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LncRNA TSLNC8 inhibits proliferation of breast cancer cell through the miR-214-3p/FOXP2 axis

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Abstract. – OBJECTIVE: In multiple cancers, heterozygosity is frequently lost for the tumor-suppressive long noncoding RNA (lncRNA). The expression, function, and molecular mechanisms of tumor suppressive lncRNA on chromosome 8p12 (TSLNC8) in breast cancer are still unknown.

MATERIALS AND METHODS: QRT-PCR assays were carried out to evaluate the level of TSLNC8 in breast cancer tissues and cell lines. MTT, colony formation, and anchorage-independent growth assays were performed to investigate the effect of TSLNC8 on cell proliferation, and flow cytometry assays were conducted to detect cell percent of different phases. Luciferase reporter assays were used to confirm the interaction of different molecules.

RESULTS: TSLNC8 is significantly increased in breast cancer tissues and cell lines. Up-regulation of TSLNC8 reduces the proliferation capacity of breast cancer cells and the transition from G1 to S phase of the cell cycle. Further analysis indicated that TSLNC8 could directly bind to miR-214-3p. Up-regulation of miR-214-3p may attenuate the suppressive role of TSLNC8 on the proliferation capacity of breast cancer cells. Moreover, miR-214-3p was found to directly interact with the 3'-untranslated region (UTR) of Forkhead box P2 (FOXP2) in luciferase assays, suggesting that FOXP2 may be one of the downstream targets of miR-412-3p.

CONCLUSIONS: TSLNC8 was found to inhibit the proliferation and G1/S phase transition of breast cancer cells, an effect mediated by miR-214-3p/FOXP2 axis. Our study provides evidence that TSLNC8 may act as a suppressive lncRNA and represent a novel therapeutic target for breast cancer therapy.

Key Words:
TSLNC8, Proliferation, Cell cycle, FOXP2, MiR-214-3p.

Introduction

In 2018, breast cancer was evaluated as the most commonly diagnosed malignancy and the leading cause of cancer-related mortality in women, with 2.1 million newly diagnosed cases, accounting for almost 1 in 4 cancer cases among women[1]. In spite of the significant advances in early detection and use of personalized treatments, breast cancer represents a significant public health issue[2]. The detailed mechanisms that lead to breast cancer remain vague. In the absence of a clear understanding of the mechanisms underlying breast cancer, the identification and characterization of molecules involved in the tumorigenesis and progression of breast cancer are essential.

Cancer is known to alter physiological information flow, resulting in a disruption of cellular homeostasis and in enhanced cell proliferation. The findings of multiple genetic codes for coding proteins result in substantial breakthroughs on understanding how genetic mutations drive cancer. Besides, based on the above principles, therapy targeted the numerous coding protein mutations are clinically applied[3]. Only less than 2% of the genome is translated into proteins, and more than 75% of the genome is non-coding. Mutations within the non-coding genome, particularly those affecting lncRNAs, are thought to play crucial roles in both tumorigenesis and cancer progression[4-6]. LncRNAs are defined as transcripts of > 200 nucleotides with no protein-coding potential which are broadly related to cellular and biological processes. Dysregulation of lncRNAs has been implicated in tumorigenesis and cancer metastasis[7-9]. Several lncRNAs may represent biomarkers and therapeutic targets for cancers[10-12]. However, the expression and function of lncRNAs in breast cancer remain unclear. The tumor-suppressive lncRNA is located on chromosome 8p12 (TSLNC8), and its heterozygosity is known to be lost in multiple cancers. Zhang et al[13] showed TSLNC8 to be inhibited in Hepatocellular carcinoma (HCC), an effect that negatively correlated with prognosis of patients with HCC.

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Further analysis indicated that TSLNC8 inhibits tumorigenicity and metastasis by binding to transketolase and signal transducer and activator of transcription 3 (STAT3), and inhibiting the phosphorylation and transcription activity of STAT3, thus inactivating IL-6/STAT3 signaling in HCC[13]. Chen et al[14] found TSLNC8 to be down-regulated in glioma and its levels were found to be inversely related with tumor stage. In addition, TSLNC8 was found to promote the cleavage of caspase-3 and -9[14]. These results indicate that TSLNC8 may play an essential role in inhibiting HCC progression. However, the expression, function, and molecular mechanisms of TSLNC8 in breast cancer are unknown.

