Original Paper

MicroRNA-101 Inhibits Growth, Proliferation and Migration and Induces Apoptosis of Breast Cancer Cells by Targeting Sex-Determining Region Y-Box 2

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Key Words

Breast cancer • MiR-101 • SOX2 • Invasion • Migration

Abstract

Background: Increasing evidence has demonstrated that microRNAs play a critical role in breast cancer (BC) progression. microRNA-101 (miR-101) has been considered a tumor suppressive miRNA in different cancer types. This study aimed to investigate miR-101 expression in BC tissues and to investigate its roles in BC progression that are mediated by Sex-determining region Y-box 2 (SOX2), a critical oncogene in various cancers. **Methods:** qRT-PCR and immunohistochemistry were performed to detect miR-101 and SOX2 expression in BC tissues and paired normal tissues or BC cell lines. MTT, transwell migration, wound healing, colony formation, flow cytometric and xenograft assays were performed to determine the influence of miR-101 and SOX2 on the malignant behaviors of BC cells *in vitro* and *in vivo*. **Results:** miR-101 was significantly downregulated in BC tissues and cell lines. miR-101 overexpression inhibited the malignant behaviors of BC cells, both *in vitro* and *in vivo*. miR-101 downregulation had the converse effect. A miR-101 binding site was identified by luciferase reporter assay in the 3'UTR of SOX2. SOX2 was upregulated in BC tissues and cell lines, and its upregulation was associated with lymph node metastasis, pathological grade and TNM classification. SOX2 knockdown mimicked the effects of miR-101 overexpression on the malignant behaviors of BC cells, while SOX2 overexpression mitigated the miR-101-induced inhibition of these effects. **Conclusions:** Our study revealed that miR-101 plays a critical role in suppressing tumor progression by directly targeting SOX2.

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Published by S. Karger AG, Basel

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Introduction

Worldwide, breast cancer (BC) is the most frequently diagnosed tumor in women, for whom it is the second leading cause of malignancy-related deaths [1]. Moreover, BC is the primary cause of cancer-related deaths in women aged 20 to 59 [1]. Although BC treatments are varied and developed, the mortality rate still ranges from 20% to 40% in patients treated according to the National Comprehensive Cancer Network guidelines, due to local recurrence and distant metastasis [2]. Even though many studies have shown that BC recurrence and metastasis are related to the dysregulation of oncogenes and tumor suppressors, a clear and effective treatment target with known molecular mechanisms is still needed.

MicroRNAs (miRNAs) are a class of small (19–24 bp) non-coding single stranded RNA molecules encoded by endogenous genes that regulate various cellular processes including early embryonic development, proliferation and apoptosis, differentiation, and fat metabolism via targeting mRNAs involved in downstream signaling pathways [3]. Recent studies have demonstrated that miRNA dysregulation plays critical roles in carcinogenesis and the metastasis of many cancers, including BC [4–9]. More recently, microRNA-101 (miR-101) has been reported to be downregulated in carcinoma tissues compared with adjacent healthy tissues in many types of cancers, including laryngeal squamous cell carcinoma [10], osteosarcoma [11], glioma [12], papillary thyroid carcinoma [13], hepatocellular carcinoma [14] and gallbladder carcinoma [15], suggesting it functions as a tumor suppressor. A correlation between miR-101 expression and BC has also been reported. In 2014, Wang et al. found that miR-101 was downregulated in BC tissues and BC cell lines. They further showed that restored miR-101 expression could promote BC apoptosis by directly targeting Jak2 [16]. It has also been reported that the miR-101-AMPK axis functions as an important regulator of tumor metabolism in triple negative BC [17]. Meanwhile, Li et al. found that miR-101 expression in BC inhibited growth and metastasis and could be identified as a prognostic indicator [18]. These results indicated that miR-101 plays key roles in BC progression. Although miR-101 has been reported to target many downstream genes, including CDK8 [10], c-FOS [11], CXCR7 [18], SOX9 [14, 19] and GSK3 β [20], the potential correlation between miR-101 and Sex-determining region Y-box 2 (SOX2) in BC is still unknown and needs to be further explored.

SOX2 is a member of the Sex-determining region Y-related high-mobility group box (SOX) gene family, proteins of which contribute significantly to tumorigenesis in various tissues [21], including lung cancer, laryngeal cancer, hepatocellular carcinoma and ovarian cancer [22–25]. Recently, studies have demonstrated that SOX2 expression is regulated by miRNAs. Yang et al. found that miR-126 overexpression can suppress cell proliferation, migration and invasion and promote apoptosis via targeting SOX2 in Osteosarcoma [26]. Furthermore, similar conclusions were obtained by Zhao et al., who showed that miR-126 suppresses proliferation and promotes apoptosis in hepatocellular carcinoma cells that overexpress SOX2 [27]. Additionally, Wang et al. found that miR-625 was one of the upstream regulators of SOX2, and miR-625 down-regulation could promote proliferation and invasion in esophageal cancer by activating SOX2 [28]. However, whether SOX2 is a target of miR-101 that regulates cell proliferation, migration and invasion of BC cells still remains to be explored.

