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Long Noncoding RNA FOXC2-AS1 Predicts Poor Survival in Breast Cancer Patients and Promotes Cell Proliferation

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Breast cancer (BC) is the most common malignant tumor in women. Recently, long noncoding RNAs (lncRNAs) have been proposed as critical regulators in biological processes, including tumorigenesis. FOXC2-AS1, a single antisense oligonucleotide RNA transcribed from the negative strand of forkhead box protein C2 (FOXC2), has been identified as an oncogene in osteosarcoma. In the present study, we investigated the prognosis value and biological role of FOXC2-AS1 in BC. Our findings revealed that FOXC2-AS1 was significantly increased in BC tissues and cell lines, and Kaplan–Meier survival analysis indicated that a high level of FOXC2-AS1 was associated with poor prognosis of BC patients. Loss of function revealed that silenced FOXC2-AS1 significantly suppressed the proliferation ability, and flow cytometric analysis illustrated the influence of FOXC2-AS1 on cell cycle and apoptosis rate. Finally, we found that cyclin D1, cyclin D2, and cyclin D3 were all partly positively modulated by FOXC2-AS1 in BC. Collectively, FOXC2-AS1 may serve as a promising prognostic biomarker and therapeutic target for BC patients.

Key words: Breast cancer (BC); FOXC2-AS1; Prognosis; Proliferation

INTRODUCTION

Breast cancer (BC) is the most common malignant tumor in women[1,2]. Despite recent advances in multimodality therapies, the prognosis remains dismal. The pathogenesis and progression of BC are a long process involving activation of oncogenes and/or inactivation of tumor suppressor genes. Therefore, investigating in more detail the molecular mechanisms underlying BC development and progression will be helpful in exploiting useful prognostic biomarkers and therapeutic targets for BC therapy.

Long noncoding RNAs (lncRNAs), a newly discovered class of ncRNAs with length longer than 200 nt, have been identified as being widely involved in multiple physiological and pathological processes acting as enhancers, scaffolds, decoys, and guides in gene regulation[3–6]. Many studies have demonstrated the functional role of lncRNAs in tumorigenesis. For instance, Lai et al. reported that lncRNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation[7]. The lncRNA MEG3 inhibits cell proliferation of endometrial carcinoma by repressing Notch signaling[8], while the lncRNA H19 competitively binds miR-17-5p to regulate YES1 expression in thyroid cancer[9]. Additionally, many novel lncRNAs have been identified as dysregulated in cancers, such as CASC2, SPRY4-IT1, DUXAP8, and AK126698[10–13]. Despite the numerous lncRNAs that have been reported in cancers, the biological role of lncRNAs in BC still needs to be investigated. FOXC2-AS1, a single antisense oligonucleotide RNA transcribed from the negative strand of forkhead box protein C2 (FOXC2), has been shown to be upregulated in doxorubicin-resistant osteosarcoma cell lines and tissues, and the level correlated with poor prognosis[14]. However, its expression level and biological function in BC have not been studied.

In our present study, the expression levels of FOXC2-AS1 in human BC tissues and adjacent normal tissues were examined by quantitative real-time PCR (qRT-PCR), and the correlation between FOXC2-AS1 dysregulation and clinical characteristics and prognosis were analyzed. Moreover, the functional assay indicates that FOXC2-AS1 promoted BC cell proliferation through influencing cell cycle and apoptosis.

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MATERIALS AND METHODS

Clinical Samples

A total of 56 pairs of BC tissue and matched adjacent normal tissue specimens were collected from patients who underwent surgery between 2013 and 2015 in the Department of Breast Surgery at the Affiliated Hospital of Guizhou Medical University. The fresh tissue specimens were collected and immediately placed in liquid nitrogen until use. None of the patients recruited in this study had undergone preoperative chemotherapy or radiotherapy. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Guizhou Medical University. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Cell Culture and Transfection