In the present study, we present evidence of significantly reduced levels of TSLNC8 in breast cancer tissues and cell lines. In addition, overexpression of TSLNC8 significantly inhibited the proliferation and G1/S transition of breast cancer cells, and TSLNC8 was found to directly bind to miR-214-3p, which in turn reduced levels of FOXP2 by targeting its 3'UTR. Our study thus provides evidence that TSLNC8 acts as a suppressive lncRNA and may represent a novel therapeutic target in breast cancer.

Materials and Methods

Cell Lines and Tissue Specimens

Normal breast epithelial cell (NBEC) lines were purchased from the China Center for Type Culture Collection (Wuhan, China). Breast cancer cell lines (MDA-MB-231, HCC1559, BT-549, UACC-812, and MDA-MB-453), were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, South-Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The breast cancer tissues and normal adjacent tissues were collected from Weihai Municipal Hospital (Weihai, Shandong, China). Informed consent was obtained from patients, and use of tissues was approved by the Institutional Ethics Committee.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was collected using TRIzol (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR kits (Invitrogen, Carlsbad, CA, USA). The expression of TSLNC8 and FOXP2 were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, and that of miR-214-3p to U6. The primers used for qRT-PCR are listed in Table I.

Vector and Lentivirus Infection

LncRNA TSLNC8 overexpression was achieved by amplifying full-length TSLNC8 from cDNA and cloning the construct into a lentiviral vector. Knock-down of lncRNA TSLNC8 was achieved by targeting TSLNC8 shRNA, which was cloned into vectors. Transfection of constructs was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Primer sequences and protocols used were previously described[13].

MTT Assays

Cells were seeded in 96-well plates. At the designated time point, cells were incubated with 100 μL of sterile MTT reagent (final concentration, 0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 4 h after which 150 μL of dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well to dissolve the precipitation formed by formazan. The absorbance was evaluated at 490 nm.

Colony Formation Assay

Cells were seeded into 6-well plates and maintained for 10 days. The colonies were fixed with 10% formaldehyde for 5 min, and subsequently dyed using 1% crystal violet for 30 s.

Table I. Primers used in this study.

Name	Forward primer	Reverse primer
TSLNC8	CACCTCCATTCAACCAATAAGC	ACCCTGTCCCCAATAACCC
miR-214-3p	GCATCCTGCCTCCACATGCAT	GCGCTGAGGAATAATAGAGTATGTAT
FOXP2	TGGATGACCGAAGCACTGCTCA	TGGGAGATGGTTTGGGCTCTGA
U6	TGACTTCCAAGTACCATCGCCA	TTGTAGAGGTAGGTGTGCAGCAT
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

Anchorage-Independent Growth Ability Assay

Plates were coated with complete medium containing 1% agar. The complex containing 500 cells and 2 mL complete medium including 0.3% agar were added into the layer of the bottom mixture. Ten days later, the colonies with a diameter > 0.1 mm were counted.

Flow Cytometry Assay

Cells were harvested, treated with 75% ethanol, and with 20 μg/mL RNase A and 50 μg/mL propidium. Cells were then incubated at 37°C in the dark for 30 min, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle phases were evaluated using Cell Quest 3.3 software.

Luciferase Reporter Assay

A total of 1×10^3 cells was seeded into each well of 48-well plates and maintained in a humid incubator for 24 h. Luciferase reporter plasmids (100 ng) and *Renilla* plasmids (10 ng) were transfected into cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The luciferase and *Renilla* signals were detected using a Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted using the software package SPSS 22.0 (IBM, Armonk, NY, USA). Two-tailed paired Student's *t*-tests were used to evaluate the statistical significance (i.e., with $p < 0.05$) between groups. Bivariate correlation was examined using Spearman's rank correlation coefficients. All experiments were repeated at least three times.

Results

TSLNC8 Is Significantly Reduced in Breast Cancer Tissues and Cell Lines

We first evaluated TSLNC8 expression in 10 pairs of breast cancer tissue (Tumor) and normal adjacent tissue (ANT) using qRT-PCR. The results indicate that relative TSLNC8 expression is much lower in breast cancer tissue compared to adjacent normal tissue (Figure 1A). We also evaluated TSLNC8 expression in breast cancer cell lines and normal breast epithelial cells (NBEC), and found a dramatic inhibition of TSLNC8 in breast cancer cell lines compared with normal cells (Figure 1B). Altogether, we found that TSLNC8 is significantly decreased in breast cancer tissues and cell lines.

