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MYBL2 Is Targeted by miR-143-3p and Regulates Breast Cancer Cell Proliferation and Apoptosis

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Breast cancer remains a public health issue on a global scale. The present study aimed to explore the functional role of MYB proto-oncogene like 2 (MYBL2) in breast cancer, as well as underlying mechanisms. The regulatory relationship between miR-143-3p and MYBL2 was analyzed, and the effects of dysregulation of miR-143-3p and MYBL2 on cell proliferation and apoptosis were investigated. The results showed that MYBL2 and miR-143-3p were inversely expressed in breast cancer tissues and cells: MYBL2 was highly expressed, whereas miR-143-3p was lowly expressed. MYBL2 was confirmed as a target gene of miR-143-3p. Suppression of MYBL2 inhibited proliferation and induced apoptosis of breast cancer cells, which was similar to the effects of overexpression of miR-143-3p. Our findings reveal that MYBL2 is targeted by miR-143-3p and regulates breast cancer cell proliferation and apoptosis.

Key words: MYB proto-oncogene like 2 (MYBL2); Breast cancer; miR-143-3p; Cell apoptosis; Cell proliferation

INTRODUCTION

Breast cancer is one of the most common malignancies of women, with high morbidity and increased mortality^{1–3}. This disease seriously endangers the patient's survival and quality of life and thus has been considered an increasing public health problem^{4–6}. Histologically, breast cancer can be divided into three categories: usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), and ductal carcinoma in situ (DCIS). DCIS early malignant lesions always develop into invasive ductal carcinoma (IDC)^{7–9}. Progress has been made regarding the pathological mechanism of breast cancer, but its occurrence, development, and metastasis remain high¹⁰. In view of this, the exploration of the mechanism of breast cancer would be very meaningful for early diagnosis and treatment of breast cancer.

Genome-wide association studies have identified more than 70 common variants that are involved in breast cancer susceptibility, and several exhibit significant heterogeneity in their associations with different breast cancer subtypes^{10,11}. MYB proto-oncogene like 2 (MYBL2) is

one of the members of the family of MYB transcription factors and interacts with cell cycle protein cyclin A, promoting cell cycle progression^{12,13}. Many studies have shown that MYBL2 is highly expressed in several human tumors and plays an important role in the genesis and progression of tumors^{14,15}. In addition, a previous study has shown that MYBL2 promotes cell proliferation and metastasis in many tumors¹⁶.

In this study, we investigated the regulatory relationship between miR-143-3p and MYBL2 in breast cancer cells. We analyzed the MYBL2 expression levels in clinical breast cancer tissues, and then performed an in-depth analysis of the effects of abnormal expression of MYBL2 and miR-143-3p on breast cancer cell proliferation and apoptosis. Our study confirms that MYBL2 is the target gene of miR-143-3p. Inverse expression and roles of miR-143-3p and MYBL2 in regulating breast cancer cell proliferation and apoptosis are found. The results clarify the role of MYBL2 in the development of breast cancer and provide effective theoretical guidance for clinical treatment.

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MATERIALS AND METHODS

Patients and Specimens

We selected breast cancer patients with UDH (benign), ADH (pre-malignant), DCIS (primary), or IDC by the evaluation of gene expression. Patients with UDH and ADH were diagnosed during regular breast cancer screening. Specimens from women with breast mass were pathologically examined for the determination of UDH or ADH. Histopathologically normal breast ductal tissues were collected from the UDH patients far from the UDH tissue and were used as controls. Patients with IDC and DCIS were recruited at the Pathology Department of Shenzhen Second Hospital. The tumor grade and pathological stage of the IDC and DCIS specimens were respectively determined according to the WHO tumor grading system and the AJCC Cancer Staging System⁴. The details were as follows: IDC grade 1 ($n=12$), grade 2 ($n=34$), grade 3 ($n=14$); IDC stage I ($n=23$), stage IIa ($n=19$), stage IIb ($n=16$), stage IIIa ($n=2$); DCIS grade 1 ($n=12$), grade 2 ($n=11$), grade 3 ($n=2$). All DCIS patients were stage 0. Pre-malignant ADH and flat epithelial atypia were diagnosed according to the WHO recommendation⁵.

Cell Culture and Transfection

Normal breast cell line MCF-10A and breast cancer cell line MDA-MB-435 used in the present study were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% antibiotics (both from Invitrogen, Grand Island, NY, USA). Transfection of the cells with miR-143-3p mimic, mimic control, miR-143-3p inhibitor, or inhibitor control (GenePharma, Shanghai, P.R. China) was performed using Lipofectamine™ 2000 (Invitrogen).

MTT Assay

MCF-10A and MDA-MB-435 cells were plated in 96-well culture plates. After 24 h of incubation, the cells were transfected with miR-143-3p mimic or mimic control for 12, 24, and 48 h. Then MTT solution was added to each well (20 μ l/well). The solution was discarded, and 200 μ l of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added, and the plates were shaken gently after 4 h of additional incubation. The absorbance was measured on a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm.

