miR-185 Suppresses Tumor Proliferation by Directly Targeting E2F6 and DNMT1 and Indirectly Upregulating BRCA1 in Triple-Negative Breast Cancer

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Abstract

Breast cancer is a major public health problem all over the world, and the current treatment strategies are not potent enough for some patients, especially those with triple-negative breast cancer (TNBC). Recent studies have demonstrated that microRNAs (miRNA) play vital roles in the development of TNBC. In this study, we found that miR-185 was strongly downregulated in TNBC tissues and cell lines and that its expression levels were associated with lymph node metastasis, clinical stage, overall survival, and relapse-free survival in TNBC. We also found that ectopic expression of miR-185 inhibited TNBC cell proliferation in vitro and in vivo. We further identified that miR-185 directly targeted DNMT1 and E2F6, which resulted in a marked increase in the expression of BRCA1 at the mRNA and protein levels in TNBC. Our data suggest that miR-185 functions as a tumor suppressor in TNBC development. It is a promising prognostic biomarker and potential therapeutic target for TNBC.

Introduction

Breast cancer is the most common malignancy in the female population throughout the world, and it is also the most common cause of cancer-related death among women. Breast cancer has five distinct subtypes, including luminal A [estrogen receptor (ER)+ and/or progesterone receptor (PR)+, human epidermal growth factor receptor-2 (HER2)+], luminal B (ER+ and/or PR+, HER2-), basal-like [ER-, PR-, HER2-], cytokeratin (CK)5/6+, and/or epidermal growth factor receptor (EGFR)+], HER2-over-expressing (ER-, PR-, and HER2+), and unclassified (negative for all five markers; ref. 1). Basal-like breast cancer is also known as triple-negative breast cancer (TNBC; ref. 2). TNBC accounts for approximately 15% of all breast cancers, with a higher morbidity being observed among younger women (3). TNBC is characterized by an aggressive behavior, poor prognosis, and a lack of targeted therapies (4). The BRCA1 gene is one of the best-characterized genes related to cancer susceptibility and is associated with hereditary breast and ovarian cancer (5, 6). In particular, downregulation of BRCA1 has frequently been observed in more aggressive breast cancers, such as the basal-like breast cancer and TNBC subtypes. These two groups of tumors show a high degree of overlap and frequently display a phenotype defined as “BRCAness” (7) that is characterized by traits similar to those of BRCA1-mutated breast tumors, including lack of ER, high-grade, aggressive, and frequent TP53 mutations (8). Despite this role in malignancies, the molecular basis of BRCAness remains largely unclear.

An increasing array of studies has demonstrated that microRNAs (miRNA) can function as oncogenes or tumor suppressors and are often dysregulated in tumors (9). Accumulating evidence suggests that correlations exist between miRNA expression and clinical recurrence, metastasis development, and/or survival (10). miR-185, located on chromosome 22q11.21, is a tumor-suppressor miRNA. miR-185 has been reported to be downregulated in a variety of cancers, including colorectal cancer (11, 12), hepatocellular carcinoma (13), epithelial ovarian cancer (14), and prostate cancer (15). We previously reported that miR-185 is strongly downregulated in glioma and that it suppresses tumor proliferation and invasion by directly targeting DNMT1, RhoA, and CDC42. Although miR-185 has been reported to be involved in breast cancer (16), its role in breast cancer, especially in TNBC, is not yet fully understood. Thus, we aimed to investigate the effect of miR-185 on the carcinogenesis and progression of breast cancer and to determine its prognostic significance.
In this study, we showed that miR-185 was downregulated in TNBC cell lines and specimens. Moreover, the miR-185 level was associated with clinical stage and lymph node metastasis. Decreased expression of miR-185 was significantly correlated with poor relapse-free survival (RFS) and overall survival (OS). We hypothesized that downregulation of the BRCA1 gene could be achieved through the action of miRNA downregulated in TNBC. Through further study, we identified miR-185, which positively regulates BRCA1 expression by targeting the DNMT1 and E2F6 genes in TNBC. These results support a previously unreported role of miRNAs in BRCA1 downregulation in TNBC and provide a pharmacologic rationale for the use of synthetic miR-185 for TNBC therapy.