TSLNC8 Suppresses the Proliferation Capacity of Breast Cancer Cells

To explore the function of TSLNC8 in breast cancer, we examined the correlation between the levels of TSLNC8 and Ki-67, a proliferative marker, using breast cancer tissue specimens.

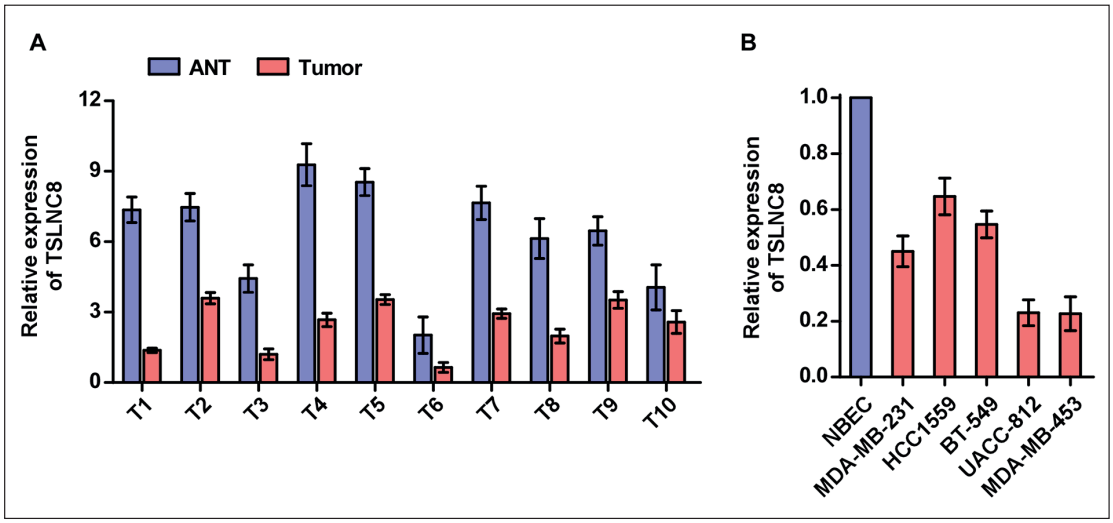

Figure 1. LncRNA TSLNC8 is significantly decreased in breast cancer tissues and cells. **A**, qPCR assay showed that TSLNC8 is significantly down-regulated in breast cancer tissues (Tumor) compared with the normal adjacent tissues (ANT). **B**, qPCR showed significant reduction in TSLNC8 expression in breast cancer cells compared with normal breast epithelial cell (NBEC).

TSLNC8 expression was found to be negatively correlated with Ki-67 expression (Figure 2A), suggesting a possible involvement of TSLNC8 in breast cancer cell proliferation. Subsequently, we screened stable cells in which TSLNC8 was up-regulated or down-regulated using MDA-MB-231 that expresses average levels of TSLNC8 (Figure 2B). MTT and colony formation assays indicated that up-regulation of TSLNC8 significantly inhibited the proliferation of breast cancer cells, while its down-regulation significantly increased their proliferation (Figure 2C and 2D). We investigated the capacity of *in vitro* tumorigenicity using an anchorage-independent growth assay. The size and number of colonies were significantly decreased in TSLNC8-overexpressing cells, while the results were opposite in cells in which TSLNC8 was silenced