Colony Formation Assay

Cells were counted and seeded in 12-well plates (in triplicate) at 100 cells/well. Fresh cultured medium was replaced every 3 days. The number of viable cell colonies was determined after 14 days, and the colonies were fixed

with methanol, stained with crystal violet, photographed, and counted under a microscope (Ix71; Olympus, Tokyo, Japan). Each experiment was performed in triplicate.

Cell Cycle and Apoptosis Analysis

Transfected MCF-10A and MDA-MB-435 cells were seeded into six-well plates for 24 h in complete medium. The cells were deprived of serum for 48 h followed by returning the complete medium for an additional 24 h. After that, the cells were collected by centrifugation, fixed in 95% ethanol, incubated at -20°C overnight, and washed with phosphate-buffered saline (PBS). The cells were then resuspended in 1 ml of fluorescence-activated cell sorting (FACS) solution [PBS, 0.1% Triton X-100, 60 μ g/ml propidium iodide (PI), 0.1 mg/ml DNase-free RNase, and 0.1% trisodium citrate] with a final incubation on ice for 30 min. The cells were analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Fullerton, CA, USA). A total of 10,000 events were counted for each sample. For the annexin V assay, the MCF-10A and MDA-MB-468 cells were transfected. After 48 h, the DNA content was determined by PI staining, and annexin V staining was performed with the Vybrant Apoptosis Assay kit (Invitrogen, Carlsbad, CA, USA).

Luciferase Assay

The full-length 3'-untranslated region (3'-UTR) of *MYBL2* was cloned by standard procedures into the pMIR-Report vector (Ambion, Austin, TX, USA), immediately downstream of the stop codon of the luciferase gene to generate the pMIR-MYBL2-3'UTR luciferase reporter plasmid. Mutagenesis of the pMIR-MYBL2-3'UTR was performed using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The two binding sites of miR-143-3p on the MYBL2 3'-UTR were mutated simultaneously to generate pMIR-MYBL2-3'UTR-mut and to analyze their functional role. Cells were cotransfected with 2 mg of wild-type pMIR-MYBL2-3'UTR or pMIR-MYBL2-3'UTR-mut, 50 pmol of miRNA mimic, and 0.01 mg of *Renilla* in 24-well plates. Forty-eight hours after transfection, the cells were washed and lysed with passive lysis buffer (Promega, Madison, WI, USA), and the luciferase activity was measured using a luminometer (Sirius; Titertekberthold, Pforzheim, Germany).

Western Blot

Cells were plated at a concentration of 10^6 cells/ml. After transfection with the miR-143-3p mimic or miR-143-3p inhibitor for 48 h, the cells were lysed with RIPA buffer (1 \times PBS, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1% PMSF). After centrifuging at 12,000 rpm

for 15 min at 4°C to remove the cell debris, the supernatant was transferred, and the protein concentration was determined using Bradford protein dye reagent (Bio-Rad, Hercules, CA, USA). The samples were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk at room temperature for 1 h, the membrane was incubated with the MYBL2 or GAPDH antibody (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing, HRP-conjugated rabbit secondary monoclonal antibody (#7074; dilution 1:1,000; Cell Signaling Technology, Beverly, MA, USA) was added and incubated at room temperature for 1 h. Densitometric analysis of the band intensity was performed using the NIH ImageJ software (version 1.32J).

qPCR

The small RNA fraction was isolated from the cells using the mirVana miRNA isolation kit (Ambion). Quantitative RT-PCR was performed using the mirVanaq RT-PCR miRNA Detection kit and the miR-143-3p and U6 snRNA primer sets (Ambion) in a Roche LightCycler (Roche, Basel, Switzerland). The LightCycler software package version 5.3.2 was used to determine the expression of miR-143-3p relative to that of U6 snRNA. Relative changes in miR-143-3p levels were calculated using a standard curve constructed from serial dilutions of control RNA.

Statistical Analysis

A Student's *t*-test was performed to analyze the significance of differences between the sample means obtained from three independent experiments. Differences were considered statistically significant at a value of $p < 0.05$.

RESULTS

miR-143-3p Is Lowly Expressed in Breast Cancer Tissues and Cell Lines

We separated normal breast tissues and disease tissues from breast cancer patients and divided disease tissues into UDH, ADH, DCIS, and IDC. In addition, we chose the normal cell line MCF-10A and the breast cancer cell line MDA-MB-435 for the research. By qPCR, miR-143-3p expression levels of these tissues and cell lines were detected (Fig. 1A and B). We found that the expression level of miR-143-3p in normal tissue was significantly higher than that of each type of breast cancer tissue and was lowest in IDC tissues. The level of miR-143-3p expression in normal breast MCF-10A cells was significantly higher than in the breast cancer MDA-MB-435 cells. These data indicate that miR-143-3p is lowly expressed in breast cancer tissues and cells and may play an important role in the development of breast cancer.