Materials and Methods

Cell lines and culture

The following cell lines were obtained from the American Type Culture Collection and were passaged in our laboratory for less than 6 months after thawing frozen aliquots: human breast cancer cell lines (MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, MCF-7, T47D, BT-474, BT-20, and BT-483) and normal mammary epithelial cell lines (HBL-100, 184A1, and MCF-10A). All cells were maintained according to the supplier’s instructions. Before use, all cell lines were authenticated by short tandem repeat DNA profiling, and were found to be free of Mycoplasma infection.

Clinical samples

The tissue microarrays (TMA) consisted of 51 cases of TNBC and 51 cases of normal mammal tissues diagnosed by histopathology diagnosis from October 2001 to September 2006. Specimens stored in the Department of Specimens and Resources of Sun Yat-Sen University Cancer Center (Guangzhou, Guangdong, China) were obtained during surgery and were formalin-fixed and embedded in paraffin by standard methods. Immunohistochemistry (IHC) of P53, ER, PR, and HER2 status was performed in the Pathology Department of Sun Yat-Sen University Cancer Center. Positive staining for p53 was defined by IHC. Positive staining for ER and PR was defined as nuclear staining in more than 1% of tumor cells. HER2 was evaluated by an experienced pathologist and scored as 0, 1+, 2+, and 3+ according to the American Society of Clinical Oncology/College of American Pathologists guidelines. HER2 negativity was considered as HER2 0 or 1+, whereas cases with 2+ or 3+ would be tested using fluorescence in situ hybridization (FISH). An amplification ratio HER2/C17 of >2 on FISH test was considered as HER2 positivity. None of the patients included in this study received any chemotherapy or radiotherapy before the surgery, and their complete clinical data were available and reviewed, including age, histologic type, lymph node status, tumor size, stage, local relapse, distant metastatic relapse, ER status, PR status, and HER2 status. Histologic type was based on the tumor-node-metastasis (TNM) staging system, reclassified according to the World Health Organization (WHO) classification and tumor stage (American Joint Committee on Cancer classification). Follow-up included the review of records and telephone calls. The date of death and the date of relapse were used to calculate OS and DFS. The patients were grouped according to TN status, age, histologic type, lymph node status, tumor size, stage, local relapse, distant metastatic relapse, ER status, PR status, and HER2 status. This study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center Health Authority. The collection and use of tissues followed the procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration.

Tissue samples from 20 TNBC tissues and the corresponding paired normal adjacent tissues (Normal 1), 21 non-TNBC (NTNBC) tissues, and the corresponding paired normal adjacent tissues (Normal 2) were subjected to quantitative real-time PCR (qRT-PCR) analysis. Resected cancerous tissues and paired matched normal mammary tissues were immediately cut and stored in RNAlater (Ambion).

Stable cell lines expressing firefly luciferase

MDA-MB-231, MDA-MB-468, and MDA-MB-435 cells were transfected with pcDNA-Luc-Neo and selected with G418 (1 mg/mL; Invitrogen). Ten individual G418-resistant clones with a high-level of luciferase activity, designated as MDA-MB-231-Luc, MDA-MB-468-Luc, and MDA-MB-435-luc, were collected, identified, and maintained.

Quantitative RT-PCR analysis

Total RNA was extracted from cells with TRizol reagent (Invitrogen). Reverse transcription and qRT-PCR reactions were performed by means of a qSYBR-Green-containing PCR kit (Qiagen). Fold change was determined as 2^{-\Delta C_t}. C_t is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔC_t was calculated by subtracting the C_t of snRNA U6 from the C_t of the miRNA of interest. The ΔΔC_t was calculated by subtracting the ΔC_t of the reference sample (paired nontumorous tissue for surgical samples, and MCF-10A cells for nine breast cancer cell lines) from the ΔC_t of each sample. The primers for qRT-PCR detection of DNMT1, E2F6, and BRCA1 mRNA were synthesized by Invitrogen. All RT-PCR was performed with the Bio-Rad IQTM5 Multicolor Real-Time PCR Detection System.