Normal human breast epithelial cell line MCF-10A and BC cell lines MDA-MB-468, MDA-MB-231, MDA-MB-436, and MCF-7 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All of the cells were cultured in RPMI-1640 medium (Life Technologies, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) in a 5% CO_2 incubator at 37°C. FOXC2-AS1-siRNA or control-siRNA (synthesized by Shanghai GenePharma Co., Ltd., Shanghai, P.R. China) were transfected into the MDA-MB-231 and MCF-7 cells according to the manufacturer's instructions. Also, for knockdown of FOXC2-AS1, shRNA targeting FOXC2-AS1 was constructed in U6/GFP/Neo plasmids, and a nontarget sequence served as its negative control (NC; GenePharma). The transfection efficiency was harvested after about 2 days. All transfections were accomplished using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from BC specimens or BC cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized from 200 ng of extracted total RNA using the PrimeScript RT Reagent Kit (Takara Bio Company, Shiga, Japan) and amplified by qRT-PCR with an SYBR Green Kit (Takara Bio Company) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the housekeeping gene GAPDH as an internal control. The $2^{-\ Ct}$ method was used to determine the relative quantification of gene expression levels. The primer sequences were synthesized by RiboBio (Guangzhou, P.R. China) as follows: FOXC2-AS1, 5 -TTCATCGGCTGCGTATTCG-3 (forward) and 5 -TTGCCTTCTAGTCGCCTCC-3 (reverse); GAPDH, 5 -CATGAGAAGTATGACAACAGCCT-3 (forward) and 5 -AGTCCTTCCACGATACCAAAGT-3 (reverse). Each experiment was performed in triplicate.

Relative Cell Viability

Cells (3 10^3–6 10^3) were inoculated in a 96-well plate (200 µl per well, six repeated wells) at 37°C, 5% CO_2 for 24–72 h, and 20 µl of MTT solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added into each well. After 4 h of incubation at 37°C and 5% CO_2, the incubation was terminated, and the culture medium was discarded. DMSO (150 µl; Sigma-Aldrich) was added to each well and gently shaken for 10 min to promote crystallization dissolution. Absorbance values (OD) were determined with an enzyme-linked immunosorbent detector at 12, 24, 48, 72, and 96 h. The experiment was performed in triplicate.

Colony Formation Assay

Cells (500 cells/well) were plated in six-well plates and incubated in RPMI-1640 with 10% FBS at 37°C. Two weeks later, the cells were fixed and stained with 0.1% crystal violet. The number of visible colonies was counted manually.

Flow Cytometric Analysis of Apoptosis

Cells transfected with indicated plasmid or NC were reaped after 48 h. Apoptosis analysis was performed by flow cytometry using Annexin-V/FITC Apoptosis Detection Kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. All samples were assayed in triplicate.

Flow Cytometric Analysis of Cell Cycle Distribution

Cells were collected directly or 48 h after transfection, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight at −20°C. Fixed cells were rehydrated in PBS for 10 min and incubated in RNase A (1 mg/ml) for 30 min at 37°C. The cells were then subjected to PI/RNase staining followed by flow cytometric analysis using a FACScan instrument (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson, San Jose, CA, USA) as described previously[15].

Western Blotting Assay

Cells were suspended and then lysed in RIPA buffer (Beyotime, Beijing, P.R. China), which was supplemented with protease inhibitor cocktail (Sigma-Aldrich). Extraction of protein was made by SDS-PAGE, and the protein was transferred to a PVDF membrane (Millipore, Boston, MA, USA). The membrane was blocked with 5% (w/v) nonfat milk (Cell Signaling Technology, Danvers,

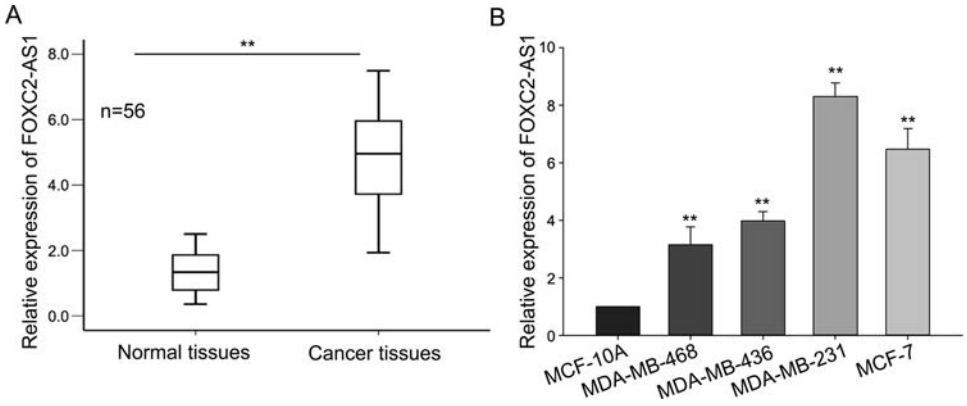

Figure 1. FOXC2-AS1 was significantly upregulated in breast cancer (BC) tissues and cell lines. The level of FOXC2-AS1 in BC tissues and cell lines was measured by quantitative real-time PCR (qRT-PCR). Mean ± standard deviation (SD) shown. $^{**}p < 0.01$.