In this study, we examined miR-101 and SOX2 expression in human BC cell lines and carcinoma tissues, as well as in adjacent healthy tissues. Furthermore, we demonstrated that miR-101 upregulation could inhibit proliferation, migration and invasion and promote apoptosis of BC cells by targeting SOX2. Meanwhile, we analyzed the relationship between miR-101 or SOX2 expression and clinical characteristics, finding that miR-101 or SOX2 was associated with lymph node metastasis, pathological grade and TNM classification. Moreover, we found that SOX2 overexpression could reverse the miR-101-induced inhibition of malignant behaviors in BC cells. Thus, target miR-101/SOX2 signaling may be a potential strategy for future metastasis BC treatments.

Materials and Methods

Cell lines and culture

The cells used in this study were all purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The human BC cell lines MCF-7 and MDA-MB-231 were maintained in DMEM (Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin-streptomycin (Beyotime, Haimen, China) at 37°C in a 5% CO₂ atmosphere. BC cell lines HBL-100 and SK-BR-3 were maintained in RPMI-1640 (Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin-streptomycin (Beyotime, Haimen, China). Cells were split with trypsin when they covered 80%–90% of the dish.

Tissue samples and clinical data

BC and paired adjacent tissue samples were obtained from 74 patients who underwent modified radical mastectomy at Jinling Hospital from 2013 to 2016. The tissues were stored in liquid nitrogen as soon as they were obtained. Clinical data were derived from a self-built follow-up database.

Total RNA extraction and reverse transcription

Total RNA from BC tissues, adjacent tissues and BC cell lines were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription to cDNA was performed using PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa, Shiga, Japan). A specific reverse transcription primer from the Bulge-loop miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) designed by RiboBio (Guangzhou, China) was used to reverse transcribe the miR-101 and U6 RNA.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed to precisely detected the expression of miR-101, U6 (internal control), SOX2 and GAPDH (internal control) in BC tissues and cell lines with SYBRGreen reagents (TaKaRa) in an ABI step-one plus quantitative PCR instrument. The specific qRT-PCR primers for miR-101 and U6 were from the Bulge-loop miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) designed by RiboBio. The qRT-PCR primers for SOX2 and GAPDH were as follows: SOX2 forward: 5'-CAGGAGTTGTCAAGGCAGAGA-3' and SOX2 reverse: 5'-CAGGAGTTGTCAAGGCAGAGA-3'; GAPDH forward: 5'-ACAACTTTGGTATCGTGAAGG-3' and GAPDH reverse: 5'-GCCATCACGCCACAGTTTC-3' (Invitrogen). The relative expressions of miR-101 and SOX2 were calculated by the 2^{-ΔΔCt} method.

Transfection of miRNA mimics and plasmid vectors

The miR-101 mimic (miR-101) and corresponding negative control oligonucleotide (miR-NC) were purchased from Ribobio. The sequences of miR-101 mimics: 5'-UACAGUACUGUGAUACUGAA-3' ; miR-NC: 5'-UCACAACCUCCUAGAAAGAGUAGA-3'. The small hairpin RNA (shRNA) plasmid vector targeting SOX2 (pSil/sh-SOX2) and control vector (pSil/sh-NC) were purchased from Genechem (Shanghai, China). All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were transfected with those recombinant DNA vectors containing a G418 selection marker and were selected G418 (Sigma) at 400 mg/ml for 4 weeks. Then single clones were obtained and maintained in G418 at 100 mg/ml.

Total protein extraction and western blotting

Total protein of tissues and cells were extracted in RIPA buffer (Beyotime) containing 1% PMSF and 1% protease inhibitor cocktail. After shaking on ice for 30 min, the protein extracts in SDS loading buffer were heated to 100°C for 10 min. Proteins were electrophoresed in SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibody at 4°C overnight after blocking for 1 h in 5% non-fat milk. After washing, the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China) at 37°C for 1 h. Immunoreactive bands were detected by chemiluminescence. The primary antibodies were as follows: anti-SOX2 (Ab75485, Abcam, Cambridge, UK), anti-GAPDH (No.5174), anti-Bcl2 (No.15071), anti-Bax (No.5023), anti-cleaved caspase3 (No.9664), anti-total caspase3 (No.14220) (all from Cell Signaling Technology, Danvers, MA, USA); all primary antibodies were diluted at 1:1000.