RNA interference

The sense sequences of siRNA oligonucleotides targeting the BRCA1, DNMT1, and E2F6 transcripts, respectively, were as follows: si-BRCA1: 5’-GGGACCUCUGUCACAAAGdTdT-3’; si-DNMT1: 5’-UUGUAUAAUGGGAUUGCGGTT-3’; and si-E2F6: 5’-AGGAGACUGUGAUAAUCCTT-3’ (Invitrogen). Scrambled siRNA was used as a negative control. Cells were plated in culture dishes or in 96- or 6-well plates for 24 hours, and
transfected with siRNA using Lipofectamine 2000. After 48 hours, the cells were harvested for use in other assays or for RNA and protein extraction.

**In situ hybridization and immunohistochemical analysis**

*In situ* hybridization (ISH) procedures were carried out as previously described (17, 18). miR-185 miRCURYTM LNA custom detection probes (Exiqon) were used for ISH. The 5’-3’ sequence (enhanced with LNA) was TCAGGAACTGCCTTTCTCTCCA with digoxigenin at the 5’ and 3’ ends. Hybridization, washing, and scanning were carried out according to the manufacturer’s instructions. IHC studies were performed using the standard streptavidin/peroxidase staining method and intensities as described previously (19). The intensities of miR-185 staining was scored by 0–4, according to the standards of 0–1 (no staining), 1–2 (weak staining), 2–3 (medium staining), and 3–4 (strong staining). The percentages of miR-185 cells in three representative high-power fields of individual samples were analyzed. Those expression scores equaled to scores of the intensities × the percentages of positive cells, and the maximum was 4 and the minimum was 0. Individual samples were evaluated by at least two pathologists in a blinded manner, and those expression scores of greater or less than 2 were defined.

**Overall and disease-free survival**

DFS was defined as the time from diagnosis to disease progression or death, no matter which occurred first. Patients who were alive and disease free were censored at the date of last follow-up visit. OS was calculated from the date of diagnosis to the date of death for any cause, and patients who were alive were censored at date of last follow-up visit. In 51 patients, with a median follow-up of 74 months, recurrence or distant organs metastasis was observed in 13 patients, and 38 patients died.

**Cell proliferation assay**

Cell viability analysis was carried out according to the methods described previously using 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (20, 21). To assess the incorporation of BrdUrd, cells (treated and control) were incubated with culture media containing 10 nmol/L BrdUrd for 16 hours before fixation with cold methanol and acetone (1:1). Immunocytochemistry was subsequently carried out.

**MTT assay**

Cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells transfected with either scrambled, miR-185 mimics, control, or siRNA were seeded at a density of 5,000 cells per well in 96-well plates and incubated at 37°C for 24 hours. Cells were then incubated an additional 48 hours, and the MTT assay was performed according to the manufacturer’s instructions (Molecular Probes). Absorbance values were determined at 570 nm on a Spectra Max 250 spectrophotometer (Molecular Devices).

**Luciferase assays**

The full-length (2102bp) of 3’-untranslated regions (UTR) of the E2F6 genes was amplified by PCR from MDA-MB-231 genomic DNA and inserted into the pGL3 control vector (Promega) using the Xba1 site immediately downstream from the stop codon of luciferase. We also generated several inserts with deletions of 4 bp from the site of perfect complementarity of the E2F6 gene using the QIAGEN XL-Site-Directed Mutagenesis Kit (Qiagen). MDA-MB-231 cells were cotransfected using nucleoporation (Amaza Biosystems) according to the manufacturer’s protocol (solution V, program T-016) using 5 μg of the firefly luciferase report vector and 0.5 μg of the control vector containing Renilla luciferase, pRL-TK (Promega). Each nucleoporation used 50 nmol/L of the miR-185 or a scrambled oligonucleotide and LNA-modified anti-miR-185 oligonucleotide or a control oligonucleotide. Firefly and Renilla luciferase activities were measured consecutively using the Dual-Luciferase Assay (Promega) 48 hours after transfection.