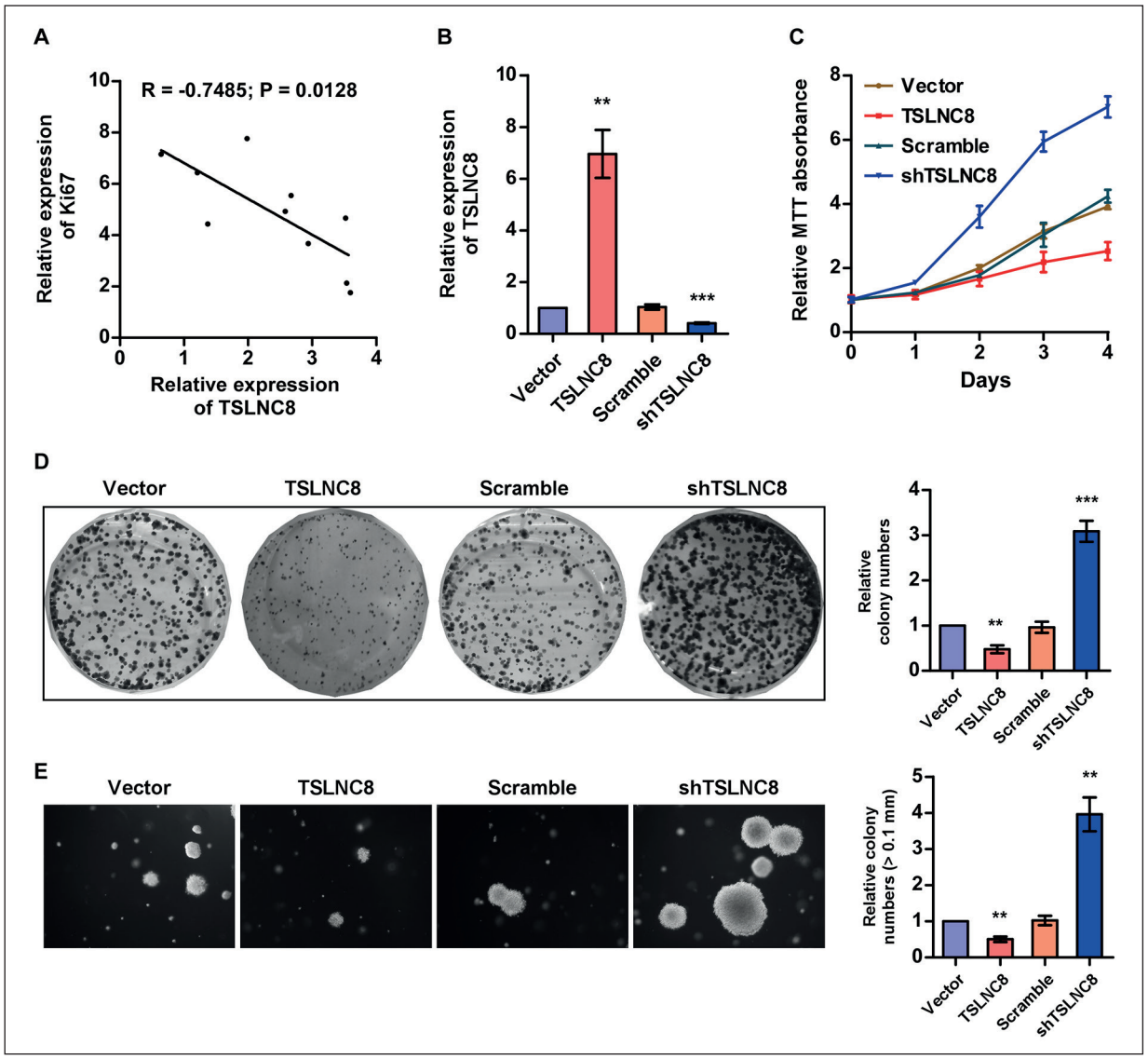

Figure 2. Up-regulation of lncRNA TSLNC8 can inhibit the proliferation of breast cancer cells. **A**, Correlation analysis between TSLNC8 and Ki67 in breast cancer tissues. **B**, Relative expression of TSLNC8 in indicated stable cell lines. **C**, MTT assay showed that overexpression of TSLNC8 (TSLNC8 condition) significantly decreased, while TSLNC8 down-regulation with shTSLNC8increased the proliferation of breast cancer cells. **D**, Colony formation experiments indicated that overexpression of TSLNC8 significantly decreased the proliferation of breast cancer cells, while its down-regulation increased their proliferation (magnification: 100×). **E**, Anchorage-independent growth ability assay indicated that the colonies had increased size and number in TSLNC8-silenced cells, while decreased in TSLNC8-overexpressed cells (magnification: 100×). *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

(Figure 3E). These results suggest that TSLNC8 inhibits the tumorigenic ability of breast cancer cells *in vitro*.