Cell viability Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability. After transfecting miR-101 mimics or miR-NC and/or SOX2 plasmid or empty plasmid, cells were plated in 96-well plates (2×10^3 cells per well) and incubated in 100 μ l/well culture medium. Every 24 h until the 4th day, 20 μ l MTT solution was added to the medium, and the cells were incubated for 4–6 h at 37°C. Then, the medium was removed, and 200 μ l DMSO was added into every well. After 10 min lysis, absorbance at 450 nm was detected using a microplate reader.

Wound healing assay

Wound healing assays were performed to evaluate the migration capacity of cells following a common protocol [18]. Briefly, MCF7, MDA-MB-231 or HBL-100 cells were seeded into 6-well plates (1×10^6 cells per well) and transfected with miR-101 mimics or miR-NC, miR-101 inhibitors or miR-101 NC and/or SOX2 plasmid or empty plasmid. When cells were 70%–80% confluent, an artificial wound of the same width was scratched using a sterile plastic micropipette tip on the bottom of the plate. After wounding, cell debris were washed with phosphate-buffered saline. Cell migration was observed and photographed at 48 h after wounding and compared with the 0 h images.

Transwell assay

Transwell assays were performed to measure cell invasion. Matrigel invasion chambers (BD Bioscience, San Diego, CA, USA) were used for the invasion assay. Briefly, 3×10^4 cells were plated in the upper chambers in serum free medium. The lower chambers were filled with culture medium supplemented with 10% FBS. After 24 h incubation, cells on the bottom of membrane were fixed and stained with 0.1% crystal violet. 5 visual fields of invaded cells were selected randomly and counted and photographed under a microscope.

Colony formation assay

Colony formation assays were performed to evaluate cell proliferation. Cells after stable transfection with miR-101 or miR-NC plasmid and/or SOX2 plasmid or empty plasmid were seeded in 6-well plates at approximately 1000 cells per well. After 14-d incubation, culture medium was removed, and cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were then stained with 0.5% crystal violet for 15 min, and visible colonies were counted.

Flow cytometric apoptosis assay

The early apoptosis rates of BC cells after transfection with miR-NC or miR-101 mimics, miR-101 NC or miR-101 inhibitors and/or sh-NC or sh-SOX2 were detected by flow cytometry. Cells were harvested using 0.25% trypsin and 10^6 cells were detected per group. After washing in cold PBS, cells were treated with the Vybrant Apoptosis Assay Kit #2 (Invitrogen) according to the instructions supplied by the manufacturer. Then, early apoptosis rates of the cells were analyzed by flow cytometry within 1 h of staining.

Xenograft tumorigenicity assay

Xenograft tumorigenicity assays were performed to demonstrate the effect of miR-101 on tumorigenesis and apoptosis *in vivo*. After stable transfection of miR-101 or miR-NC plasmid, 2×10^7 MCF-7 cells were subcutaneously injected into the right hind limb of nude mice. Tumor volumes were measured and recorded every 4 d. After 32 d, pictures of mice with subcutaneous tumors were taken, and then tumors were removed for immunohistochemistry and TUNEL.

Luciferase reporter assays

To confirm the presence of miR-101 binding sites in the SOX2 3'UTR, SOX2-wild-type 3'UTR (wt-SOX2) and SOX2-mutant 3'UTR (mut-SOX2) Luciferase reporter vectors were constructed. Binding sites of miR-101 in the 3'-UTR regions of SOX2 were shown in Fig. 4A. Cells were seeded in 24-well plates the day before transfection. After 24 hours, cells were co-transfected with 50 ng pGL-3 firefly luciferase reporter, 10 ng pRL-TK Renilla luciferase reporter (for normalization) and 50 nM miR-101 mimics or scramble mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). All plasmid vectors were purchased from Promega, USA. Then, Luciferase reporter assays were performed according to the manufacturers' instructions (Promega, Cat #E1980). Experiments were repeated at least three times.

Immunohistochemistry

Immunohistochemistry was performed to detect SOX2 expression in BC tumor tissues and xenograft tumor tissues. Tissues were fixed in 4% paraformaldehyde, and then processed for standard (4 μ m) paraffin sectioning. For immunohistological staining, sections were dewaxed with xylene and rehydrated in 100%, 95%, 80% and 70% ethanol. Hydrogen peroxide was used to block endogenous peroxidase activity, and then samples were heated to 96°C for antigen retrieval. After blocking with 1% goat serum, the sections were incubated with anti-SOX2 primary antibody (Cell Signaling Technology) overnight at 4°C, and then HRP-linked second antibody. Diaminobenzidine was applied to the sections to produce a brown staining indicating immunoreactivity, and the samples were counterstained with hematoxylin. Images were captured by light microscopy.

