

Overexpression of microRNA-365 inhibits breast cancer cell growth and chemo-resistance through GALNT4

J. ZHANG, Z. ZHANG, Q. WANG, X.-J. XING, Y. ZHAO

Department of General Surgery, Shidong Hospital, Yangpu District, Shanghai, China

Jun Zhang and Zhou Zhang contribute equally to this work

Abstract. – OBJECTIVE: A role of microRNA-365 (miR-365) in the carcinogenesis of breast cancer remains undetermined. In the current study, we addressed this question.

PATIENTS AND METHODS: We examined the levels of miR-365 in breast cancer tissue, compared to the paired adjacent non-tumor breast tissue from the patients. We also examined the effects of miR-365 modification on the breast cancer growth and sensitivity to Fluorouracil, as well as the underlying mechanisms.

RESULTS: The miR-365 levels in breast cancer tissue were significantly lower than those in control normal breast tissues. Transfection with the miR-365 mimic decreased the breast cancer cell growth and increased their sensitivity to Fluorouracil, while transfection with the anti-sense of miR-365 (as-miR-365) increased breast cancer cell growth and decreased their sensitivity to Fluorouracil. Bioinformatics analyses showed that GALNT4 was a potential target gene of miR-365. The luciferase activities assay and Western blot verified that miR-365 targeted GALNT4 mRNA to modulate its protein levels.

CONCLUSIONS: Our study suggests that downregulation of miR-365 may facilitate carcinogenesis of breast cancer cells via GALNT4, and thus miR-365 appears to be a promising target for breast cancer therapy.

Key Words:

Breast cancer, miR-365, GALNT4, Chemo-sensitivity.

Introduction

Breast cancer is a very commonly occurred cancer with high malignancy in women¹. The molecular regulation of the carcinogenesis of breast cancer has been extensively studied, showing the involvement of epidermal growth factor receptor signaling pathway², Wnt signaling pathway³, insulin-like growth factor receptor signaling pathway⁴, Notch signaling pathway⁵ and transforming growth factor β superfamily receptor signaling pathway⁶⁻⁹. However, more and

more molecules have been found to play roles in the tumorigenesis of breast cancer, and comprehension of this complex regulation system appears to be important to fully understand the tumor biology of breast cancer.

GALNT4, a member of the family of N-acetyl galactosaminyl transferases, catalyzes the transfer of GalNAc to serine or threonine residues in the initial step of mucin-type O-linked protein glycosylation¹⁰⁻¹², as a post-translational modification of proteins that play critical roles in cellular proliferation, differentiation and other pathological disorders. Of note, GALNTs has been recently shown to be targeted by a miRNA cluster for the control of glycosylation, resulting in increased tumor invasion and immunosuppression^{13,14}.

MicroRNAs (miRNAs) are some small endogenous RNAs, which are noncoding¹⁵⁻²¹. MiRNAs display abnormal expression and functions in various kinds of malignancies, which act as different kinds of tumor related genes²²⁻²⁹. Among all miRNAs, miR-365 has been recently shown as a potential inhibitor for some cancers. For example, miR-365 targets NRP1 to inhibit tumor growth, proliferation and metastasis in malignant myeloma^{30,31}, and miR-365-targeted nuclear factor I/B transcriptionally represses cyclin-dependent kinase 6 and 4 to inhibit the progression of cutaneous squamous cell carcinoma³². Most interestingly, miR-365 has been shown to regulate Cyclin D1 to inhibit proliferation of smooth muscle cells^{33,34}. However, a role of miR-365 in the tumorigenesis of breast cancer, unlikely some other miRNAs³⁵⁻³⁹, has not been defined.