**Immunofluorescence assay**

For immunofluorescence assay, cells were seeded on coverslips, fixed with 3% paraformaldehyde for 20 minutes, and then permeabilized with 0.1% Triton X-100. And incubated with antibodies against γH2AX (#05-636, 1:2,000; Millipore) for 2 hours at room temperature or overnight at 4°C. Then, cells were incubated with species-specific Alexa Fluor 488-conjugated secondary antibodies (1:2,000; Invitrogen) for 1 hour at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL).

**Western blot analysis**

Protein was extracted from TNBC cell lines using RIPA lysis buffer with a proteinase inhibitor. The protein concentration in the lysates was measured with the Protein BCA Assay Kit (BioRad), and 20 μg of protein mixed with 2× SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). To block nonspecific binding, membranes were incubated at room temperature for 1 hour with 5% skim milk powder. Next, the membranes were incubated for 12 hours at 4°C with an antisera containing antibodies against BRCA1, DNMT1, and E2F6 purchased from Santa Cruz Biotechnology. A peroxidase-conjugated secondary antibody (1:5,000 dilution) and ECL Western blot detection reagents were used to visualize the target proteins (ECL New England Biolabs), which were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BiolImage Ltd.). An anti–β-actin antibody (Boster) was used as a protein loading control.
Statistical analysis
Comparisons between groups were analyzed with the Student t test, one-way ANOVA, and \( \chi^2 \) tests. OS and DFS curves were plotted according to the Kaplan–Meier method, with the log-rank test used for comparison. Survival was counted from the day of the surgery. Variables with \( P < 0.05 \) by univariate analysis were used in a subsequent multivariate analysis based on the Cox proportional hazards model. Survival curves were plotted using SPSS 16.0 to see the power of miR-185 and other categorical factors on DFS and OS. The power analysis is for DFS and OS. All differences were statistically significant at the \( P < 0.05 \) level. Statistical analyses were performed using SPSS 16.0 software.

Results

miR-185 is downregulated in TNBC

miR-185 has been reported to be downregulated in a variety of cancers, including breast cancer (16). Using a qRT-PCR method, miR-185 was detected in different subtypes of mammary cell lines. We found that miR-185 is highly expressed in normal mammary cell lines, and expressed at low levels in some tumor cell lines, particularly those classified as basal-like (Fig. 1A; ref. 22).

Then, we detected miR-185 in 20 pairs of TNBC tissues and their matched adjacent tissues, as well as in 21 pairs of NTNBC tissues and their matched adjacent tissues. Among 20 patients with TNBC, approximately 85% (\( P = 0.006; 17 \) of 20 patients) of tumors revealed notable reductions in the miR-185 levels (Fig. 1B). However, the miR-185 level was only slightly reduced in approximately 43% (\( P = 0.090; 9 \) of 21 patients) of NTNBC tumors (Fig. 1B). Meanwhile, the miR-185 level was notably reduced in TNBC compared with NTNBC (\( P = 0.0006; \) Fig. 1B). To further verify the biologic role of miR-185 in TNBC, we performed ISH to evaluate miR-185 levels in 51 TNBC and 51 normal mammary tissues with TMA and found that miR-185 was strongly downregulated in TNBC compared with normal tissues (\( P < 0.001; \) Supplementary Fig. S1). These data indicate overt downregulation of miR-185 in TNBC.

Decreased miR-185 levels are correlated with advanced clinical stage, lymph node metastasis, and poor clinical outcomes

Next, we determined the potential clinicopathologic implications of altered miR-185 expression. Clinical samples were divided into low-expression and high-
expression groups based on miR-185 expression scores greater or less than 2. Among TNBC specimens, 73% (37 of 51) exhibited low to negative expression of miR-185 (Table 1). Thus, miR-185 is underexpressed in TNBC compared with normal mammary tissues. This is consistent with the data presented above. Among the 51 individuals with TNBC, the miR-185 level was inversely correlated with clinical stage, distant metastatic relapse, and lymph node metastasis (P = 0.025, 0.009, and 0.009, respectively; Table 1). However, the miR-185 levels in patients with TNBC were not correlated with age, histologic type, tumor size, or P53 status. Our results suggest that miR-185 could play critical roles in the carcinogenesis and progression of TNBC.