TSLNC8 Inhibits the Transition of G1/S Phase in Breast Cancer Cells

To further clarify the mechanisms of TSLNC8-mediated proliferation, the flow cytometer assays were carried out. Overexpression of TSLNC8 significantly increased the percentage of cells in G0/G1-phase, and reduced the percentage of cells in S-phase. Conversely, down-regulation of TSLNC8 significantly decreased the percentage of cells in G0/G1-phase, and increased the percentage of cells in S-phase (Figure 3). Altogether, results from flow cytometric assays indicate that TSLNC8 may inhibit the G1/S phase transition in breast cancer cells.

LncRNA TSLNC8 is Targeted by MiR-214-3p

Several lncRNAs have been reported to regulate cancer progression via competing endogenous RNAs (ceRNAs)[15-17]. To examine the role of TSLNC8 in proliferation of breast cancer and the possible involvement of ceRNAs, we predicted potential miRNA binding sites on lncRNA TSLNC8 using RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/). The prediction analysis suggested that two miRNAs, miR-214-3p (Figure 4A), and miR-4647, may target TSLCN8. There is evidence[18-20] of dysregulation of miR-214-3p in several cancers. In breast cancer, the up-regulation of miR-214-3p was found to be beneficial to osteolytic bone metastasis, suggesting that miR-214-3p may play an essential role in the progression of breast cancer. Luciferase reporter assays indicated that up-regulation and knock-down of miR-214-3p suppress and elevate, respectively, the transcription activity of wild-type lncRNA TSLNC8. However, up- or down-regulation of miR-214-3p was not found to modify luciferase reporter activity of mutant lncRNA TSLNC8 (Figure 4B), suggesting that miR-214-3p may directly interact with TSLNC8. Moreover, we evaluated the level of miR-214-3p in breast cancer tissues (Tumor) and corresponding adjacent normal tissues (ANT) with qRT-PCR. Here, miR-214-3p levels were found to be aberrantly increased in cancer tissues, in contrast with TSLNC8 levels (Figure 4C). In addition, correlation analysis indicated that miR-214-3p expression levels were inversely correlated with TSLNC8 expression levels in breast cancer tissues (Figure 4D). MiR-214-3p was found to be significantly up-regulated in breast cancer cells relative to normal cells (NBEC; Figure 4E). Overexpression of TSLNC8 was found to inhibit miR-214-3p expression, and down-regulation of TSLNC8 led to increased levels of miR-214-3p (Figure 4F).

Moreover, MTT, colony formation, and anchorage-independent growth assays indicated that miR-214-3p can counteract the suppressive effect of TSLNC8 on the proliferation of breast cancer cells (Figure 5A-C). The percentage of cells in G0/G1-phase in cells transfected with TSLNC8 was found to be significantly increased compared with cells in which TSLNC8 was overexpressed in the absence of miR-214-3p (Figure 5D). Altogether, our results demonstrated that TSLNC8 can directly target miR-214-3p, and miR-214-3p can counteract the suppressive effect of TSLNC8 on the proliferation of breast cancer cells.

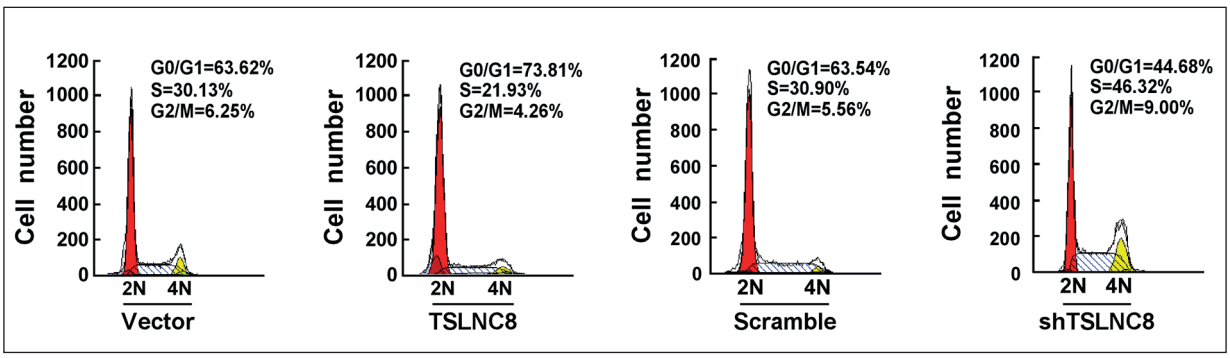

Figure 3. Up-regulation of lncRNA TSLNC8 can inhibit the transition of G1/S phase in breast cancer cells.