In this paper, we studied the role of miR-365 in the regulation of breast cancer cell growth and chemo-sensitivity. We found that the levels of miR-365 significantly down-regulated in breast cancer tissue, compared to the paired adjacent non-tumor breast tissue. The miR-365 levels in breast cancer cell lines were significantly lower

than those in control normal breast tissues. Transfection with the miR-365 mimic decreased the breast cancer cell growth and increased their sensitivity to Fluorouracil, while transfection with the antisense of miR-365 (as-miR-365) increased breast cancer cell growth and decreased their sensitivity to Fluorouracil. Bioinformatics analyses showed that GALNT4 was a potential target gene of miR-365. The luciferase activities assay and Western blot verified that miR-365 targeted GALNT4 mRNA to modulate its protein levels. Together, our study suggests that down-regulation of miR-365 may facilitate carcinogenesis of breast cancer cells via GALNT4, and thus miR-365 appears to be a promising target for breast cancer therapy.

Patients and Methods

Clinical Specimens

Total 40 breast cancer (BC) and adjacent normal breast tissues (NT) were from Shidong Hospital from 2012 to 2015. BC vs NT was determined based on pathological and cytological evidence. The Ethics Committees of Shidong Hospital approved this study, and the patient's permission for this study was obtained. After surgery, the tissue samples were stored at -80°C, after which the pathological information was obtained.

Cell Lines and Reagents

The human breast cancer cell lines MCF7⁴⁰, BT474⁴¹, HCC38⁴², HTB-121⁴¹ and normal human breast tissue cells, Hs 861.T, were all purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and was cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) in a humidified chamber with 5% CO₂ at 37°C. Fluorouracil (5-FU; Sigma-Aldrich) was prepared in a stock of 1 mmol/l and applied to the cultured cells at 10 µmol/l.

Quantitative Real Time -PCR (RT-qPCR)

Total RNA was extracted from resected specimens from the patients or from cultured cells with miRNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was randomly primed from 2 µg of total RNA using the Omniscript reverse transcription kit (Qiagen). Real-time quantitative PCR (RT-qPCR) was subsequently performed in triplicate with a 1:4 dilu-

tion of cDNA with QuantiTect SYBR Green PCR Kit (Qiagen) in a Quantitect SyBr green PCR system (Qiagen). All primers were purchased from Qiagen. A ^{2-ΔΔCt} method was used for quantification of the relative mRNA expression levels. Values of genes were first normalized against β-actin, and then compared to controls.

Transfection

Cells at 80% concentration were seeded into 6-well plates before transfection. The specific miRNA mimic or antisense and a control null plasmid were all synthesized by Life Technologies (Carlsbad, CA, USA) and were used for transfection of breast cells or cancer cells with LipofectamineTM 3000 Reagent (Invitrogen, Carlsbad, CA, USA). The final concentration of miRNAs was 50 nmol/l. After 4 hours, cells were cultured with normal media, and harvested 48 hours later to evaluate transfection efficiency.

Cell Viability Assay

The CCK-8 detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate at a density of 5X10⁴/ml. After 24 h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml/well) was added and the plate was incubated at 37°C for 2 h. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

Cell Growth Assay

A diphenyltetrazolium bromide (MTT) assay was performed to determine cell growth. Five thousand cells per well were seeded in a 96-well plate to allow the cells to grow. Then the media were removed and washed with PBS, after which 5 g/l of thiazolyl tetrazolium (Amersco, Indianapolis, IN, USA) was added to each well. Four hours later, MTT was removed and 150 µl of dimethyl sulfoxide (Sigma-Aldrich) was added. The viability of the cells was calculated from the absorption at 570/630 nm with an enzyme-linked immunosorbent assay reader.

MicroRNA Targets Prediction and Dual-Luciferase Reporter Assay

The target gene of miR-365 was predicted by TargetScan (<http://www.targetscan.org>). The

dual-luciferase reporter plasmids, p3'-UTR-GALNT4 (containing the wild-type GALNT4 3'-UTR binding site in luciferase reporter plasmid (RiboBio Co. Ltd., Guangzhou, China) and p3'-UTR-GALNT4-mut (containing the mutant GALNT4 3'-UTR; mut) were constructed. For the luciferase assay, the constructed plasmid (500 ng) and miR-365 (mir-365) (100 nmol/l) were co-transfected into breast cancer cells using Lipofectamine™ 3000 Reagent (Invitrogen, Carlsbad, CA, USA). Then the luciferase activity was detected with the dual-luciferase reporter assay system (Promega Madison, WI, USA) after co-transfection cells for 48 hours, following the manufacturer's protocol.