MA, USA). Next, the membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with a secondary antibody. In order to visualize the protein bands, Clarity™ Western ECL substrate (Bio-Rad, Hercules, CA, USA) was employed. The level of protein was quantified using ImageJ software and normalized with GAPDH.

Statistical Analyses

SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were presented as means ± standard deviation (SD). The Pearson chi-square test was used to evaluate the links between FOXC2-AS1 expression and clinicopathological factors. The differences between two groups were analyzed by Student's *t*-test. One-way ANOVA was performed when multiple comparisons were made. Survival analysis was performed using the Kaplan–Meier method, and the log-rank test was used to compare the differences between patient groups. Cox proportional hazards regression model was generated to identify factors associated with overall survival through a multivariate survival analysis of BC. A value of $p < 0.05$ indicated statistical significance.

RESULTS

FOXC2-AS1 Was Significantly Upregulated in BC Tissues and Cell Lines

In order to clarify the biological role of FOXC2-AS1 in BC, we first determined the level of FOXC2-AS1 in 56 BC tissues and corresponding normal tissues. As demonstrated in Figure 1A, the level of FOXC2-AS1 was significantly upregulated in BC tissues, compared with that in matched adjacent normal tissue. Moreover, we measured the level of FOXC2-AS1 in four BC cell lines (MDA-MB-468, MDA-MB-436, MDA-MB-231, and MCF-7) and a normal human breast cell line (MCF-10A). As shown in Figure 1B, compared with the normal human breast cell line, FOXC2-AS1 was obviously increased in BC cell lines, especially in MDA-MB-231 and MCF-7 cells, which were chosen for the subsequent assays. These findings indicated that FOXC2-AS1 might act as an oncogene in BC.

The Correlation Between FOXC2-AS1 and Prognosis in BC Patients

Subsequently, the association between FOXC2-AS1 expression and the clinicopathological features of BC

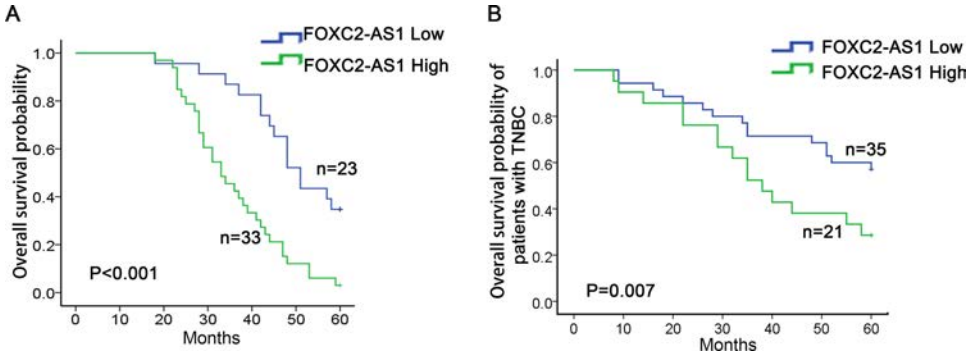

Figure 2. Kaplan–Meier method analysis (log-rank test) was employed to analyze the correlation between FOXC2-AS1 level and overall survival of BC patients.