miR-143-3p Regulates the Viability of Breast Cancer Cells

We transfected miR-143-3p mimic or inhibitor and divided them into four groups: (1) MCF-10A+control, (2) MCF-10A+miR-143-3p inhibitor, (3) MDA-MB-435+mimic control, and (4) MDA-MB-435+mir-143-3p mimic. Changes in cell viability after 12, 24, and 48 h of transfection were detected by MTT assay (Fig. 2A). We found that suppression of miR-143-3p promoted the viability of MCF-10A cells, and overexpression of miR-143-3p inhibited MDA-MB-435 cell viability, which showed significant differences in 24 h after transfection. So the subsequent testing was conducted at 24 h after transfection. First, the miR-143-3p level of each group was detected by qPCR detection in this time point (Fig. 2B).

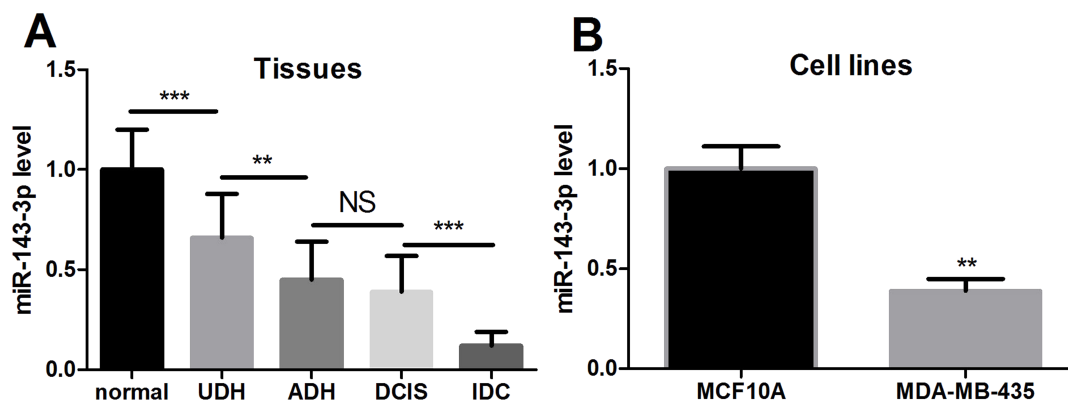


Figure 1. Expression of miR-143-3p in breast cancer tissues and cell lines. (A) Levels of miR-143-3p were measured in breast cancer and normal tissues. (B) Levels of miR-143-3p were measured in breast cancer and normal cell lines. The results showed that miR-143-3p was highly expressed in breast cancer. UDH, usual ductal hyperplasia; ADH, atypical hyperplasia of catheter; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma. ** $p < 0.01$, *** $p < 0.001$. NS, not significant.