To further analyze the significance of miR-185 in terms of clinical prognosis, a Kaplan–Meier survival analysis was performed using patient OS and disease-free survival. The results demonstrated that patients with low miR-185 expression had shorter mean months of OS and DFS than patients with high miR-185 expression (P = 0.004 for OS; P = 0.005 for DFS; Fig. 1C and D). The value of power is 34% for DFS and OS. Our results indicated that the expression levels of miR-185 were significantly associated with patient OS and DFS.

We used a Cox proportional-hazards regression to further evaluate the association between miR-185 expression and prognosis. In the univariate analysis, the miR-185 level was significantly associated with prognosis (Supplementary Tables S1 and S2). The final multivariate model revealed that reduced miR-185 levels in tumors were independent predictors of shorter survival (Supplementary Tables S1 and S2). Lymph node metastasis (P < 0.05) was also an independent significant prognostic factor (Supplementary Tables S1 and S2). A similar trend was observed for clinical stage (P < 0.05; Supplementary Tables S1 and S2).

### E2F6 is a target gene of miR-185

We used two algorithms (TargetScan and Miranda) to help identify miR-185 targets in human TNBC. Among these candidate target genes, both algorithms predicted E2F6 (Fig. 2A). We confirmed this finding in breast cancer basal-like cell lines by performing luciferase reporter assays. The full-length E2F6 3’-UTRs were cloned.

### Table 1. Analysis of the correlation between miR-185 expression and clinicopathologic parameters in TNBC

<table>
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<th>Viable</th>
<th>Cases</th>
<th>miR-185</th>
<th>P</th>
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<td>DFS</td>
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miR-185 directly targets E2F6 and DNMT1 and upregulates the expression of BRCA1. A, predicted binding of miR-185 to the E2F6 3'-UTR by TargetScan. The mutant UTR contains five bases in the complementary seed sequences, as indicated by the arrows (top). Luciferase assay of MDA-MB-231 cells cotransfected with miR-185 mimics, a scrambled control, miR-185 inhibitors, control, and a luciferase reporter containing E2F6 3'-UTR (E2F6-wt) or mutant constructs in which the five nucleotides of the miR-185 binding site were mutated (E2F6-mut). An empty luciferase reporter construct was used as a negative control. **P < 0.01 vs. scramble (bottom). (Continued on the following page.)
downstream of the firefly luciferase gene and cotransfected with miR-185 mimics or scrambled oligonucleotides and LNA-modified anti–miR-185 oligonucleotide or a control oligonucleotide. Luciferase activity was measured after 48 hours of transfection. MDA-MB-231 cells cotransfected with E2F6 reporter constructs and miR-185 exhibited an approximately 40% reduction in the luciferase activity with respect to those cotransfected with the scrambled oligonucleotide, and the relative luciferase activity of the wild-type construct of the 3′-UTR of E2F6 in MDA-MB-231 cells was significantly increased in the presence of anti–miR-185 (Fig. 2A). In addition, mutation of the putative miR-185 sites in the 3′-UTR of E2F6 abrogated luciferase responsiveness to miR-185 (Fig. 2A).

miR-185 regulates BRCA1 by targeting DNMT1 and E2F6

Oberley and colleagues (23) and Yang and colleagues (24) reported that E2F6 negatively regulated BRCA1. The expression of miR-185 led to a drastic reduction in the amount of DNMT1, a known target of miR-185. Furthermore, methylation of the BRCA1 promoter has been reported to occur sporadically in breast cancer with proportions ranging from 11% to 31% (25, 26). In addition, BRCA1 promoter methylation has been linked to reduced mRNA and protein expression in primary breast tumors and cancer cell lines (26–29). We then analyzed the expression level of the BRCA1 protein in nontumorigenic and basal-like mammary cell lines by Western blot analysis. We found much lower levels of BRCA1 in most basal-like cell lines than in the nontumorigenic MCF-10A cell line (Fig. 2B). The three basal-like cell lines with lower miR-185 expression levels had particularly low amounts of BRCA1.