Western Blot Analysis

A total of 20 mg of protein was used for western blotting. After SDS-PAGE gels electrophoresis, samples were transferred to PVDF membranes. After blocked, membranes were incubated with primary polyclonal against GALNT4 or β -actin (Cell Signaling, San Jose, CA, USA) and then secondary anti-rabbit antibody (Cell signaling). Protein signals were enhanced by chemiluminescence detection kit. The protein quantification was performed using Image J software (NIH, Bethesda, MA, USA).

Statistical Analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). All values are depicted as mean $\pm$ standard deviation. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test to compare two groups. $p < 0.05$ was considered to be statistically significant and indicated by (*). Bivariate correlations were calculated by Spearman's rank correlation coefficients.

Results

MiR-365 is Down-Regulated in Breast Cancer Tissues and Cell Lines

To research potential effects about miR-365 in breast cancer, the miR-365 levels were estimated in breast cancer tissues and cells specimens. Lower mRNA levels of miR-365 were observed in breast cancer tissues compared with the paired non-tumor breast tissue (CTL) (Figure 1A).

Moreover, MiR-365 levels in breast cancer cell lines HTB-121, BT474, MCF7, HCC38 were all much lower than that a normal human breast tissue cells, Hs861.T (Figure 1B). We then aimed to investigate whether miR-365 influences the breast cancer cell growth and chemo-sensitivity. We chose to use the breast cancer cell line, MCF7, since it expressed medium levels of miR-365 among all examined cell lines (Figure 1B). As-miR-365 or miR-365 mimic or a control null plasmids was transfected into MCF7 cells to inhibit or increase the miR-365 expression, respectively (Figure 1C).

MiR-365 Suppresses Breast Cancer Cell Growth And Decreases Survival Against 5-FU

Then, we examined cell growth in an MTT assay. We found that overexpression of miR-365 in MCF7 cells significantly decreased cell growth, while miR-365 depletion significantly increased cell growth (Figure 2A). Next, we examined cell survival in a CCK-8 assay at the presence of 5-FU. We found that overexpression of miR-365 in MCF7 cells significantly decreased cell survival, while miR-365 depletion significantly increased MCF7 cell survival (Figure 2B). These data demonstrate that miR-365 may suppress NSCLC cell growth and decrease survival against 5-FU treatment.

MiR-365 Functionally Targets GALNT4 in Breast Cancer Cells

Due to the important function of miR-365 gene in breast cancer, we studied the underlying mechanisms. Using the publicly available databases TargetScan, we found a conserved miR-365 binding site in the 3'-UTR of GALNT4 (Figure 3A). The GALNT4 levels were up-regulated significantly in breast cancer tissues, compared to CTL (Figure 3B). Furthermore, the levels of miR-365 and GALNT4 inversely correlated in breast cancer tissue (Figure 3C).

To further investigate whether GALNT4 was a target gene of miR-365, the luciferase reporter plasmids containing either wild-type or mutant 3'-UTRs of GALNT4 were constructed. The luciferase reporter assay was set up to identify the direct miR-365-GALNT4 interaction. The relative luciferase activity was significantly lower in cells after 48 hours co-transfection with miR-365-modified plasmids and p3'-UTR-GALNT4 or miR-365 (Figure 4). There was a statistically difference between cells co-transfected with p3'-