TUNEL assay

By using the TUNEL assay, we detected apoptosis in subcutaneous transplanted tumor tissues. The TUNEL assay kit (KeyGen, Nanjing, China) was used according to the manufacturer's guidelines.

Statistical analysis

A two-tailed student's t test was used to determine the statistical significance between the mean values of the experimental and control groups. The significance of SOX2 expression in BC as correlated with clinicopathological characteristics was investigated using the Chi-squared test. All statistical calculations were performed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA) or Prism v5.0 (GraphPad, San Diego, CA, USA). Each experiment was repeated three times. All graphs were made using GraphPad Prism v5.0. P values <0.05 were considered to indicate statistically significant differences.

Results

Mir-101 is significantly downregulated in BC tissues and cell lines

To investigate miR-101 expression in BC, qRT-PCR was performed to detect miR-101 in 74 BCs and paired tumor-adjacent tissues. The results showed that miR-101 was significantly downregulated in 64 (86.5%) BC tumor tissues compared with the paired tumor-adjacent tissues ($P < 0.05$) (Fig. 1A). In addition, we analyzed the relationship between miR-101 and clinical characteristics after dividing the 74 patients into a low miR-101 expression group and high miR-101 expression group in BC tissues according to the mean value as the cut off; the results of statistical analysis are shown in Table 2. According to the results, we found that BC patients who expressed miR-101 at lower levels had lymph node metastasis ($P = 0.024$), and higher pathological grade ($P = 0.034$) and TNM stage ($P = 0.013$), but no association was found with other clinicopathological factors, including age, T stage, ER status, Her-2 status or molecular typing ($P = 0.273, 0.121, 0.979, 0.721$ and 0.843 , respectively). qRT-PCR was also performed to compare miR-101 expression in the BC cell lines HBL-100, MCF7, MDA-MB-231 and SK-BR-3 and the normal human mammary epithelial cells MCF-10A. The results

Fig. 1. miR-101 was significantly downregulated in BC tissues and cell lines. (A) By qRT-PCR, miR-101 was expressed at lower levels in 64 BC samples than paired carcinoma-adjacent samples. (B) By qRT-PCR, miR-101 was downregulated in the BC cell lines MCF7, MDA-MB-231 and SK-BR-3 compared with normal breast cell line MCF-10A. * $P < 0.05$. ** $P < 0.01$.

Fig. 2. miR-101 upregulation inhibits BC cell growth, proliferation and migration, and induces apoptosis. (A) By qRT-PCR, miR-101 was upregulated by miR-101 mimics in MCF-7 and MDA-MB-231 cells. (B) The effects of miR-101 mimics on the viability of BC cells was determined using the MTT assay. (C) MCF-7 and MDA-MB-231 cells were transfected with miR-101 mimics or negative control and subjected to a colony formation assay. The number of colonies was quantitatively analyzed. (D) The ability of cells to invade through Matrigel was decreased in the high miR-101 expression groups compared with control groups. (E) The wound healing rates of the high miR-101 expression groups were lower than control groups. (F) miR-101 overexpression induced apoptosis of BC cells. * $P < 0.05$. ** $P < 0.01$.

showed that miR-101 was suppressed to various degrees compared with MCF-10A in the BC cell lines (Fig. 1B).

miR-101 overexpression suppresses BC cell growth, proliferation and migration and induces cell apoptosis in vitro

To further explore the effects of miR-101 on oncogenic BC phenotypes, functional assay were performed on BC cells expressing miR-101 mimics. Using qRT-PCR, we confirmed that miR-101 was significantly increased in BC cells after transfection with miR-101 mimics compared with cells transfected with miR-NC (Fig. 2A). Then, we performed an MTT assay and observed that miR-101 overexpression significantly inhibited BC cell viability (Fig. 2B). Similarly, we also found that miR-101 upregulation decreased colony formation compared with the control group (Fig. 2C). Additionally, we performed wound healing and transwell migration assays to investigate the effects of miR-101 on the migration of BC cells. As shown in Fig. 2E, the wound healing rate of cells overexpressing miR-101 was significantly slower than the control group. Likewise, miR-101 upregulation strikingly inhibited the invasion

Fig. 3. miR-101 inhibitors increase BC cell growth, proliferation and migration, and suppress apoptosis. (A) By qRT-PCR, miR-101 was downregulated by miR-101 inhibitors in HBL-100 cells. (B) The effects of miR-101 inhibitors on the viability of BC cells was determined using the MTT assay. (C) HBL-100 cells were transfected with miR-101 inhibitors or negative control and subjected to a colony formation assay. The number of colonies was quantitatively analyzed. (D) The ability of cells to invade through Matrigel was increased in the low miR-101 expression groups compared with control groups. (E) The wound healing rates of the low miR-101 expression groups were faster than control groups. (F) miR-101 downregulation suppressed apoptosis of BC cells. ** $P < 0.01$.