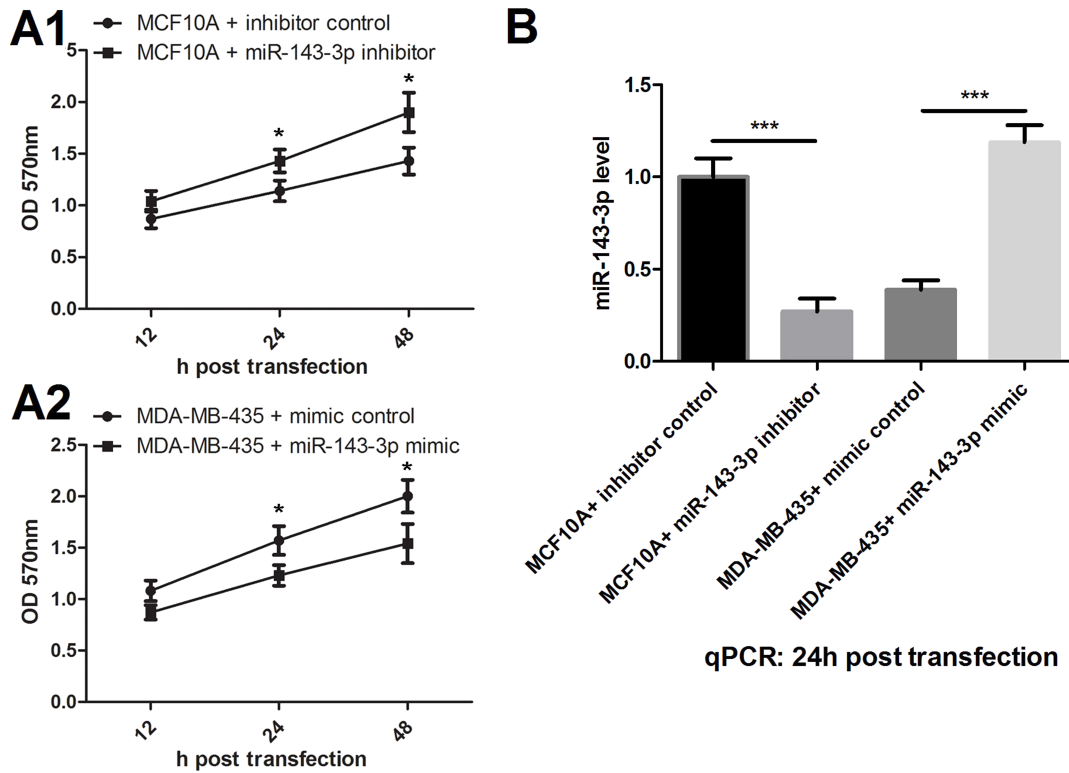


Figure 2. Effects of miR-143-3p on cell viability. (A1) miR-143-3p suppression promoted the viability of MCF-10A cells. (A2) miR-143-3p overexpression inhibited MDA-MB-435 cell viability. (B) miR-143-3p inhibitor suppressed miR-143-3p expression, and miR-143-3p mimic promoted miR-143-3p expression. * $p < 0.05$, *** $p < 0.001$.

The results showed that inhibition and overexpression of miR-143-3p significantly inhibited or increased the levels of miR-143-3p, respectively.

miR-143-3p Suppresses Proliferation and Induces Apoptosis of Breast Cancer Cells

We tested the cell proliferation at 24 h after transfection through a tablet cloning experiment (Fig. 3A). The suppression of miR-143-3p significantly promoted the proliferation of MCF-10A, and increased miR-143-3p expression inhibited the proliferation of MDA-MB-435 