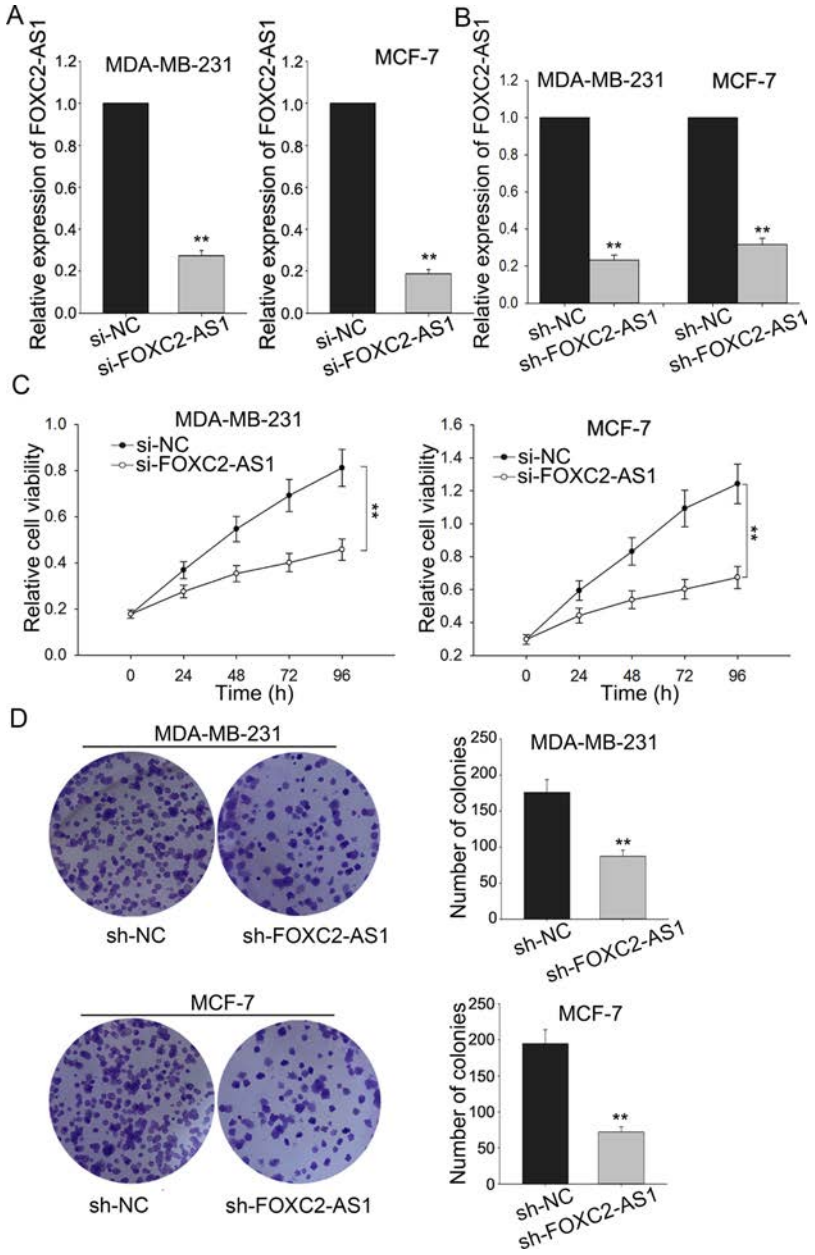

Figure 3. Knockdown of FOXC2-AS1 impaired BC cell proliferation ability. (A) MDA-MB-231 and MCF-7 cells were transfected with si-FOXC2-AS1 and si-NC. Transfection efficiency was measured by qRT-PCR after 48 h. (B) sh-FOXC2-AS1 or a control shRNA (sh-NC) was transfected into these two BC cell lines. Transfection efficiency was obtained after 48 h. The effect of FOXC2-AS1 knockdown on cell proliferation was measured by (C) MTT and (D) colony formation assays. Mean ± SD shown. $^{**}p < 0.01$.

was analyzed. By statistical analyses, we found that FOXC2-AS1 expression was significantly correlated with differentiation grade ($p = 0.030$), lymph node metastases ($p = 0.029$), Her-2/neu status ($p = 0.006$), and TNM stage ($p = 0.029$) of BC patients, but was not associated with other clinicopathological factors of BC patients, including age, tumor size, estrogen receptor (ER), as well as progesterone receptor (PR) ($p > 0.05$). These data indicated that upregulation of FOXC2-AS1 might play a critical role in BC progression. In addition, association between FOXC2-AS1 expression and clinicopathological features of triple-negative breast cancer (TNBC) was also statistically analyzed. The results of the analysis revealed that FOXC2-AS1 expression was significantly correlated with lymph metastasis ($p = 0.029$) and TNM stage ($p = 0.006$), but not correlated with age, gender, and other elements ($p > 0.05$). Proportional hazards method analysis revealed that a high level of FOXC2-AS1 could act as

an independent prognostic factor ($p = 0.028$). The same result was obtained in TNBC ($p = 0.007$).

