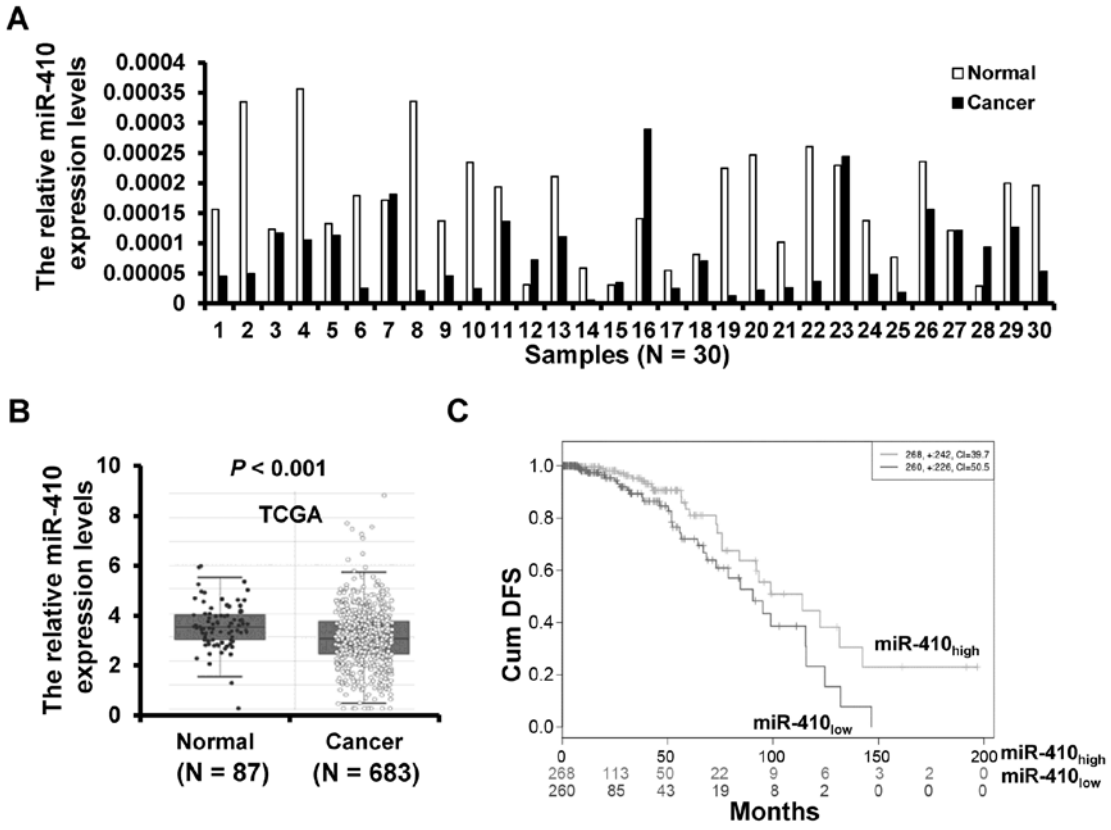

Figure 1. miR-410-3p is downregulated in breast cancer tissues. (A) Expression of miR-410-3p in breast cancer and paired normal breast tissues by RT-qPCR. (B) Expression of miR-410-3p in breast cancer tissues and normal breast tissues based on gene expression profiling data from TCGA. (C) Kaplan-Meier analysis of breast cancer patients with different miR-410-3p expression by starBase v2.0.

upper chamber, and medium containing 20% FBS was added into the lower chamber. Twenty-four hours later, the migrating cells that had attached to the lower surface were fixed with 20% methanol and stained for 20 min with crystal violet. The membranes were then carved and embedded under coverslips with the cells on the top. The number of migrating cells was counted under a microscope in five predetermined fields.

Statistical analysis. All the experiments were performed at least twice independently, and data are presented as mean ± standard error mean. All statistical analyses were performed using SPSS18.0 software system for Windows (SPSS Inc.). Statistical significance of difference was calculated using the Student's t-test with significant differences defined as at least a P-value of <0.05.