cells. Next, cell apoptosis was detected by flow cytometry. The suppression of miR-143-3p reduced the apoptosis of MCF-10A cells, and miR-143-3p overexpression promoted MDA-MB-435 apoptosis (Fig. 3B). The expression levels of apoptotic proteins BAX and BCL2 were consistent with apoptosis (Fig. 3C). Furthermore,

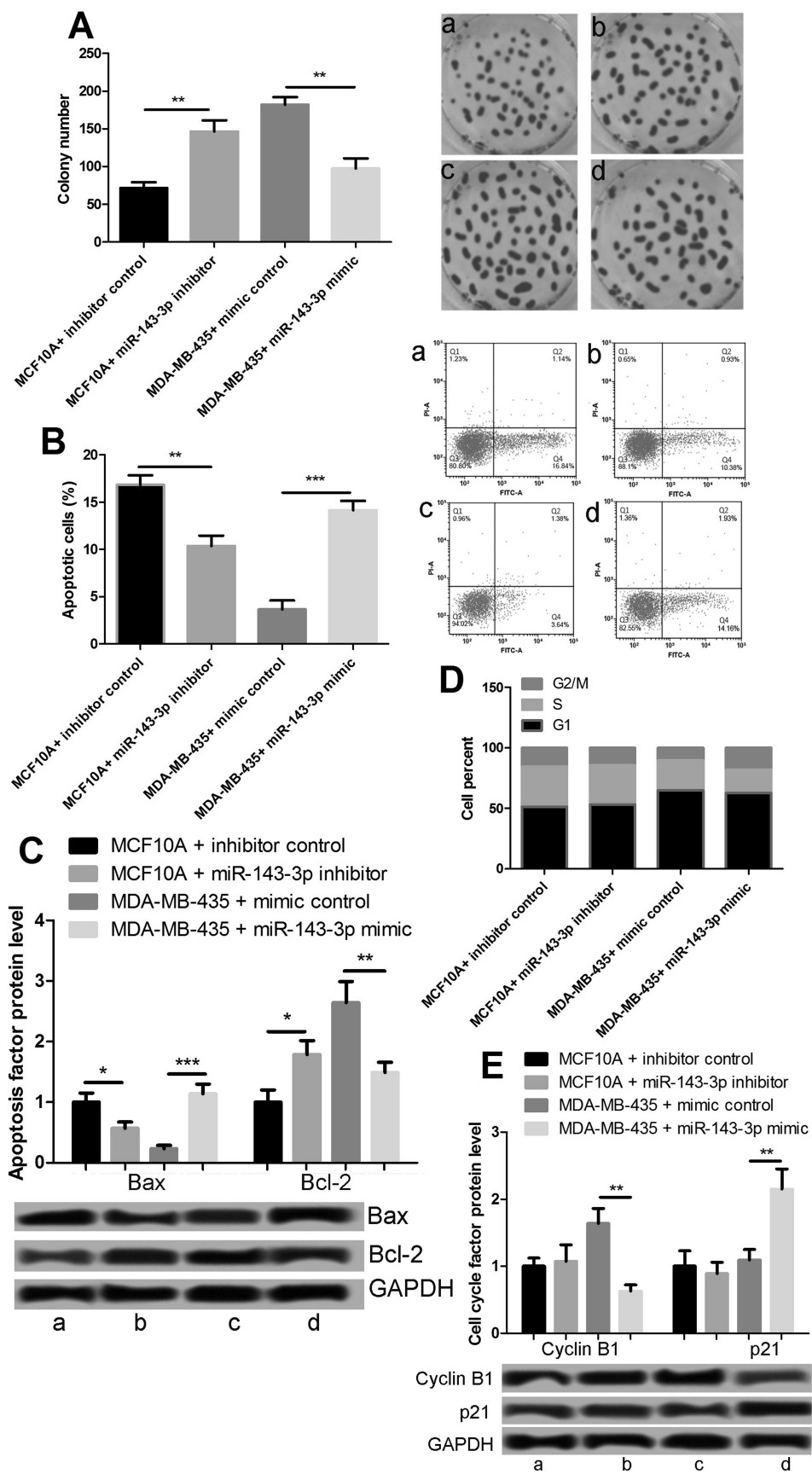
we detected the cell cycle by flow cytometry. The cell cycle of MCF-10A did not change notably after the inhibition of miR-143-3p, but overexpression of miR-143-3p in MDA-MB-435 cells induced G₂/M arrest and inhibited cell cycle progression (Fig. 3D). We examined the G₂/M-associated proteins and found that miR-143-3p inhibited cyclin B1 expression and promoted p21 expression (Fig. 3E). These results showed that miR-143-3p inhibited the proliferation of breast cancer cells and induced cell cycle G₂/M arrest and cell apoptosis.