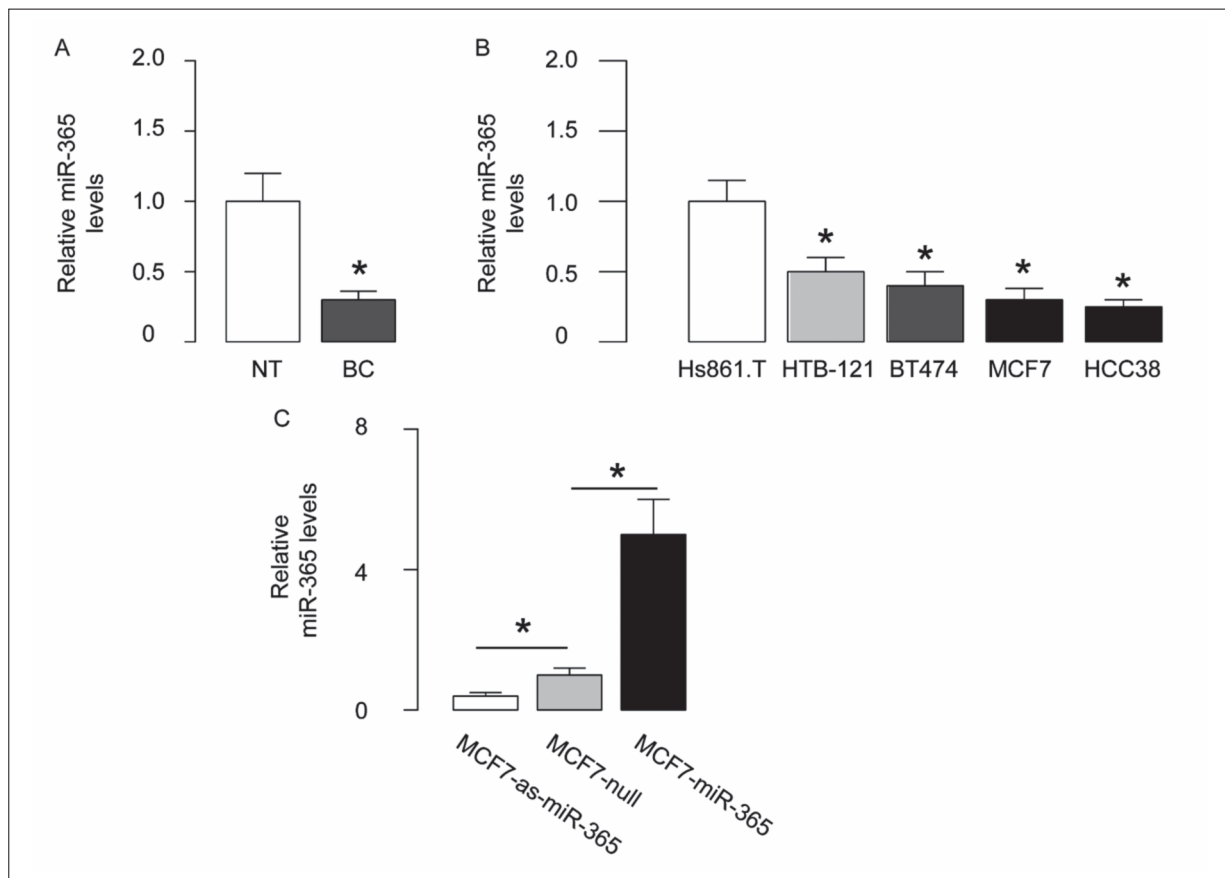


Figure 1. MiR-365 down-regulates in breast cancer tissues and cell lines. **A**, The miR-365 levels were estimated in breast cancer tissues and cells specimens. Lower mRNA levels of miR-365 were observed in breast cancer tissues compared with the paired normal breast tissue (CTL). N = 40. **B**, MiR-365 levels in breast cancer cell lines were all much lower than that in normal human breast tissue cells. N = 5. **C**, As-miR-365 or miR-365 mimic or null control plasmid was transfected into MCF7 cells to modulate the miR-365 expression. RT-qPCR for miR-365 was performed. N = 5. * $p < 0.05$.

UTR-mut and cells co-transfected with the miR-365 (Figure 4). Those consequences indicated that miR-365 could specifically bind to seed zone of GALNT4 3'UTR to inhibit its expression, while mut vector could not combine with miR-365 to decrease the relative luciferase activity. GALNT4 is thus a specific and direct target gene of miR-365.