To determine whether miR-185 affected endogenous BRCA1 expression, we compared the level of the BRCA1 protein in three basal-like cell lines after transfection with miR-185 mimics or a scrambled oligonucleotide, finding a marked reduction in the protein and mRNA levels of DNMT1 and E2F6 and a marked increase in the protein and mRNA levels of BRCA1 (Fig. 2C and D). On the contrary, knockdown of miR-185 expression significantly increased the protein and mRNA levels of DNMT1 and E2F6, and reduced the protein and mRNA levels of BRCA1. Furthermore, DNMT1 and/or E2F6 RNA interference synergistically increased the protein levels of BRCA1 in three basal-like cell lines (Fig. 2E). Those results demonstrated that miR-185 affects the endogenous BRCA1 gene. Taken together, these results show that miR-185 upregulates BRCA1 expression by targeting DNMT1 and E2F6 in basal-like cell lines.

Phosphorylated histone H2AX (γH2AX) forms nuclear foci at sites of DNA damage and facilitates DNA-damage response and repair. To investigate the extent to which miR-185 may impact on DNA-damage response and repair, we first analyzed its effect on histone H2AX phosphorylation. Overexpression of miR-185 in MDA-MB-231 cells caused a strong increase in the number of γH2AX foci and knockdown of miR-185 expression significantly decreased the number of γH2AX foci (Fig. 2F) and in the extent of H2AX phosphorylation upon DNA damage (Fig. 2G). Importantly, this effect was comparable with that observed upon DNMT1 or E2F6 RNA interference (Fig. 2G).

miR-185 is positively correlated with BRCA1 expression in TNBC

To evaluate the relevance of the endogenous expression of miR-185 and BRCA1, we assessed their expression in human TNBC tissues.ISH analysis of miR-185 and IHC analysis of BRCA1 showed that all 51 TNBC samples exhibited decreased levels of miR-185 (except for four cases) and decreased levels of BRCA1 (except for six cases), compared with levels detected in the 51 corresponding paired normal adjacent tissues (Fig. 3A). We found that the expression of miR-185 was positively correlated with the expression of BRCA1 proteins in TNBC tissues (Fig. 3B).

miR-185 inhibits BRCA1-mediated proliferation in vivo and in vitro

To assess the biologic effects of miR-185 overexpression in cells, ectopic miR-185 mimics were transfected into four breast cancer basal-like cell lines (BT-20, MDA-MB-231, MDA-MB-435, and MDA-MB-468). qRT-PCR analysis showed that the transfection and knockdown were successful. We determined that the overexpression of miR-185 in four breast cancer basal-like cell lines markedly attenuated cell proliferation compared with the scrambled control (Fig. 4A). BRCA1 has been repeatedly shown to inhibit cellular proliferation when overexpressed in different cell types (30, 31). Conversely, BRCA1 depletion through RNA interference has been shown to stimulate proliferation. Therefore, we studied the effect of the
knockdown of miR-185 expression on the proliferation of four breast cancer basal-like cell lines. As expected, knockdown of miR-185 expression significantly promoted cell proliferation compared with the control (Fig. 4A), as did BRCA1 siRNA (Fig. 4A). Moreover, BRCA1 siRNA antagonized the role of miR-185 mimics (Fig. 4A). Similarly, BRCA1 siRNA antagonized the role of miR-185 mimics as demonstrated by luciferase imaging and BrdUrd incorporation tests (Fig. 4B and C).

Next, we investigated whether miR-185 could induce apoptosis in three breast cancer basal-like cell lines by upregulating BRCA1. Three breast cancer basal-like cell lines were transfected with miR-185 mimics and/or BRCA1 siRNA for 48 hours. The apoptosis rate following transfection with miR-185 mimics was higher than that of the scrambled control, and BRCA1 siRNA acted synergistically with the role of miR-185 mimics (Fig. 4D).