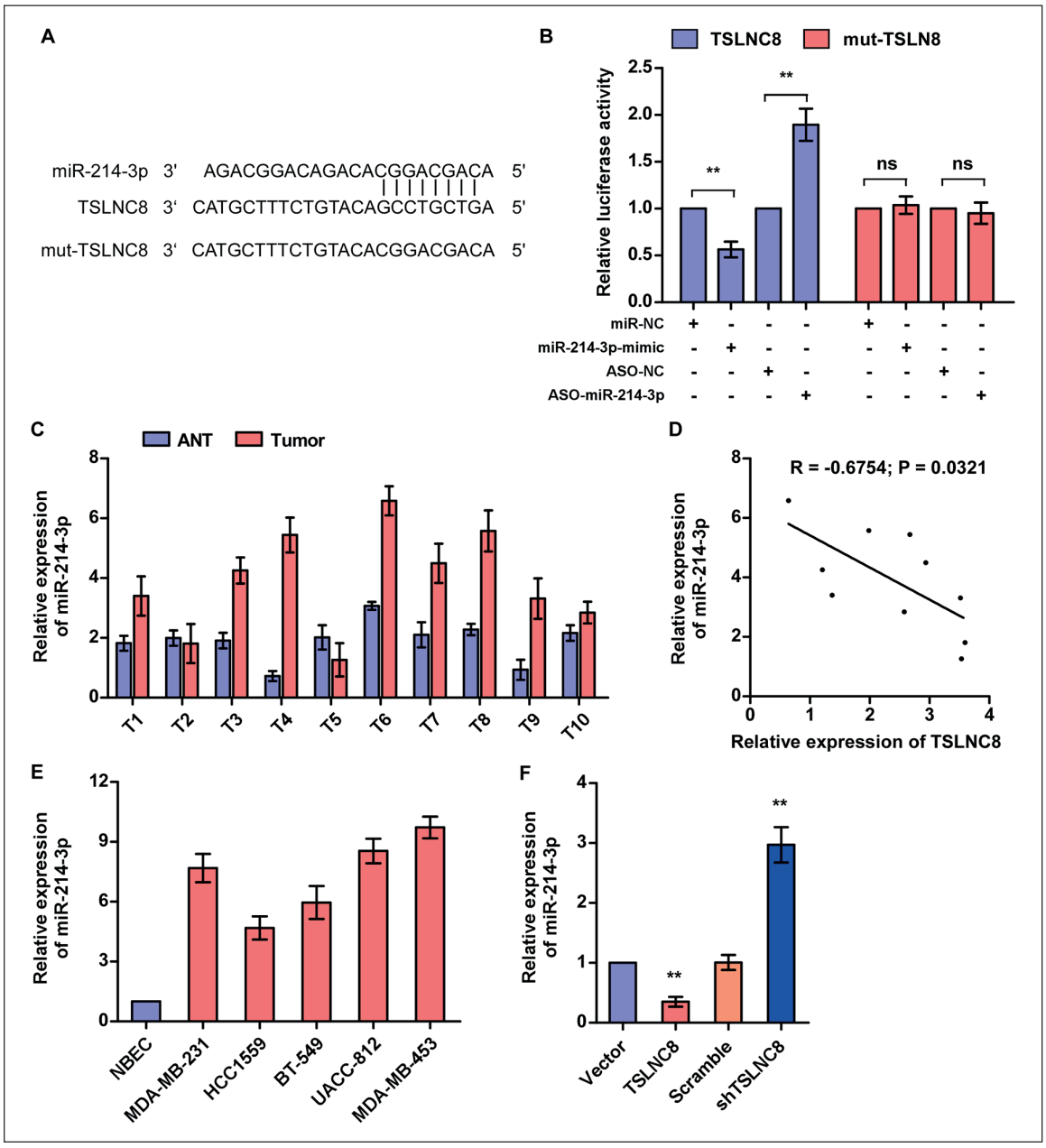

Figure 4. TSLNC8 can be a direct target of miR-214-3p. **A**, Binding sites of miR-214-3p on TSLNC8 were predicted. **B**, Luciferase reporter assays showed that miR-214-3p can directly target with the wild-type TSLNC8, but can't bind with the mutant TSLNC8. **C**, Relative expression of miR-214-3p in breast cancer tissues (Tumor) and corresponding adjacent normal tissues (ANT) by qPCR assay. **D**, Correlation assay demonstrated that the level of miR-214-3p is negatively related with the expression of TSLNC8 in breast cancer tissues. **E**, Level of miR-214-3p is significantly increased in breast cancer cells compared with NBEC. **F**, Relative expression of miR-214-3p in indicated cells. ns: no significant; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$. miR-NC: miRNA negative control; ASO-NC: negative control antisense oligonucleotides; ASO-miR-214-3p: antisense oligonucleotides against miR-214-3p.

FOXP2 Mediates the Suppressive Role of TSLNC8 in Breast Cancer Cells

We further investigated the downstream targets of miR-214-3p. A publicly available algorithm (http://www.microrna.org/microrna/home.do) predicted that FOXP2 may be a possible target of miR-214-3p (Figure 6A). Luciferase reporter assay showed that the up-regulation of miR-214-3p may attenuate the transcription activity of TSLNC8 driven by the wild-type 3'UTR