miR-143-3p Targets MYBL2 3'-UTR and Inhibits MYBL2 Expression

In order to study the mechanism of miR-143-3p in breast cancer regulation, the online database was applied to predict its target mRNA. A sequence on the 3'-UTR of MYBL2 mRNA was likely to be the binding site of

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Figure 3. Effects of miR-143-3p on cell proliferation and apoptosis. (A) miR-143-3p inhibited the proliferation of MDA-MB-435 cells. (B) miR-143-3p promoted the apoptosis of MDA-MB-435 cells. (C) Expression of apoptotic proteins BAX and BCL2. (D) miR-143-3p inhibited cell cycle progression. (E) Expression of G₂/M-associated proteins cyclin B1 and p21. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Lane a: MCF-10A+ inhibitor control; lane b: MCF-10A+ miR-143-3p inhibitor; lane c: MDA-MB-435+ mimic control; lane d: MDA-MB-435+ miR-143-3p mimic.



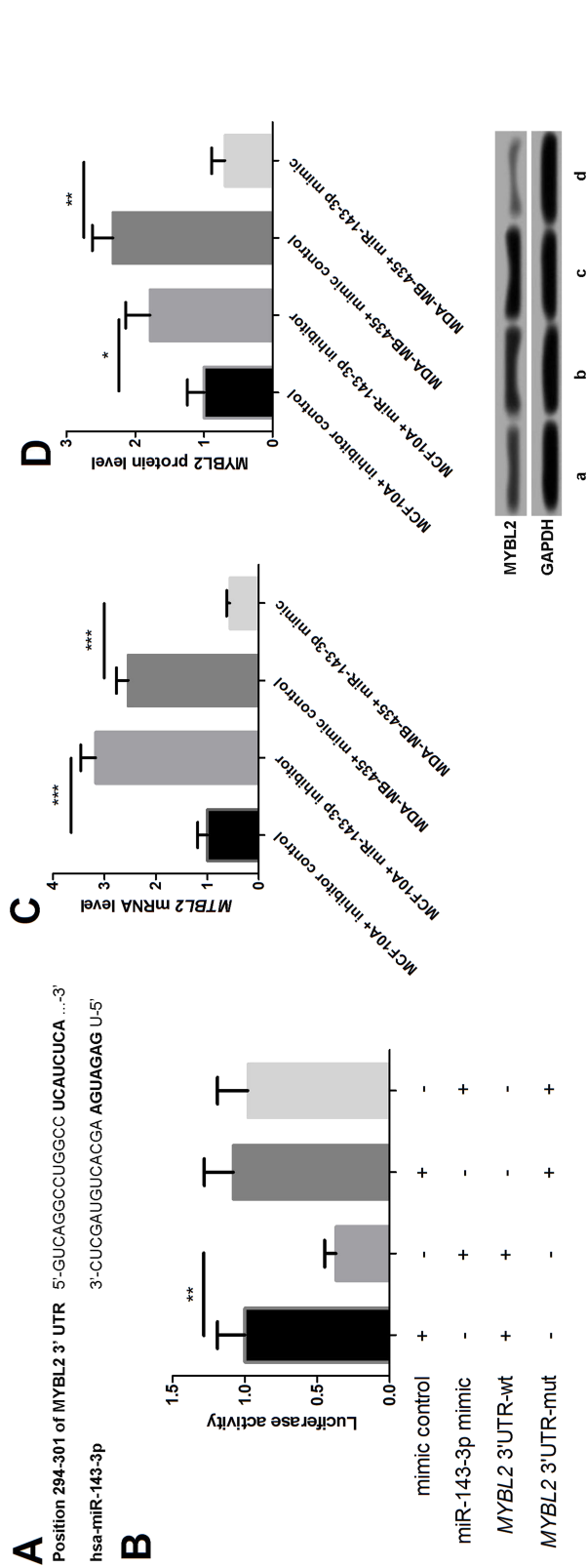


Figure 4. Connection of miR-143-3p with MYBL2. (A) MYBL2 was a target gene of miR-143-3p. (B) miR-143-3p controlled luciferase activity of wild-type MYBL2 mRNA 3'-untranslated region (3'-UTR) carrier in MDA-MB-435 cells. (C) miR-143-3p inhibited MYBL2 at the mRNA level. (D) miR-143-3p inhibits MYBL2 at the protein level. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Lane a: MCF-10A + inhibitor control; lane b: MCF-10A + miR-143-3p inhibitor; lane c: MDA-MB-435 + mimic control; lane d: MDA-MB-435 + miR-143-3p mimic.

miR-143-3p (Fig. 4A). MYB belongs to a family of transcription factors that include the closely related family members MYBL1 and MYBL2¹⁷. Research has found that MYBL2 is related to the development of breast cancer¹⁸. We built the expression vector that carried wild-type MYBL2 3'-UTR and mutated MYBL2 3'-UTR, and the interactivity of miR-143-3p with MYBL2 mRNA in the MDA-MB-435 cells was analyzed by luciferase analysis. miR-143-3p can only control luciferase activity of the wild-type MYBL2 mRNA 3'-UTR carrier, while there was no obvious effect on the activity of mutant MYBL2 (Fig. 4B). These data indicate that miR-143-3p may be a direct target of MYBL2 mRNA 3'-UTR. Next, the expression of MYBL2 in the four groups of transfection cells was examined. The qPCR results showed that the miR-143-3p inhibitor increased MYBL2 mRNA level, while miR-143-3p mimics inhibited the MYBL2 mRNA level (Fig. 4C). The Western blot results showed that the changes in MYBL2 protein were consistent with the mRNA level (Fig. 4D). Taken together, our data indicate

that miR-143-3p may inhibit its mRNA and protein levels by directly binding to MYBL2 3'-UTR.

MYBL2 Was Highly Expressed in Breast Cancer Cells

To determine the correlation between MYBL2 and the development of breast cancer and its role in the miR-143-3p regulatory mechanism, we examined the expression of MYBL2 in breast cancer tissues. The qPCR results showed that MYBL2 expression was higher in the breast cancer tissues than in normal tissues, and its expression was highest in the IDC (Fig. 5A). Western blot showed similar results (Fig. 5B).

The breast cancer MDA-MB-435 cells were transfected and divided into four groups: (1) si-control, (2) si-mybl2, (3) si-mybl2+inhibitor control, and (4) si-mybl2+ miR-143-3p inhibitor. At 24 h after transfection, we detected the protein level of MYBL2 in each group of cells. The results showed that si-MYBL2 significantly inhibited MYBL2 expression; on the basis of this, the miR-143-3p inhibitor markedly increased the protein level of MYBL2 (Fig. 5C).