To directly evaluate the role of miR-185 in tumor formation and growth in vivo, MDA-MB-231 cells were subcutaneously injected into nude mice. Intratumoral injection of 40 μL of scrambled, miR-185 mimics, or miR-185 inhibitors and control in PBS was performed at 2-day intervals beginning 7 days after the first injection. At 28 days after injection, the mean volume and weight of the tumors generated from MDA-MB-231 cells treated with miR-185 mimics was significantly lower than those of tumors from mice injected with the scrambled control (Figs. 5A and B). Tumor growth (including tumor volume and weight) was significantly higher in mice treated with the miR-185 inhibitors than in the control group (Fig. 5A and B). ISH analysis was used to detect the expression of miR-185. IHC was used to detect the protein expression of E2F6, DNMT1, and BRCA1. The results showed that all the tumors from the mice injected with the miR-185 mimics expressed high levels of miR-185 and BRCA1 and low levels of E2F6 and DNMT1 (Fig. 5C). On the contrary, all of the tumors from the mice injected with the miR-185 inhibitors expressed low levels of miR-185 and BRCA1 and high levels of E2F6 and DNMT1 (Fig. 5C). These data suggested that miR-185 can significantly inhibit the growth and tumorigenicity by upregulating BRCA1 in human TNBC cells.

Discussion
Breast cancer is a heterogeneous disease that comprises a range of distinct tumor types differing in their biologic and clinical features. The most aggressive breast cancer subtypes, such as basal-like and TNBC, are characterized by high rates of relapse and of chemoresistant metastasis, and greatly suffer from a lack of therapeutic options (32, 33). Therapeutic strategies have been proposed on the basis of ectopic expression or the inhibition of cancer-related miRNAs (34). An alternative strategy exploiting miRNA deregulation in tumors would be able to take advantage of cancer-related miRNAs to predict the clinical response to available treatments. We previously reported that miR-
216b is greatly downregulated in nasopharyngeal carcinoma and inhibits tumor growth by targeting KRAS (35). We also showed that miR-34a is greatly downregulated in breast cancer cells and tissues and that it inhibits breast cancer proliferation and invasion (36, 37). miR-185 is known to play a significant role in growth and invasion in human cancers (11–14, 16, 38–40). In this study, we showed that miR-185 levels in TNBC tissues were significantly lower than those in noncancerous tissues by qRT-PCR and ISH. Moreover, the miR-185 levels were

![Figure 4.](image)

Figure 4. miR-185 inhibits BRCA1-mediated proliferation in vitro. A, MCF-10A, BT-20, MDA-MB-231, MDA-MB-435, and MDA-MB-468 cells were transfected with miR-185 mimics, a scrambled oligonucleotide, miR-185 inhibitors, control, si-BRCA1, or si-control. Cell viability was determined with the MTT assay at 48 hours after transfection. B, MDA-MB-231-luciferase, MDA-MB-361-luciferase, and MDA-MB-435-luciferase cells were transfected with si-control, si-BRCA1, scramble, miR-185 mimics, scramble + si-control, and si-BRCA1 + miR-185 mimics. Cell viability was determined with luciferase imaging at 48 hours after transfection. C, MDA-MB-231 cells were transfected with si-control, si-BRCA1, si-BRCA1 + miR-185 mimics, and miR-185 mimics. BrdUrd incorporation was assessed by IHC after treatment with si-control or the si-BRCA1 and/or miR-185 mimics to evaluate cell proliferation at 48 hours after transfection (left). Image analysis and total gray value of BrdUrd are estimated by the GSM-2000P pathology image analysis system. Data are presented as mean ± SD. * P < 0.05 compared with the scramble. The above experiments were repeated three times with similar results (right). D, MDA-MB-231, MDA-MB-435, and MDA-MB-468 cells were transfected with miR-185 mimics, a scrambled oligonucleotide, scramble + si-control and si-BRCA1 + miR-185 mimics. Apoptotic cells were determined with Annexin V-FITC/propidium iodide staining and FACS quantification by flow cytometry at 48 hours after transfection.
associated with clinical stage, distant metastatic relapse, and lymph node metastasis. Kaplan–Meier survival analyses revealed that patients with TNBC whose primary tumors displayed low miR-185 expression had shorter OS and RFS. In addition, multivariate analysis showed that reduced miR-185 levels in tumors were a strong independent predictor of both shorter OS and RFS. Imam and colleagues (16) reported that the novel tumor-suppressor miR-185 was downregulated in breast cancer cells and miR-185 may be one of the central events, which led to dysregulation of Six1 in human breast cancers. Zhi and colleagues (13) found that miR-185 might be a biomarker of hepatocellular carcinoma in early stage, and the upregulation of miR-185 might be considered to be a potentially important molecular treatment strategy for patients with hepatocellular carcinoma. Tan and colleagues (39) report that miR-185 is an independent prognosis factor and suppresses tumor metastasis in gastric cancer. On the basis of these data, miR-185 may be a useful prognostic marker to predict survival and relapse in patients with TNBC.