In order to confirm the regulative effects of miR-365 on endogenous GALNT4 expression, we examined GALNT4 levels in miR-365-modified MCF7 cells. The western result showed that compared with control, the GALNT4 protein level was significant suppressed with miR-365 enhancement and was significantly increased in the examined breast cancer cells with miR-365 silence (Figure 5A), whereas the RT-qPCR result showed that endogenous GALNT4 mRNA levels were not significantly altered in these cells (Fig-

ure 5B). Together, miR-365 negatively regulates endogenous GALNT4 expression at post-translational level.

MiR-365 Suppresses Growth of Breast Cancer Cells In Vivo

Finally, the same number of MCF7-null cells or MCF7-miR-365 or MCF7-as-miR-365 cells was implanted subcutaneously into nude mice and the tumor was dissected out after 8 weeks. The tumor mass by MCF7-miR-365 cells was significantly smaller than control, while the tumor mass by MCF7-as-miR-365 was significantly greater than control, shown by quantification (Figure 6A), and by representative images (Figure 6B). These data suggest that miR-365 suppresses the growth of breast cancer cells *in vivo*.

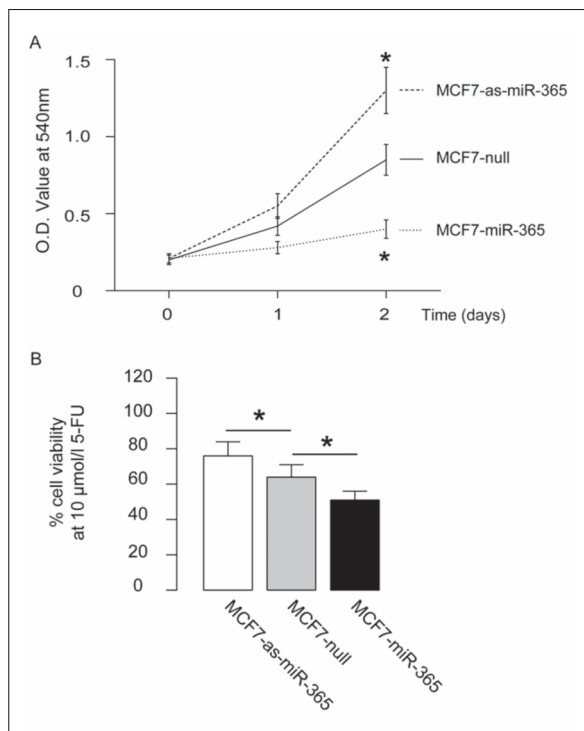


Figure 2. MiR-365 suppresses cell growth and decreases cell survival against 5-FU in breast cancer cells. **A**, An MTT assay in miR-365-modified MCF7 cells. **B**, An CCK-8 assay at presence of 5-FU in miR-365-modified MCF7 cells. N = 5. * $p < 0.05$.

Discussion

Some cases of breast cancer have the vigorous proliferation potential and are strongly resistant to the chemotherapy, resulting in malignant tumor growth and high recurrence after resection. Since aberrant miRNA expression has been shown to regulate critical biological behaviors, such as cell apoptosis and proliferation, we hypothesized that the regulation of breast cancer growth and chemo-sensitivity may involve the participation of miRNAs.

Recently, miR-365 has been shown to target NRP1 to inhibit tumor growth, proliferation and metastasis in malignant myeloma^{30,31}, and target nuclear factor I/B to inhibit the progression of cutaneous squamous cell carcinoma³². In addition, miR-365 has been shown to regulate Cyclin D1 to inhibit proliferation of smooth muscle cells^{33,34}. Specifically, miR-365 expression was found to be downregulated in human colon cancer tissues, and its downregulation was correlated with cancer progression and poor survival in colon cancer patients⁴³. In this study, restoration of miR-365 expression was found to inhibit cell cycle progression, promoted 5-FU-induced apoptosis and repressed tumorigenicity in colon can-