ability of BC cells through Matrigel (Fig. 2D). Furthermore, to investigate the influence of miR-101 on apoptosis, we performed an apoptosis assay by co-staining with Annexin V and PI. As shown in Fig. 2F, we found that ectopic miR-101 expression significantly promoted apoptosis compared with the miR-NC group. These results indicated that miR-101 functions as a tumor suppressor in BC cells, inhibiting BC cell proliferation, migration, invasion and inducing apoptosis.

miR-101 inhibitors increase BC cell growth, proliferation and migration and suppress cell apoptosis in vitro

In order to explore the influence of miR-101 inhibitors on oncogenic BC phenotypes, we chose HBL-100 cells with high expression of miR-101 to perform the study. By qRT-PCR, we confirmed that miR-101 was downregulated in HBL-100 cells after transfection with miR-101 inhibitors compared with cells transfected with miR-101 NC (Fig. 3A). Then, we performed an MTT assay and observed that miR-101 inhibition significantly increased BC cell viability (Fig. 3B). Similarly, we also found that miR-101 inhibition increased colony formation compared with the control group (Fig. 3C). Additionally, we performed transwell migration assays and wound healing to investigate the effects of miR-101 inhibitors on the migration of BC cells. As shown in Fig. 3D, miR-101 downregulation increased the invasion

Fig. 4. SOX2 is a direct target of miR-101 in BC. (A) Sequences of the miR-101 binding site within the SOX2 3'UTR and its mutated version are as shown. (B) miR-101 overexpression suppressed SOX2 expression in MCF-7 and MDA-MB-231

cells and miR-101 inhibitors increased SOX2 expression in HBL-100 cells. (C) Relative Luciferase activity was repressed by adding the miR-101 binding site from the SOX2 3'UTR to the reporter construct. **P<0.01.

ability of BC cells through Matrigel. Likewise, the wound healing rate of cells low-expressing miR-101 was significantly faster than the control group (Fig. 3E). Furthermore, to investigate the influence of miR-101 inhibitors on apoptosis, we performed an apoptosis assay by co-staining with Annexin V and PI. As shown in Fig. 3F, we found that downregulation of miR-101 expression significantly suppressed apoptosis compared with the miR-101 NC group.

SOX2 is a direct target of miR-101 in BC

In our previous study, we found that SOX2 was a significant marker in tumorigenesis of breast cancer. And by using starBase v2.0 (<http://starbase.sysu.edu.cn/mirMrna.php>) we found that there were potential miR-101 binding site of SOX2 located in chr3:181431954-181431974[+]. Thus, we chose SOX2 as the target of miR-101 and did the further researches even though the interaction between miR-101 and SOX2 was predicted only by miRanda. The potential miR-101 binding site of SOX2 was shown in Fig. 4A. SOX2 is a transcription factor that is considered an oncogene in various cancers and has been proven to be involved in tumor cell invasion and metastasis [22-25]. However, whether SOX2 is a direct target of miR-101 and/or plays a role in BC tumorigenesis is unknown. To examine the influence of miR-101 on the expression of SOX2 in BC cells, western blotting was performed. As shown in Fig. 4B, miR-101 overexpression significantly decreased SOX2 expression in MCF-7 and MDA-MB-231 cells. In addition, miR-101 inhibitors could increase SOX2 expression in HBL-100 cells.

To confirm that the predicted miR-101 binding sites in the SOX2 3'UTR were responsible for mediating the miR-101-induced SOX2 downregulation, the wt-SOX2 and mut-SOX2 Luciferase reporter vectors were co-transfected with miR-101 mimics into HEK293 cells. The results demonstrated that the Luciferase activity of cells transfected with miR-101 mimics and the wt-SOX2 reporter was notably lower than the control group, whose Luciferase activity was not inhibited by the mutant putative binding site (Fig. 4C). These results suggested that miR-101 directly targets the SOX2 3'UTR.

miR-101 overexpression suppresses BC growth and proliferation and enhances apoptosis in vivo

To investigate the influence of miR-101 on tumorigenesis *in vivo*, xenograft tumorigenicity assays were performed. As shown in Fig. 5A and 5B, miR-101 overexpression significantly suppressed xenograft growth *in vivo*. Immunohistochemical staining showed that SOX2 expression in the miR-101 overexpression groups was decreased compared with the control groups (Fig. 5C). Furthermore, the results of TUNEL assays showed that miR-101 overexpression caused a remarkable induction of apoptosis *in vivo* (Fig. 5D). Together, these results demonstrate that miR-101 inhibited cell growth and induced apoptosis *in vivo*.