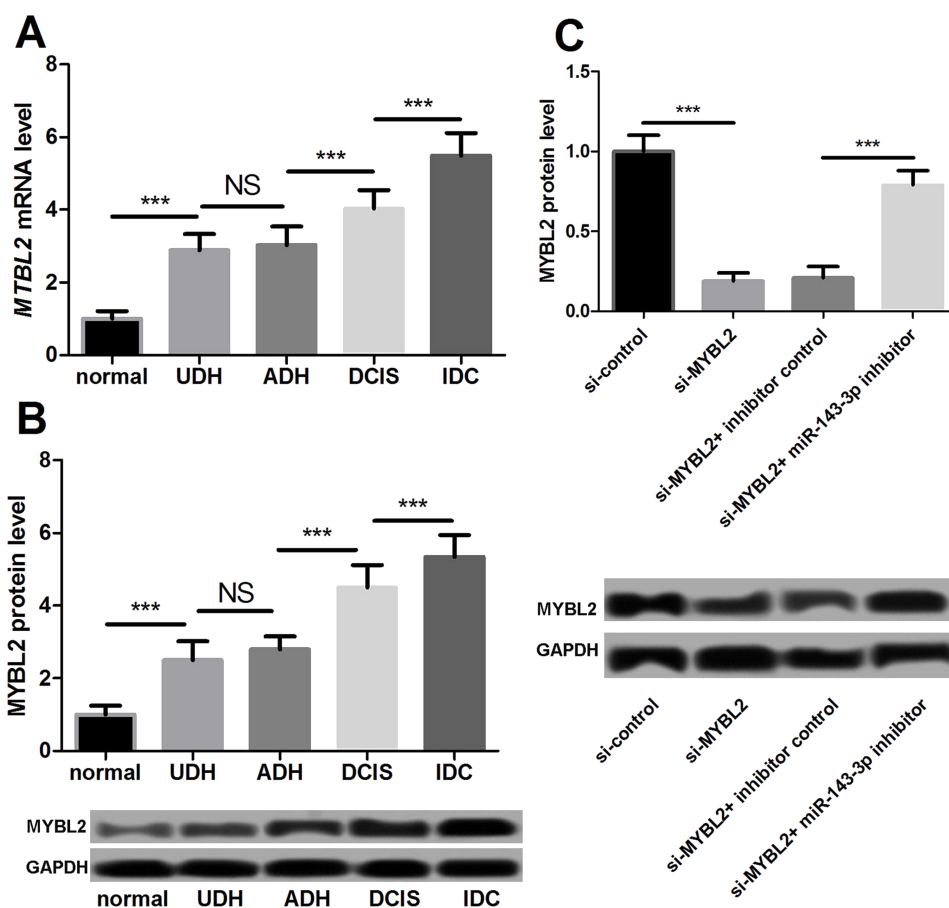


Figure 5. Expression of MYBL2 in breast cancer. (A) MYBL2 was highly expressed in breast cancer tissues at the mRNA level. (B) MYBL2 was highly expressed in breast cancer tissues at the protein level. (C) The breast cancer MDA-MB-435 cells were transfected and divided into four groups: si-control, si-mybl2, si-mybl2+inhibitor control, and si-mybl2+ miR-143-3p inhibitor. miR-143-3p inhibitor increased the MYBL2 expression at the protein level. *** $p < 0.001$.