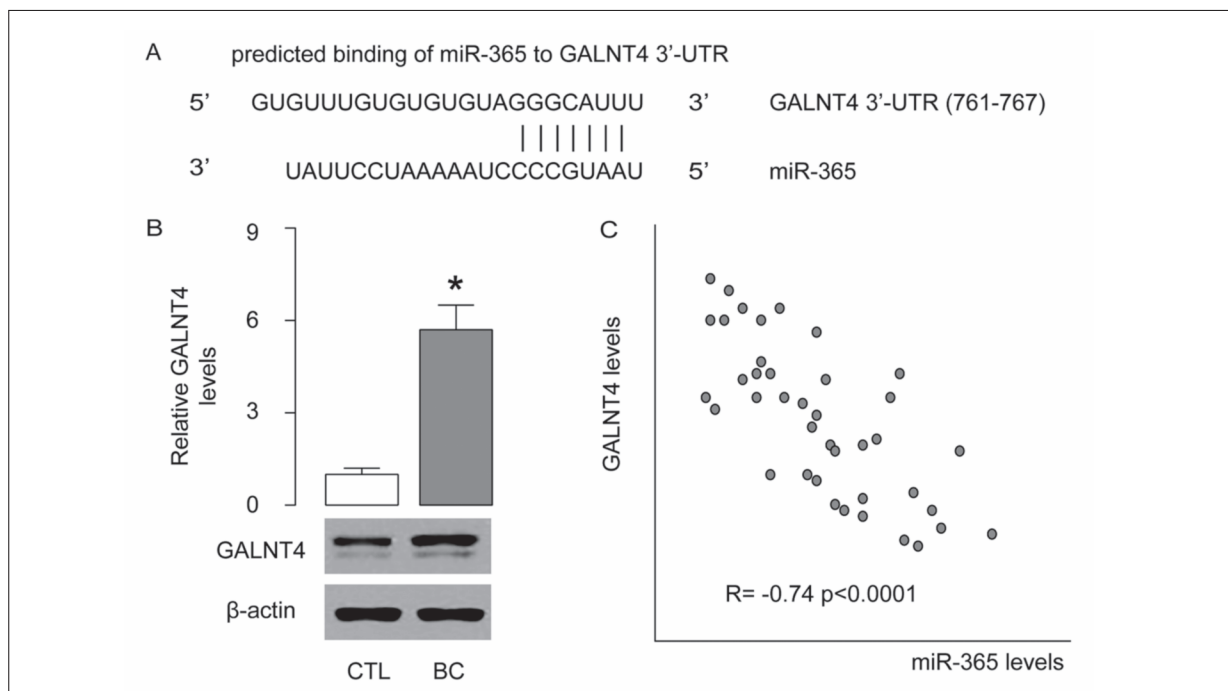


Figure 3. MiR-365 and GALNT4 inversely correlate in breast cancer. **A**, Using the publicly available databases TargetScan, we found a conserved miR-365 binding site in the 3'-UTR of GALNT4. **B**, The GALNT4 levels were up-regulated significantly in breast cancer tissues (BC), compared to CTL. **C**, The levels of miR-365 and GALNT4 inversely correlated. N = 40. * $p < 0.05$.