Fig. 5. miR-101 overexpression suppresses the growth of subcutaneous transplanted tumors and induces apoptosis. (A) Subcutaneous tumor volumes of the miR-101 overexpression groups were smaller than control groups in the xenograft tumorigenicity assay. (B) Tumor volumes were measured every 4 days for 32 days. The results demonstrated that miR-101 upregulation inhibited tumor growth in vivo. (C) SOX2 expression in transplanted tumors. (D) Apoptosis in transplanted tumors was determined by TUNEL assay. miR-101 upregulation induced apoptosis in vivo. **P<0.01.

Table 1. The expression of Sox2 in BC tumor tissues and adjacent healthy tissues

Groups	N	Expression of Sox2 protein		χ^2	P value
		Negative	Positive		
Tumor adjacent tissues	74	68(92%)	6(8%)	8.14	0.004
Tumor tissues	74	55(74%)	19(26%)		

SOX2 is upregulated in BC tumor tissues and cell lines, and is associated with poor clinical characteristics

Through immunohistochemistry and western blotting, we detected SOX2 protein expression in 74 BC tumor tissues and 74 matched tumor-adjacent tissues. The results demonstrated that SOX2 expression in BC tumor tissues (26%) were significantly upregulated compared with tumor-adjacent tissues

Table 2. Correlation of Sox2 expression with clinicopathological factors of BC patients. *P<0.05 was considered statistically significant. (*P<0.05, **P<0.01)

Factors	Expression of Sox2		P value	Expression of miR-101		P value
	Negative N=55	Positive N=19		Low N=44	High N=30	
Age						
≤50	29	9	0.687	21	17	0.273
>50	26	10		23	13	
T stage						
1	15	6	0.874	9	12	0.121
2	34	12		29	17	
3	5	1		5	1	
4	1	0		1	0	
ER status						
(+)	34	11	0.763	27	18	0.979
(-)	21	8		17	12	
Her-2 status						
(+)	31	15	0.08	25	21	0.721
(-)	24	4		19	9	
Lymph node metastasis						
(+)	31	17	0.009**	30	18	0.024*
(-)	17	2		14	5	
Pathological grading						
II	26	3	0.015*	16	13	0.034*
III	29	16		28	17	
Molecular typing						
Luminal A	13	2	0.125	11	4	0.843
Luminal B	21	7		19	9	
Her-2 Positive	12	9		6	15	
Triple negative	9	1		8	2	
TNM stage						
1, 2	31	3	0.002**	16	18	0.013*
3, 4	24	16		28	12	

Fig. 6. SOX2 is overexpressed in BC tissues and cell lines. (A) Positive SOX2 expression was detected in the nuclei of paraffin-sectioned BC tissues. Strong positive SOX2 expression is shown in case1 and weak positive expression is shown in case2. Western blotting was performed in BC tissue samples and paired adjacent tissues. Four cases of strong positive SOX2 expression in BC tumor tissues are as shown. (B) At both the protein and mRNA levels, SOX2 was upregulated in BC cells. (C) In BC tissues, the expression of miR-101 and SOX2 were negative correlated. ** $P < 0.01$.

(8%) (Fig. 6A, Table 1). Similarly, SOX2 levels in BC cell lines were higher than in normal breast cells by western blotting and qRT-PCR (Fig. 6B). Furthermore, to analyze the relationship between SOX2 and clinical characteristics, we divided the 74 patients into a negative SOX2 expression group and a positive SOX2 expression group; the results of statistical analysis are shown in Table 2. According to the results, we found that BC patients who expressed SOX2 at higher levels had lymph node metastasis ($P=0.009$), and higher pathological grade ($P=0.015$) and TNM stage ($P=0.002$), but no association was found with other clinicopathological factors, including age, T stage, ER status, Her-2 status or molecular typing ($P=0.687$, 0.874 , 0.763 , 0.08 and 0.125 , respectively). Additionally, we used q-PCR in order to investigate the correlation between the expression of miR-101 and SOX2. As shown in Fig. 6C, the expression of miR-101 and SOX2 were negative correlated in BC tissues. These results demonstrated that SOX2 functioned as an oncogene in BC and indicated that its expression was associated with a higher degree of malignancy and unfavorable prognoses.

Silencing SOX2 significantly inhibits BC cell growth, proliferation and migration, and enhance apoptosis

To further investigate the influence of SOX2 expression on the growth, proliferation and migration of BC cells, functional assays were performed in SOX2-silenced BC cells. Sh-SOX2 was transiently transfected into BC cells to knockdown SOX2 expression; sh-NC was used as a control (Fig. 7A). Using the MTT assay, we found that silencing SOX2 significantly suppressed BC cell viability (Fig. 7B). Using the colony formation assay, we similarly demonstrated that SOX2 downregulation decreased colony formation compared with the control group (Fig. 7C). Additionally, the effects of silencing SOX2 on BC cell migration were determined using wound healing and transwell migration assays. The results showed that SOX2-silenced BC cells had lower wound healing rates and poorer migration abilities through Matrigel (Fig. 7E and 7D). We next examined apoptosis by Annexin V staining and flow cytometry. As shown in Fig. 7F, SOX2-silenced BC cells had significantly increased apoptosis rates compared with the control group.