Interestingly, we characterized the role of miR-185 in the regulation of BRCA1 in TNBC. Our data showed that miR-185 targets DNMT1 and E2F6, thus resulting in the reexpression of BRCA1 in TNBC. Epigenetic regulation, especially promoter methylation, is an important mechanism of downregulating tumor-suppressor genes in human cancer cells (41, 42). The promoter regions of approximately 50% of human genes contain regions of DNA with greater than expected cytosine and guanine contents, and hypermethylation of these regions mediates transcriptional gene silencing (43, 44). Basal-like breast tumors or TNBC, a breast cancer subgroup defined through gene expression profiling and characterized by an expression signature similar to that of the basal cells of the breast (45), display lower BRCA1 expression than other breast cancer subgroups (46). In a fraction of these tumors, this low BRCA1 expression could be due to aberrant methylation of the promoter, which has been reported to occur in 11% to 14% of breast cancer cases (31, 46, 47). Our results showed that miR-185 directly targeted DNMT1 and resulted in the reexpression of the
miR-185 Is a Potential Target for TNBC Therapy

Figure 6. Schematic diagram of the working model for the miR-185 in TNBC.

Silenced tumor-suppressor gene BRCA1. Oberley and colleagues (23) and Yang and colleagues (24) reported that E2F6 negatively regulated BRCA1. Our results also showed that miR-185 directly targeted E2F6 and resulted in the reexpression of BRCA1. Most notably, we observed a positive correlation between miR-185 and BRCA1 expression in human TNBC. This mechanism of inactivation of the BRCA1 gene in breast and possibly in ovarian sporadic tumors results in the previously described effects, that is, somatic mutation, promoter methylation, haploinsufficiency, and transcriptional inhibition (31). Recent reports have indicated that another molecular mechanism of aberrant miRNA regulation results in a BRCA1-deficient-like phenotype, aberrant miRNA activity, as reported for miR-146 and miR-182, which directly target BRCA1 (31, 48).

Given the involvement of BRCA1 in ovarian cancer susceptibility and its possible involvement in sporadic ovarian cancer (49, 50), it is interesting to note that miR-185 has been found to be downregulated in ovarian cancer tissues and cell lines (16, 14). As ovarian tumors developed by individuals carrying BRCA1 mutations are mostly stage III serous carcinoma (51), these results indicate the possibility that miR-185 could also be involved in BRCA1 downregulation in sporadic ovarian cancer. Precise regulation of DNA damage response is crucial for cellular survival after DNA damage, and its abrogation often results in genomic instability in cancer. The DNA damage response process is frequently impaired in more aggressive breast cancers, as a consequence of either mutation or altered expression of critical components, such as BRCA1 (33). γH2AX forms nuclear foci at sites of DNA damage and facilitates DNA-damage response and repair (52). Our result showed that miR-138 as a potent positive regulator of γH2AX foci formation. miR-185-impaired proliferation may be a result of defective DNA repair due to upregulation of γH2AX and BRCA1.

In conclusion, we provide insights about the biologic effects of the overexpression of miR-185 in TNBC. Our in vitro and in vivo data further demonstrated that miR-185 function as tumor suppressors in TNBC. Restoring miR-185 expression in TNBC impairs proliferation. The mechanism of action of miR-185 in TNBC is summarized in Fig. 6. Therefore, miR-185 is a valuable marker of TNBC prognosis and plays an important role in the development and progression of human TNBC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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