Using the median relative expression of FOXC2-AS1 as the threshold, BC samples were divided into a high-expression group and a low-expression group. Kaplan–Meier method analysis (log-rank test) was performed to analyze the correlation between the FOXC2-AS1 level and overall survival of BC patients ($p < 0.001$) (Fig. 2A). Similarly, the correlation between FOXC2-AS1 expression and overall survival of TNBC patients was analyzed ($p = 0.007$) (Fig. 2B). These results suggested that FOXC2-AS1 might be proposed as a potential biomarker for poor prognosis of BC and TNBC.

Knockdown of FOXC2-AS1 Impaired BC Cell Proliferation Ability

To study the effects of FOXC2-AS1 on BC cell proliferation, we performed MTT and colony formation assays. Since MDA-MB-231 and MCF-7 cells exhibited high endogenous FOXC2-AS1 expression, we transfected the FOXC2-AS1-specific siRNA or a control vector (si-NC) into both cell lines to silence the level of FOXC2-AS1 (Fig. 3A). In order to demonstrate sufficient knockdown, we also stably transfected BC cells with FOXC2-AS1 shRNA or a control shRNA, and transfection efficiency was obtained after 48 h (Fig. 3B). MTT assays revealed that FOXC2-AS1 silencing significantly decreased the relative cell viability (Fig. 3C). Similarly, results from the colony formation assays demonstrated that proliferation of BC cells was reduced by transfecting shFOXC2-AS1 (Fig. 3D). These results indicated that silencing of FOXC2-AS1 could significantly inhibit BC cell proliferation.

Silenced FOXC2-AS1 Caused Cell Cycle Arrest and Induced Apoptosis

To explore the growth inhibition mechanism mediated by FOXC2-AS1 knockdown, flow cytometric analysis of apoptosis and the cell cycle was performed. As depicted in Figure 4A, deletion of FOXC2-AS1 in