Fig. 7. SOX2 knockdown inhibits BC cell growth, proliferation and migration, and induces apoptosis. (A) SOX2 was silenced by shRNA in MCF-7 and MDA-MB-231 cells. (B) The influence of SOX2 downregulation on the viability of BC cells was determined using the MTT assay. (C) BC cells were transfected with sh-SOX2 or sh-NC and then subjected to a colony formation assay. The number of colonies in the sh-SOX2 group was significantly lower than the sh-NC group. (D) The ability of cells to invade through Matrigel was decreased in the sh-SOX2 groups compared with the control groups. (E) The effect of SOX2-knockdown on cell migration were determined using the wound healing assay. The wound healing rates of sh-SOX2 groups were lower than control groups. (F). SOX2 silencing induced apoptosis of BC cells. * $P < 0.05$. ** $P < 0.01$.

SOX2 overexpression mitigates the miR-101-induced inhibition of malignant BC phenotypes

To demonstrate the potential molecular mechanisms related to the miR-101/SOX2 regulation of BC cell growth, proliferation, migration and apoptosis, we performed MTT, colony formation, transwell migration, wound healing and apoptosis assays in cells transfected with miR-NC, miR-101, miR-101+NC or miR-101+SOX2. The results of these assays all indicated that SOX2 overexpression could reverse the effect of miR-101 overexpression (Fig. 8A–E). We also detected expression of the apoptosis-related proteins Bcl2, Bax and cleaved caspase3 by western blotting. The results showed that both miR-101 upregulation and SOX2 knockdown significantly reduced Bcl2 expression, while increasing Bax and cleaved caspase3 levels (Fig. 8F). Moreover, SOX2 overexpression mitigated these changes in Bcl2, Bax and cleaved caspase 3 levels that were induced by miR-101 upregulation (Fig. 8F).

Discussion

BC metastasis significantly increases patients' tumor burden and has become the major cause of BC-associated death [29]. BC metastasis is a process that involves local invasion of the surrounding tissues, dissemination into the blood or lymph systems and spread to

Fig. 8. miR-101 inhibits BC cell malignant phenotypes and induces apoptosis by directly targeting SOX2. (A) MTT assays were performed to show the viability of BC cells. (B). The relative proliferation of BC cells was assessed using colony formation assays. (C) Transwell assays were performed to show the ability of BC cells to invade through Matrigel. (D) Wound healing assays showed the migratory ability of BC cells. (E) BC apoptosis rates were detected by flow cytometry. (F) miR-101 overexpression and SOX2 down-regulation can both regulate the levels of Bcl2, Bax and cleaved caspase3. SOX2 overexpression can mitigate the changes in Bcl2, Bax and cleaved caspase 3 levels induced by miR-101 upregulation. * $P < 0.05$, ** $P < 0.01$.

distant organs [30, 31]. Over the past decade, many molecular and genetic mechanisms that contribute to this process have been discovered, but the potential influences of epigenetics on metastasis still contain many intricacies that remain to be unraveled. In recent years, growing literature suggests that microRNAs play important roles in tumor malignancy [32]. In BC, many metastasis-inhibiting microRNAs were identified including miR-302a, miR-124-3p, miR-93, miR-107, miR-144 and miR-203 [7, 33-37]. Meanwhile, miR-101 has also been reported to function as a tumor suppressor in BC [18, 19]. In this study, we found that miR-101 was significantly downregulated in BC tissues compared with the corresponding carcinoma-adjacent tissues and the low-level expression of miR-101 was related to bad clinical features. We also found reduced miR-101 expression in BC cell lines compared with a normal breast cell line. These results were consistent with the data from Li et al. and Liu et al [18, 19]. Furthermore, through *in vitro* MTT, colony formation, wound healing, transwell migration and apoptosis assays as well as an *in vivo* xenograft tumorigenicity assay, we demonstrated that miR-101 overexpression could significantly inhibit BC cell proliferation, migration and invasion and promote apoptosis. In addition, miR-101 downregulation could increase cell proliferation, migration and invasion and suppress apoptosis. These results confirmed that miR-101 was a tumor suppressive miRNA in BC. As miRNAs usually exert their control by targeting downstream genes, the identification of miR-101 downstream target genes played an important role in understanding its potential mechanism. Previous studies have reported that miR-101 targets COX2, c-FOS, CXCR7, SOX9, EZH2 and Rac1 [11-14, 18, 19, 38].