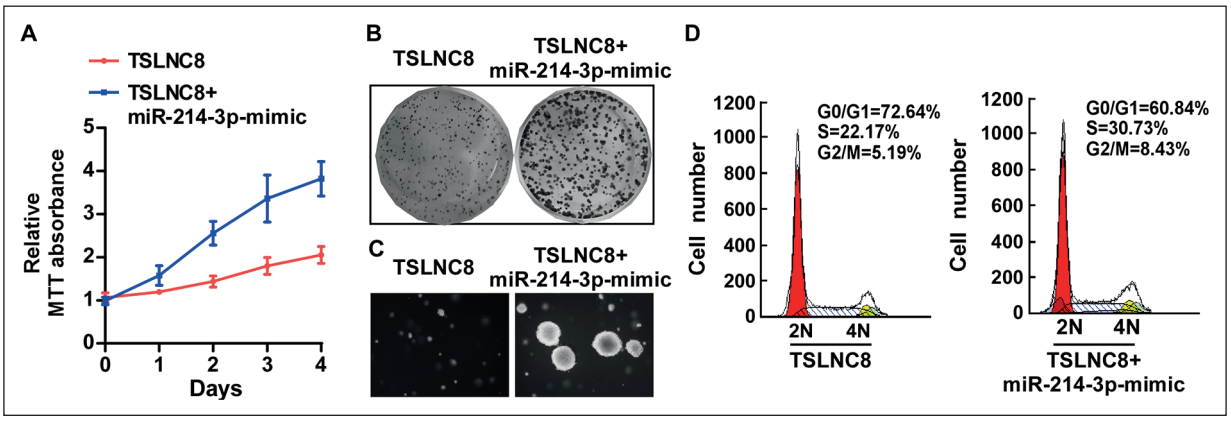

Figure 5. miR-214-3p can attenuate the inhibitory effect of TSLNC8 on proliferation and G1/S phase transition in breast cancer cells. **A**, MTT assay showed that miR-214-3p can counteract suppressive effect of TSLNC8 on proliferation of breast cancer cells. **B**, Colony formation assay showed that miR-214-3p can counteract inhibitory effect of TSLNC8 on proliferation of breast cancer cells (magnification:100×). **C**, Anchorage-independent growth assay showed the miR-214-3p can significantly promote size and number of colonies in TSLNC8-upregalated cells (magnification:100×). **D**, Flow cytometry assay showed that miR-214-3p can significantly promote the G1/S transition of cell cycle in TSLNC8-upregalated cells.

of FOXP2. Up-regulation of miR-214-3p was not found to affect the transcription activity of a mutant 3'UTR of FOXP2 (Figure 6B). FOXP2 expression was found to be significantly inhibited in TSLNC8-overexpressing cells, and to be increased in TSLNC8-silenced cells (Figure 6C). Altogether, our results demonstrated that lncRNA TSLNC8 inhibits proliferation of breast cancer cells through the TSLNC8/miR-214-3p/FOXP2 axis.