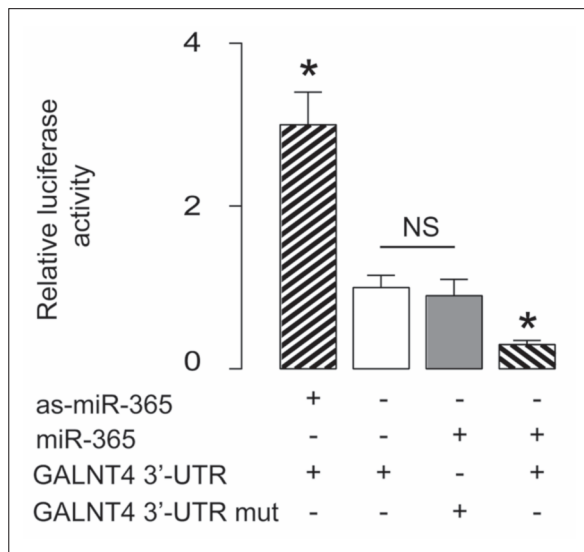


Figure 4. MiR-365 functionally targets GALNT4 in breast cancer cells. To further investigate whether GALNT4 may be a target gene of miR-365, the luciferase reporter plasmids containing either wild-type or mutant 3'-UTRs of GALNT4 were constructed. The luciferase reporter assay was set up to identify the direct miR-365-GALNT4 interaction. The relative luciferase activity was significantly lower in cells after 48 hours co-transfection with miR-365-modified plasmids and p3'-UTR-GALNT4 or miR-365. There was a statistically difference between cells co-transfected with p3'-UTR-GALNT4 -mut and cells co-transfected with the pre-miR-365. Those consequences indicated that miR-365 could specifically bind to seed zone of GALNT4 3'-UTR to inhibit its expression, while mut vector could not combined with miR-365 to decrease the relative luciferase activity. N = 5. * $p < 0.05$. NS: non-significant.

cer cell lines, through repression of Cyclin D1 and Bcl-2 expression⁴³. However, a role of miR-365 in the tumorigenesis of breast cancer has not been defined.

Conclusions

Our research showed that miR-365 was down-regulated in breast cancer. The silence of miR-365 increased the breast cancer growth and cell survival upon 5-FU treatment. Thus, we conclude that miR-365 plays an inhibitory role in the initiation, growth and development of breast cancer. For these evidences, we identified GALNT4 as a direct functional target of miR-365 using a prediction program. Computational analysis revealed that there were binding sites for miR-365 seed sequence at 3'-UTR of GALNT4. Furthermore, restoration of miR-365 expression led to decreased luciferase activity of wild-type GALNT4 3'UTR whereas the site-directed mutation abrogated miR-365 regulation. In addition, results from the RT-qPCR and protein expression analysis indicated that the ectopic expression of miR-365 suppressed protein levels of GALNT4. Taken together, these results suggest that miR-365 regulated the expression of GALNT4 by directly targeting 3'-UTR of GALNT4 in breast cancer.

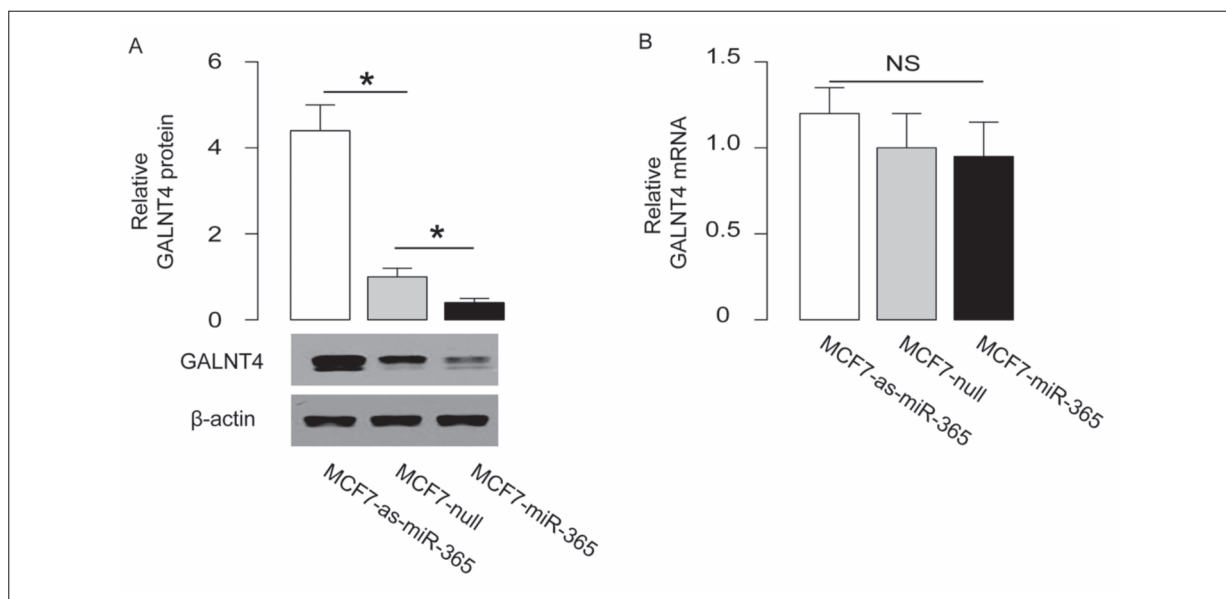


Figure 5. MiR-365 inhibits GALNT4 translation in breast cancer cells. **A**, The Western blots for GALNT4 protein in miR-365-modified MCF7 cells. **B**, The RT-qPCR for GALNT4 mRNA in miR-365-modified MCF7 cells. N = 5. * $p < 0.05$. NS: non-significant.