Results

miR-410-3p is downregulated in breast cancer. The expression of miR-410-3p in 30 breast cancer tissues and paired adjacent normal tissues was detected by RT-qPCR. The expression of miR-410-3p was downregulated in 23 (76.7%) of the 30 breast cancer samples (Fig. 1A). To further validate the expression of miR-410-3p in breast cancer, we analyzed miR-410-3p expression profiling data set from The Cancer Genome Atlas (TCGA) including 683 cases of breast cancer tissues and 87 cases of normal breast tissues. The validation data confirmed that the miR-410-3p expression was downregulated in breast cancer tissues (Fig. 1B). Moreover, we compared the cumulative disease-free survival (cum DFS) between patients with high miR-410-3p expression and low miR-410-3p expression and found that the cum DFS of patients with high miR-410-3p expression was higher (n=268) than that of patients with low miR-410-3p expression (n=260) according to the TCGA data (Fig. 1C). Taken together, these results indicate that miR-410-3p is downregulated in breast cancer.

miR-410-3p inhibits breast cancer cell proliferation and invasion. Next, we assessed the miR-410-3p expression levels in various breast cancer cell lines by RT-qPCR. We observed that the miR-410-3p expression was highly expressed in the MCF7 cells and lowly expressed in the MDA-MB-231 cells by RT-qPCR (Fig. 2A). Next, we investigated the influence of miR-410-3p on cell proliferation by transiently transfecting the miR-410-3p mimic or inhibitor, as well as their corresponding controls in the MDA-MB-231 and MCF7 cell lines (Fig. 2B). miR-410-3p overexpression reduced cell growth, colony formation and the number of EdU-positive cells in the MDA-MB-231 cells (Fig. 2C-E; left panels). In contrast, inhibition of miR-410-3p in the MCF7 cells resulted in a higher proliferation rate as assessed by MTT assay, plate colony formation and EdU assays (Fig. 2C-E; right panels). To investigate the role of miR-410-3p in cell invasion we used a Transwell assay. miR-410-3p overexpression reduced cell invasion in the MDA-MB-231 cells, while its inhibition enhanced cell invasion in the MCF7 cells as compared to the control cells (Fig. 2F). These results