Discussion

In the present research, we found that the expression of lncRNA TSLNC8 is significantly reduced in breast cancer tissues and cell lines. Overexpression of TSLNC8 was found to suppress the proliferation capacity of breast cancer cells, a phenomenon mediated through molecular interactions between miR-214-3p and FOXP2. LncRNA TSLNC8 expression was previously

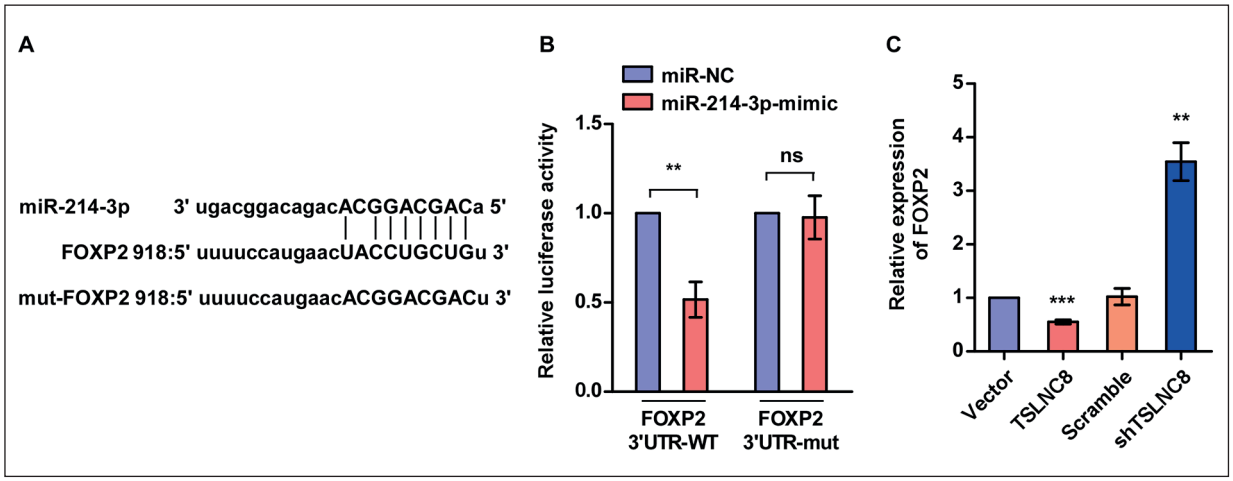

Figure 6. miR-214-3p can directly target with the 3'UTR of FOXP2. **A**, Binding sites of miR-214-3p on 3'UTR of FOXP2 were predicted. **B**, Luciferase reporter assay showed that miR-214-3p can directly target with the wild-type 3'UTR of FOXP2, but can't bind with mutant 3'UTR of FOXP2. **C**, Relative expression of FOXP2 in indicated stable cell lines. ns: no significant; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

found to be reduced in HCC and human glioma, a down-regulation that may promote cancer cell proliferation and metastasis[13-14]. Our results indicate that lncRNA TSLNC8 is significantly decreased in breast cancer, a down-regulation that was associated with a significant increase in the proliferation capacity of breast cancer cells. Whether TSLNC8 is involved in regulating the metastasis of breast cancer should be the topic of future studies. We also found that TSLNC8 may directly target miR-214-3p, and that miR-214-3p may bind to the 3'UTR of FOXP2. FOXP2 is a transcription factor and was identified as an important regulator in speech and language development, as well as developmental neurogenesis[21-23]. Several studies[24-27] have implicated FOXP2 as a transcriptional repressor, and it was found to be down-regulated in several cancers, including breast cancer. Other studies[28,29] indicated that FOXP2 was modulated by a number of miRNAs in cancer cells. Such results are in accordance with the current study in which we showed that miR-214-3p can directly bind to the 3'UTR of FOXP2, thus down-regulating its expression. Several characteristics of lncRNA TSLNC8 present the molecule as an attractive therapeutic target: its levels are much lower compared with protein-coding genes, which may be attributed to expression of lncRNAs only in some special subpopulation of cells[30]. This pattern of lncRNAs expression offers a unique mean for lncRNA-targeting therapies[31].

Conclusions

In summary, lncRNA TSLNC8 may represent an ideal therapeutic target for breast cancer, which needs more evidence.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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