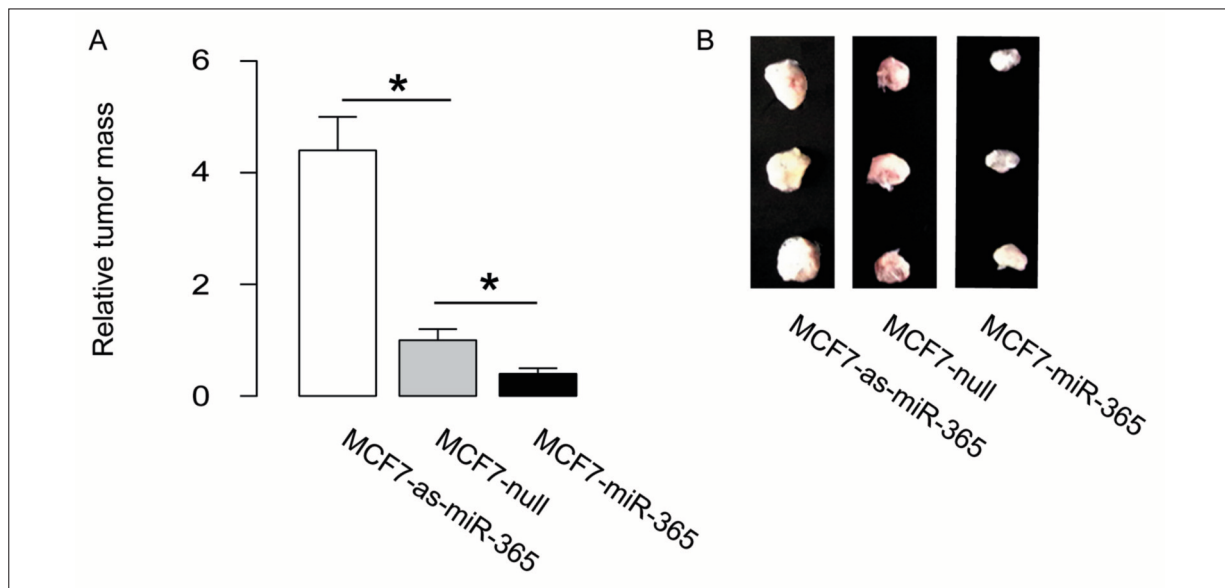


Figure 6. MiR-365 suppresses growth of breast cancer cells *in vivo*. **A-B**, Same number of MCF7-null or MCF7-miR-365 or MCF7-as-miR-365 cells were implanted subcutaneously into nude mice and the tumor was dissected out after 8 weeks for quantification of the tumor mass, shown by quantification (**A**), and by gross images. N = 3. * $p < 0.05$.

Our data from gain-of-function approaches confirmed that GALNT4 was a miR-365's direct target gene. Firstly, GALNT4 gene down-regulated in breast cancer tissues, and its expression showed a negative relationship with the expression of miR-365. Secondly, luciferase reporter assay verified the specific and direct combination between miR-365 and GALNT4 mRNA. Thirdly, over-expression of miR-365 inhibits the expression of GALNT4 protein at post-translational level, and knock-down of miR-365 could show contrary effects.

In summary, aberrantly expressed miR-365 regulates human breast cancer cell growth and chemo-sensitivity at least partially through directly down-regulating GALNT4 protein expression and this might offer a new potential therapeutic stratagem for breast cancer. Nevertheless, future research is essential to identify the detailed molecular mechanism about miR-365 in the genesis and development of breast cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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