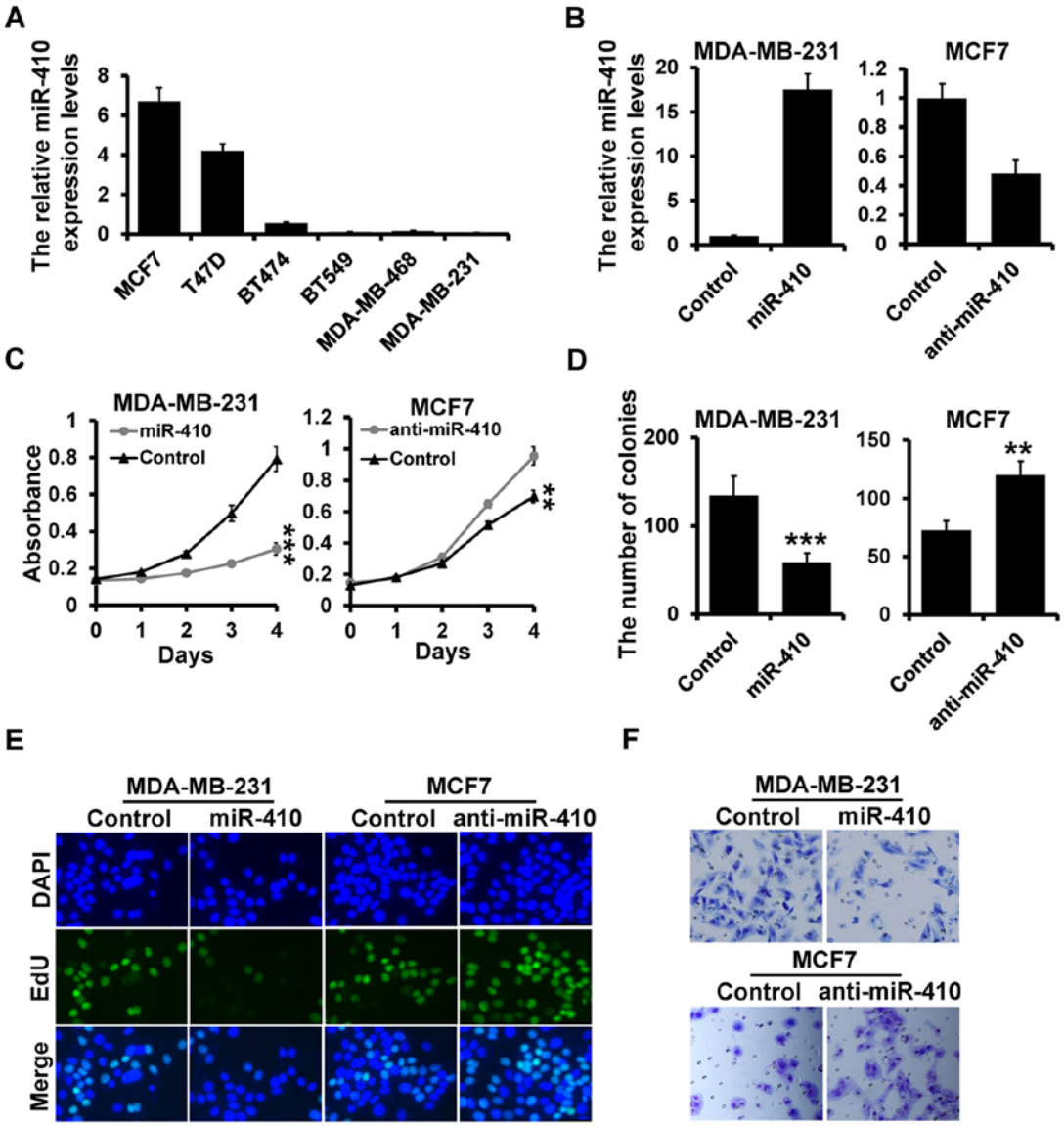

Figure 2. miR-410-3p suppresses breast cancer progression *in vitro*. (A) Expression of miR-410-3p in breast cancer cell lines by RT-qPCR. (B) Expression of miR-410-3p in MDA-MB-231 (left) and MCF7 (right) cells transfected with the miR-410-3p mimic, miR-410-3p inhibitor or appropriate controls. (C) MTT assay of cell growth in the MDA-MB-231 and MCF7 cells treated as in B. (D) Colony formation of the MDA-MB-231 and MCF7 cells treated as in B. (E) EdU analysis of the MDA-MB-231 and MCF7 cells treated as in B. (F) Transwell analysis of cell invasion in the MDA-MB-231 and MCF7 cells treated as in B. $^{***}P<0.001$, $^{**}P<0.01$.

indicated that miR-410-3p inhibits breast cancer cell proliferation and invasion, suggesting that miR-410-3p is a tumor suppressor in breast cancer.

Snail is a target of miR-410-3p. To elucidate the biological mechanisms underlying the role of miR-410-3p in the inhibition of breast cancer progression, we investigated the potential targets of miR-410-3p. Target prediction programs, miRanda and TargetScan, were applied to identify Snail as a putative miR-410-3p target (Fig. 3A). To further confirm this regulation, Snail 3'-UTR and its mutant containing the putative miR-410-3p binding sites were cloned into the downstream of the luciferase ORF (Fig. 3A). These reporter constructs were co-transfected into MDA-MB-231 cells with the miR-410-3p mimic. Overexpression of miR-410-3p significantly suppressed luciferase activity with inhibition rates of 40% compared to that of the control MDA-MB-231 cells (Fig. 3B; left panel). These effects were abolished when mutated Snail 3'-UTR, in which the binding sites for miR-410-3p were inactivated by site-directed mutagenesis (Fig. 3B; right panel). Functional regulation of Snail expression by miR-410-3p was analyzed by modulating miR-410-3p levels via overexpression in MDA-MB-231 and depletion in MCF7 cells. The Snail mRNA level was decreased in the miR-410-3p-overexpressing MDA-MB-231 cells compared with that in the control cells (Fig. 3C; left panel). Meanwhile, the protein level of Snail was also reduced in the miR-410-3p-overexpressing MDA-MB-231 cells (Fig. 3D; left panel). On the other hand, depletion of miR-410-3p in MCF7 cells resulted in elevated mRNA and protein levels of Snail (Fig. 3C and D; right panels). Collectively, these data support the bioinformatic prediction of Snail as a direct target of miR-410-3p.