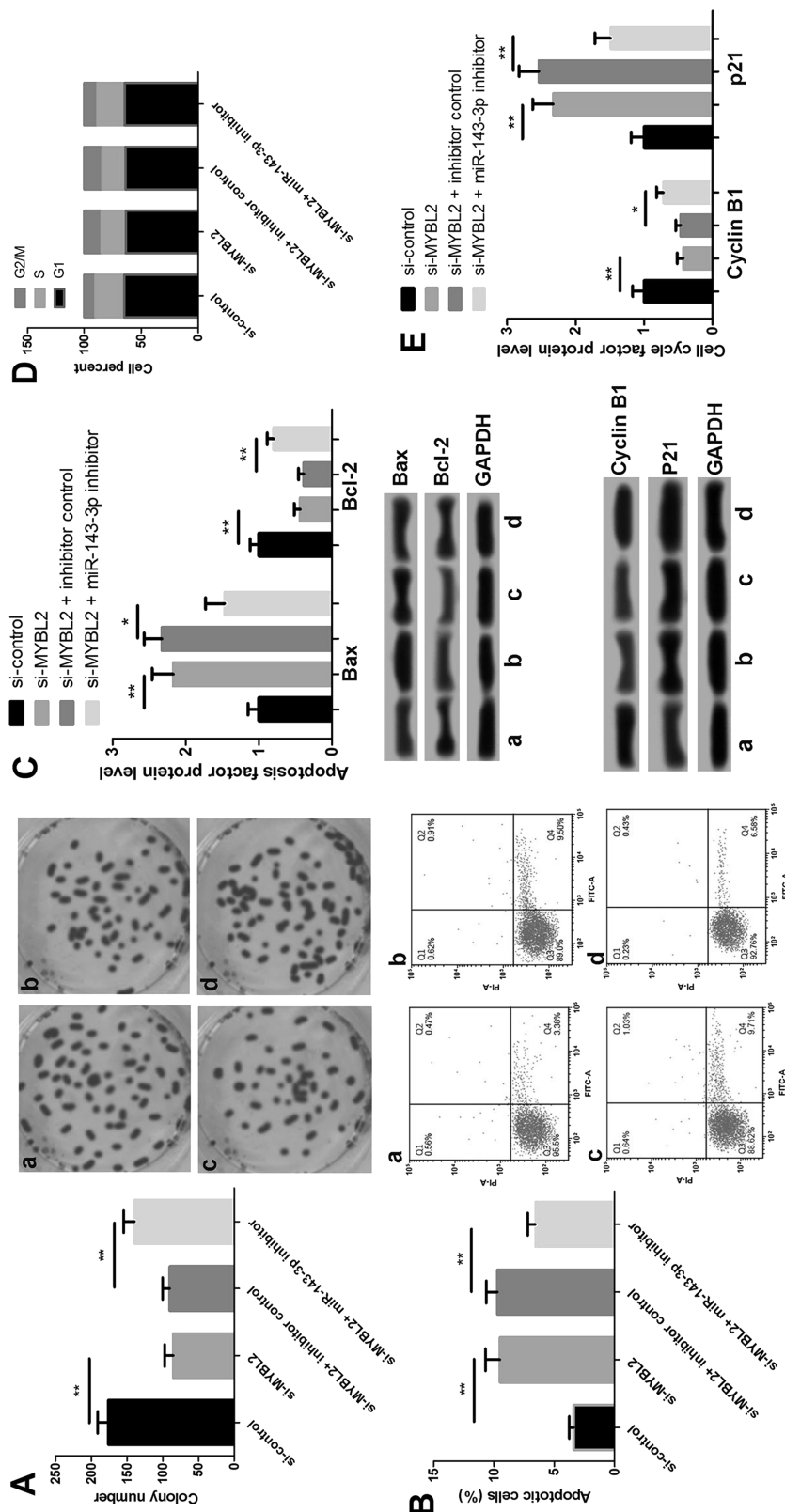


Figure 6. Effects of MYBL2 on the proliferation and apoptosis of breast cancer MDA-MB-435 cells. (A) Cell proliferation after suppression of MYBL2 and miR-143-3p. (B) Cell apoptosis after suppression of MYBL2 and miR-143-3p. (C) Expression of apoptotic factors BAX and BCL2 after suppression of MYBL2 and miR-143-3p. (D) Cell cycle after suppression of MYBL2 and miR-143-3p. (E) Expression of cell cycle-related proteins after suppression of MYBL2 and miR-143-3p. * $p < 0.05$, ** $p < 0.01$. Lane a: si-control; lane b: si-MYBL2; lane c: si-MYBL2 + inhibitor control; lane d: si-MYBL2 + miR-143-3p inhibitor.

MYBL2 Promotes Proliferation and Inhibits Apoptosis of Breast Cancer MDA-MB-435 Cells

The proliferation, apoptosis, and cell cycle of the four groups of transfection cells were detected by tablet cloning and flow cytometry. The results showed that the suppression of MYBL2 caused a significant reduction in the number of clones of MDA-MB-435 cells, and the further suppression of miR-143-3p promoted cell proliferation (Fig. 6A). Furthermore, the results shown in Figure 6B revealed that the suppression of MYBL2 caused an increase in MDA-MB-435 cell apoptosis and that further inhibition of miR-143-3p decreased apoptosis. The apoptotic factors BAX and BCL2 had corresponding changes (Fig. 6C). The reduction in MYBL2 caused the cell cycle arrest at G₂/M, and on that basis, the miR-143-3p inhibitor reduced the G₂/M arrest (Fig. 6D). The factors associated with the G₂/M arrest were regulated by cyclin B1 and p21 proteins (Fig. 6E). These results suggest that MYBL2, which has the opposite function of miR-143-3p in the MDA-MB-435 cells, may promote the proliferation and inhibit apoptosis of breast cancer cells. Because of its direct control of miR-143-3p, MYBL2 may be one of the mechanisms for miR-143p regulating proliferation and apoptosis of breast cancer cells.

DISCUSSION

Breast cancer remains a significant scientific, clinical, and societal challenge, even though recently there has been great progress in the mechanism and therapy¹⁹⁻²¹. miR-143-3p has been identified to function as a tumor suppressor in several tumors, including ovarian cancer and gastric cancer²²⁻²⁴. Li et al. explored the potential function and mechanism of miR-143-3p in triple-negative breast cancer and demonstrated that miR-143-3p functioned as a suppressor gene²⁵. However, the role and the underlying mechanism of miR-143-3p in different subtypes of breast cancer are far from sufficient. In this study, we found that the levels of miR-143-3p were decreased in breast cancer cell lines. Cells were transfected with miR-143-3p mimic or inhibitor to promote or suppress the expression of miR-143-3p. After transfection, we conducted a series of cell proliferation, cell cycle, and cell apoptosis testing experiments. The results confirmed that miR-143-3p inhibited the proliferation of breast cancer cells and induced cell cycle G₂/M arrest and cell apoptosis.

The MYBL2 gene has been reported to be involved in cell proliferation and control of cellular differentiation, playing important roles in the genesis and progression of tumors¹²⁻¹⁴. Dysregulation of MYBL2 has been associated with tumorigenesis^{15,26-28}, but the role of MYBL2 in breast cancer has not been totally identified. In the present study, we found that MYBL2 was the target gene of miR-143-3p, which indicated that it might play important

roles in breast cancer progress. So we researched the expression of MYBL2 in breast cancer and confirmed the connection of MYBL2 with miR-143-3p. We found that miR-143-3p targeted MYBL2 3'-UTR and inhibited MYBL2 expression. MYBL2 was highly expressed in breast cancer cells. Moreover, we found that MYBL2 promoted proliferation and inhibited apoptosis of breast cancer cells. The results suggest that MYBL2 plays key roles in breast cancer progress and has a close connection with miR-143-3p expression.

Our study confirms that targeted by miR-143-3p, MYBL2 promotes proliferation and inhibits apoptosis of breast cancer cells. However, there are still some limitations in this study, and additional investigation is needed to scrutinize the underlying mechanisms of MYBL2 in breast cancer.

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