However, whether SOX2 was a miR-101 target in BC was unclear. Using miRanda database, we identified SOX2, whose 3'UTR possesses a miR-101 binding sites, as a potential miR-101 target.

In previous studies, SOX2 has been validated to function as an oncogene in various cancer types [22-25], and it was reported that SOX2 overexpression is prevalent in BC. What's more, SOX2 is activated in breast cancer stem cells, indicating that SOX2 is a tumor initiation marker of BC[39]. In this study, we showed that SOX2 was upregulated in BC tissues compared with carcinoma-adjacent tissues. Moreover, high SOX2 expression was associated with lymph node metastasis, pathological grade and TNM classification, confirming the oncogenic function of SOX2 in BC. What's more the expressions of SOX2 in BC tissues were negative correlated with miR-101.

Recent studies have shown that SOX2 may be regulated by several miRNAs. Xia et al. showed that miR-638 downregulation could increase SOX2 expression, promoting invasion and proliferation in non-small cell lung cancer [40]. Furthermore, Li et al. found that miR-429 exerted its anti-apoptotic function by targeting SOX2 [41]. SOX2 has also been reported to be regulated by miR-371 and miR-145 [42-44]. In this study, we found that miR-101 could specifically bind to the 3'UTR of SOX2 using Luciferase reporter assays. Additionally, miR-101 upregulation resulted in decreased SOX2 expression. Furthermore, the results of functional assays demonstrated that SOX2 knockdown had the same effects on BC cell proliferation, migration, invasion and apoptosis as miR-101 overexpression. More importantly, we found that SOX2 overexpression could reverse the inhibition of malignant phenotypes and promotion of apoptosis induced by miR-101 overexpression in BC cell lines, further demonstrating that miR-101 functions by directly targeting SOX2. In 2017, Li and his colleagues reported the potential mechanisms of high-level-SOX2-expression induced BC metastasis [45]. They found SOX2 is critical for the proliferation, migration and metastasis of BC via SOX2/microRNA/TUSC3 axis. It may provide new views for us to further explore the downstream targets of SOX2.

In summary, our study demonstrated that the expression of miR-101 in BC tissues and cell lines was notably lower than in carcinoma-adjacent tissues and normal breast cells. Moreover, miR-101 overexpression significantly inhibited the malignant phenotypes of BC cells, including proliferation, migration and invasion as well as inducing apoptosis by directly targeting SOX2. More importantly, high SOX2 expression was associated with poor clinical characteristics, including lymph node metastasis, pathological grade and TNM classification. Therefore, miR-101/SOX2 signaling functions as a tumor suppressor involved in BC pathogenesis that may be a potential therapeutic target. Due to the tissue samples from patients were collected from 2013 to 2016 and no deaths during follow up, further exploration of longer follow up time will be needed to analyze the relevant of miR-101/SOX2 to overall survival of BC patients. What's more, it was reported that in BC miRNAs could be regulated by mRNA or lnc RNA [46, 47]. Therefore, whether miR-101/SOX2 signaling could regulate other miRNAs or mRNAs, or might be regulated by other miRNAs or mRNAs which are involved in BC metastasis merits additional investigation. Additionally, it was reported that aberrant methylation and histone modification of miRNA, which are the most common epigenetic alterations in cancer cells, are common in many types of solid tumors and hematological malignancies, including breast cancer [48, 49]. Moreover it was reported that in MCF-7/Doc and MCF-7/Adr cells the expressions of a lot of miRNAs were changed, indicating abnormal expression of miRNA could contribute to chemoresistance [50]. But whether the abnormal expression of miR-101/SOX2 in BC may be controlled by epigenetic mechanisms and may contribute to BC chemoresistance needs to be further explored.

Conclusion

In conclusion, our study revealed that miR-101 plays a critical role in suppressing tumor progression by directly targeting SOX2 and miR-101/SOX2 signaling may be a potential therapeutic target for future BC treatments.

Ethical Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Abbreviations

BC (breast cancer); miRNAs (MicroRNAs); miR-101 (microRNA-101); SOX2 (Sex-determining region Y-box 2); qRT-PCR (quantitative reverse-transcription polymerase chain reaction); TNM (tumor-nodes metastasis); shRNA (small hairpin RNA); NC (negative control).

Acknowledgements

This work was supported by National Key Basic Research Program of China (grant number 2014CB744500). Thanks to all the staff of the Department of General Surgery, Medical Oncology and Medical Imaging at Jinling Hospital for technical support. Informed consent was obtained from all participants before conducting this study.

Disclosure Statement

The authors declare that they have no conflict of interest.

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