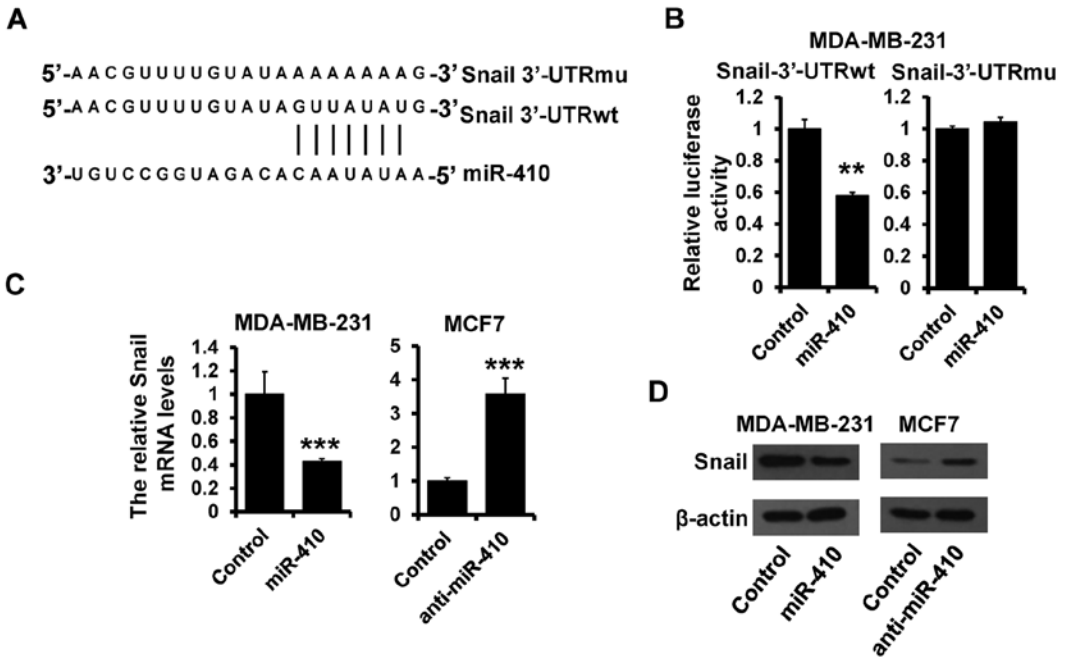

Figure 3. Snail is a target of miR-410-3p. (A) The predicted binding of miR-410-3p with Snail 3'-UTR. (B) Dual-luciferase reporter analysis was performed to validate whether miR-410-3p targets Snail. A 3'-UTR fragment containing the predicted miR-410-3p targeting sites of Snail was fused downstream of the Luc gene (Snail-3'-UTRwt). A miR-410-3p mutated binding site was also constructed (Snail-3'-UTRmu). (C and D) Expression of Snail mRNA and protein was detected in miR-410-3p-overexpressing MDA-MB-231 or miR-410-3p-depleted MCF7 cells, as well as the control cells by RT-qPCR (C) and western blot analysis (D), respectively. $^{***}P<0.001$.

miR-410-3p regulates EMT by targeting Snail in breast cancer cells. To ascertain whether miR-410-3p regulates breast cancer progression through its interaction with Snail, we performed a rescue experiment. We overexpressed Snail in the miR-410-3p-overexpressing MDA-MB-231 cells (Fig. 4A and B) and observed that Snail overexpression greatly impaired the anti-proliferative properties of miR-410-3p, as documented by MTT, colony formation and EdU assays (Fig. 4C-E). Similarly, the ability of invasion was rescued by Snail overexpression (Fig. 4F). To address whether the expression of miR-410-3p is associated with its target, Snail mRNA expression was examined in 30 cases of primary breast cancer tissues by RT-qPCR. There was a significant inverse correlation between miR-410-3p and Snail expression in the breast cancer tissues (Fig. 4G). miR-410-3p-overexpressing MDA-MB-231 cells exhibited a significant upregulation of β-catenin and E-cadherin, while mesenchymal marker vimentin was dramatically downregulated as determined by RT-PCR (Fig. 4H) and western blot analysis (Fig. 4I). Furthermore, overexpression of Snail rescued the effects of miR-410-3p overxpression on the expresssion of β-catenin and vimentin (Fig. 4H and I). Together, these data indicate that miR-410-3p regulates the EMT phenotype by targeting Snail in breast cancer cells.

Discussion

Aberrantly expressed miRNAs play a crucial role in tumor development and progression (21-25). In recent years, increasing studies have indicated that the deregulation of miRNAs is involved in many processes of carcinogenesis, functioning as either an oncogene or tumor suppressor (26,27). In the present study, we found that the expression of miR-410-3p was lower in breast cancer tissues compared with that noted in the paired normal breast tissues. Moreover, overexpression of miR-410-3p suppressed cell proliferation and invasion in breast cancer cells. Furthermore, we identified Snail as a direct target of miR-410-3p. In addition, re-expression of Snail reversed the miR-410-3p-induced inhibition of the EMT phenotype and breast cancer progression. Clinically, the expression of miR-410-3p was downregulated and was inversely correlated with expression of Snail in breast cancer tissues. These findings suggest that miR-410-3p may be involved in breast tumorigenesis and progression.

Aberrant expression of miR-410-3p is common in a variety of cancers, suggesting that miR-410-3p may play an important role in cancer development and progression (12-19). Previous research showed that high expression of miR-410-3p is associated with favorable disease-free survival in patients with non-MYCN-amplified localized neuroblastoma (28). Furthermore, miR-410-3p was reported to suppress the migration and invasion of gastric cancer and glioma cells (14,17). Other studies showed that miR-410-3p functions as an oncogene in non-small cell lung cancer, liver cancer and colorectal cancer (13,15). These data indicate that dyregulation of miR-410-3p may occur in a tissue-specific manner in different types of cancer. However, the roles of miR-410-3p in breast cancer are still unknown. In the present study, the expression of miR-410-3p was upregulated in breast cancer tissues compared with that in paired adjacent normal breast. Furthermore, miR-410-3p suppressed breast cancer cell proliferation and invasion. These results suggest that miR-410-3p may act as a tumor suppressor in breast cancer progression.

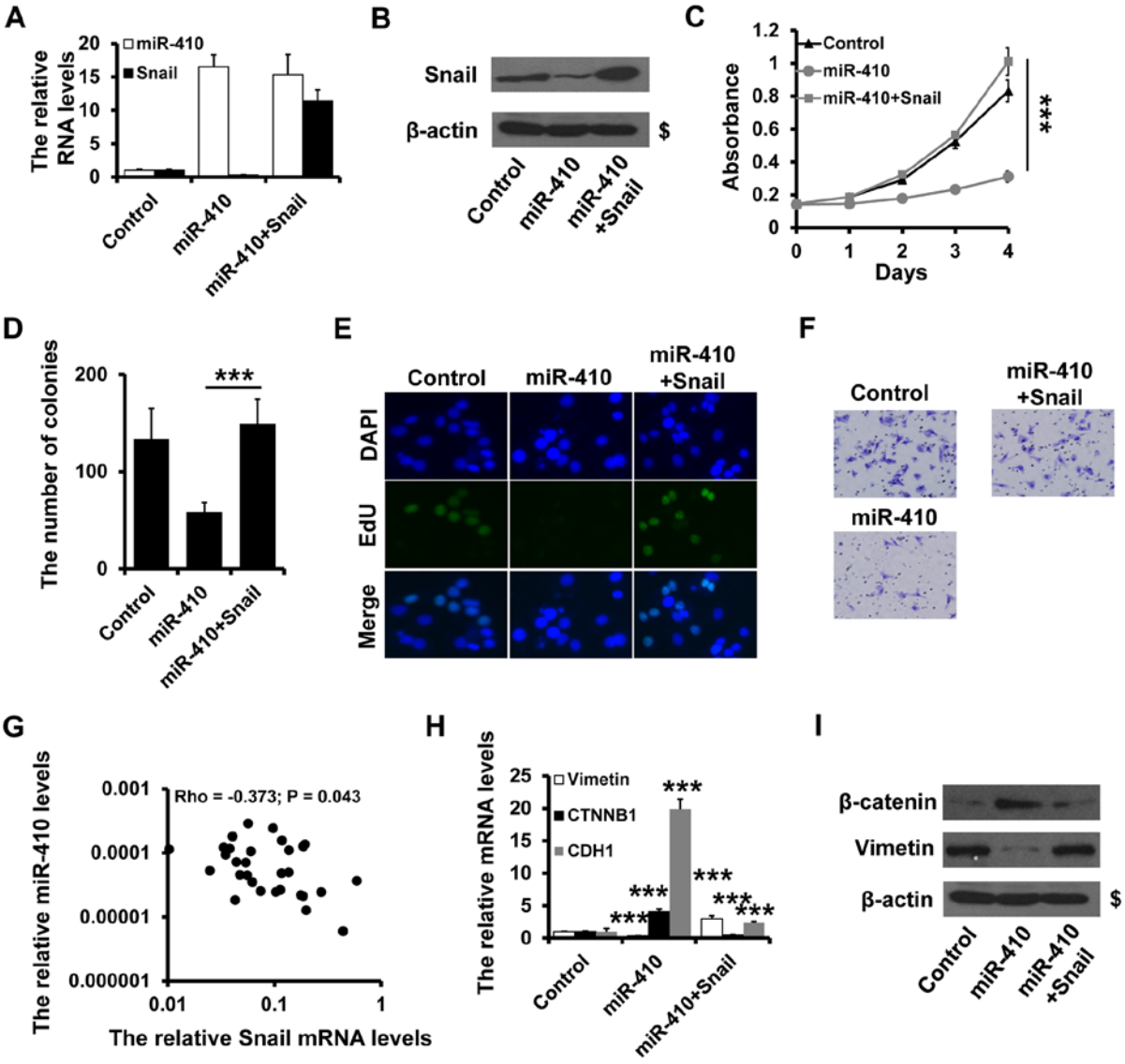

Figure 4. miR-410-3p inhibits the EMT phenotype through downregulation of Snail expression. (A) Expression of miR-410-3p and Snail in the MDA-MB-231 cells transfected with miR-410-3p mimic or/and Snail by RT-qPCR. (B) Expression of Snail in the MDA-MB-231 cells treated as in A by western blot analysis. (C) MTT assay of cell growth in the MDA-MB-231 and MCF7 cells treated as in A. (D) Colony formation analysis of the MDA-MB-231 cells treated as in A. (E) EdU analysis of the MDA-MB-231 cells treated as in A. (F) Transwell analysis of cell invasion in the MDA-MB-231 cells treated as in A. (G) The relationship between miR-410-3p and Snail 3RNA expression in 30 cases of breast cancer samples by RT-qPCR. (H and I) Expression of CTNNB1, vimetin and E-cadherin mRNA and protein levels was detected in MDA-MB-231 and MCF7 cells treated as in A by RT-qPCR (H) and western blot analysis (I), respectively. ***P<0.001.

We also demonstrated that miR-410-3p bound to the 3'-UTR of Snail. miR-410-3p downregulated Snail mRNA and protein expression. We also observed that Snail could mediate the function of miR-410-3p in breast cancer progression. EMT is a crucial process in cancer progression that causes epithelial cells to acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (29,30). Snail is overexpressed in various malignancies and is one of the master regulators that promotes EMT and mediates invasiveness as well as metastasis in many different types of malignant tumors (31-34). Our data demonstrated that miR-410-3p inhibits the EMT phenotype in breast cancer cells and the effect of miR-410-3p on EMT was rescued by over-expression of Snail. Furthermore, the expression of Snail was inversely correlated with expression of miR-410-3p in breast cancer tissues. We first demonstrated that miR-410-3p is a novel regulator of Snail in breast cancer cells, which provided one possible mechanism for the role of miR-410-3p in breast cancer progression.

In conclusion, we demonstrated for the first time that miR-410-3p acts as a tumor suppressor in breast cancer through inhibition of the expression of Snail. These data suggest a potential therapeutic application of miR-410-3p in breast cancer.

Acknowledgements

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