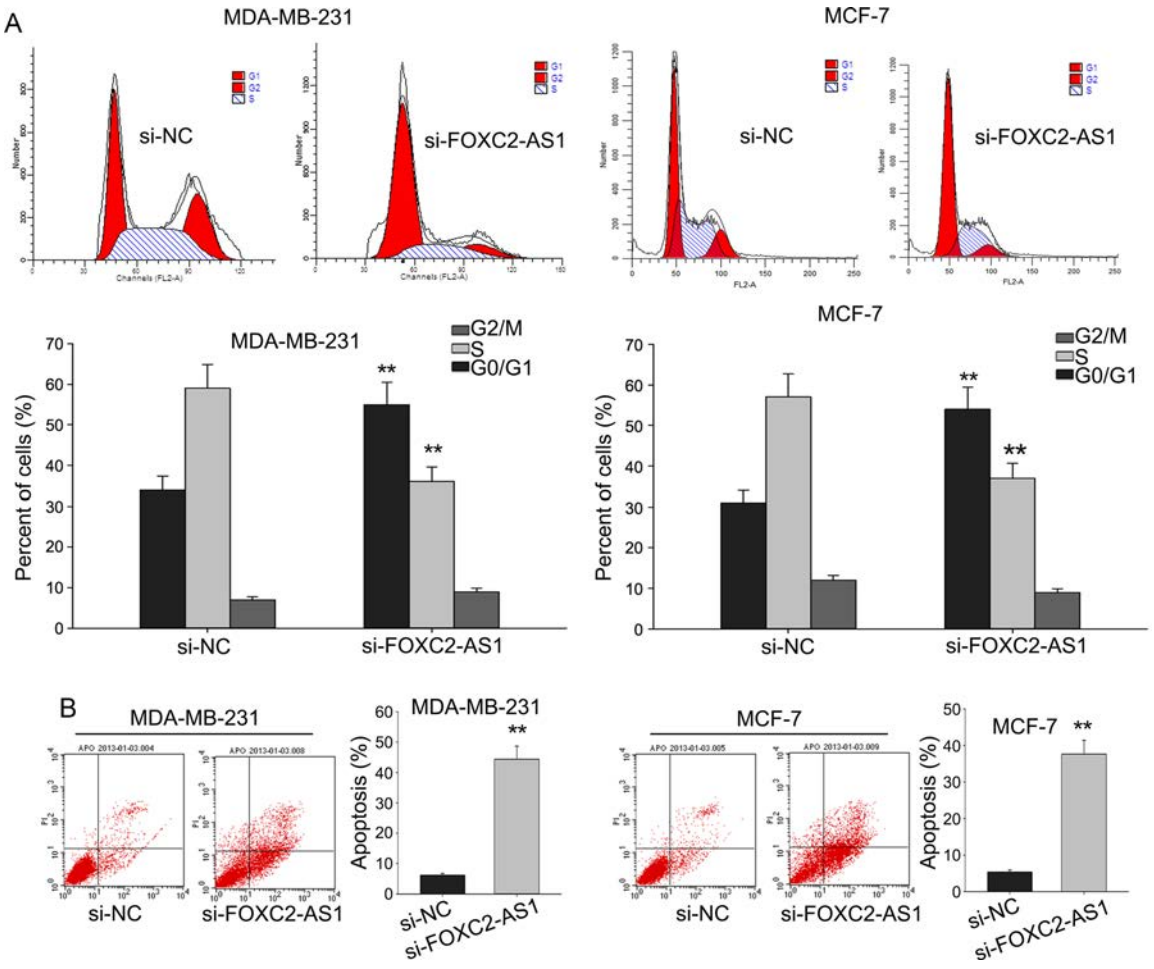

Figure 4. Silenced FOXC2-AS1 caused cell cycle arrest and induced apoptosis. The effect of FOXC2-AS1 silencing on (A) cell cycle and (B) apoptosis rate was analyzed by flow cytometric analysis. Mean ± SD shown. $^{**}p < 0.01$.

MDA-MB-231 and MCF-7 cells led to significant cell cycle arrest at G_1. Additionally, flow cytometric analysis of apoptosis uncovered that silenced FOXC2-AS1 significantly enhanced the apoptosis rate of MDA-MB-231 and MCF-7 cells (Fig. 4B). These data demonstrated that knockdown of FOXC2-AS1 mediated growth inhibition, and this might be partially due to its effect on the cell cycle and apoptosis.

Knockdown of FOXC2-AS1 Negatively Modulated Proteins Related With the Cell Cycle in BC Cells

It has previously been demonstrated that cyclin D1 is closely associated with molecular tumorigenesis of gastric cancer[16]. The cyclin family appears to be significant in the progression of cancers. Therefore, we speculated that FOXC2-AS1 could interact with cyclin D1, cyclin D2, and cyclin D3 to promote cell activities in BC. qRT-PCR analysis revealed that high mRNA levels of cyclin D1, cyclin D2, and cyclin D3 could be weakened by transfection of si-FOXC2-AS1 (Fig. 5A). The protein levels of these three proteins in BC cells were detected by Western blot analysis. The result indicated that the protein level of these three proteins was largely reduced by si-FOXC2-AS1 when compared with si-NC (Fig. 5B). According to the above results, we could conclude that knockdown of FOXC2-AS1 negatively modulates cyclin D1, cyclin D2, and cyclin D3 in BC cells to improve the progress of BC.

DISCUSSION

BC is the most common primary malignant tumor in women[17,18]. Despite extensive efforts, including chemotherapy, surgery, and radiotherapy, that have improved the diagnosis and treatment of BC, limited progress has been made, and the prognosis still remains unsatisfactory[19–21]. Currently, the detailed mechanisms of BC tumorigenesis remain largely unknown. Therefore, a better understanding of the underlying molecular mechanisms of the development and progression of BC is urgently required for improving BC treatment.

lncRNAs are emerging as valuable novel molecules, and they have been identified as both oncogenes and tumor suppressors involved in tumorigenesis[22–25]. Also, many lncRNAs are associated with the progression of BC. For example, Wu et al.[26] demonstrated that CCAT2 promotes BC growth and metastasis by regulating the TGF- signaling pathway. Deng et al.[27] reported that LINC00978 predicts poor prognosis in BC patients, and SNHG16 was identified to contribute to BC cell migration by competitively binding miR-98 with E2F5[28]. Despite so many lncRNAs being investigated recently, there are still many unknown lncRNAs that need to be uncovered. FOXC2-AS1 (Gene ID: 103752587), a single antisense oligonucleotide RNA transcribed from the negative strand of FOXC2, has been demonstrated to be dysregulated in osteosarcoma[14]. However, its expression level and biological function in BC have not been studied.

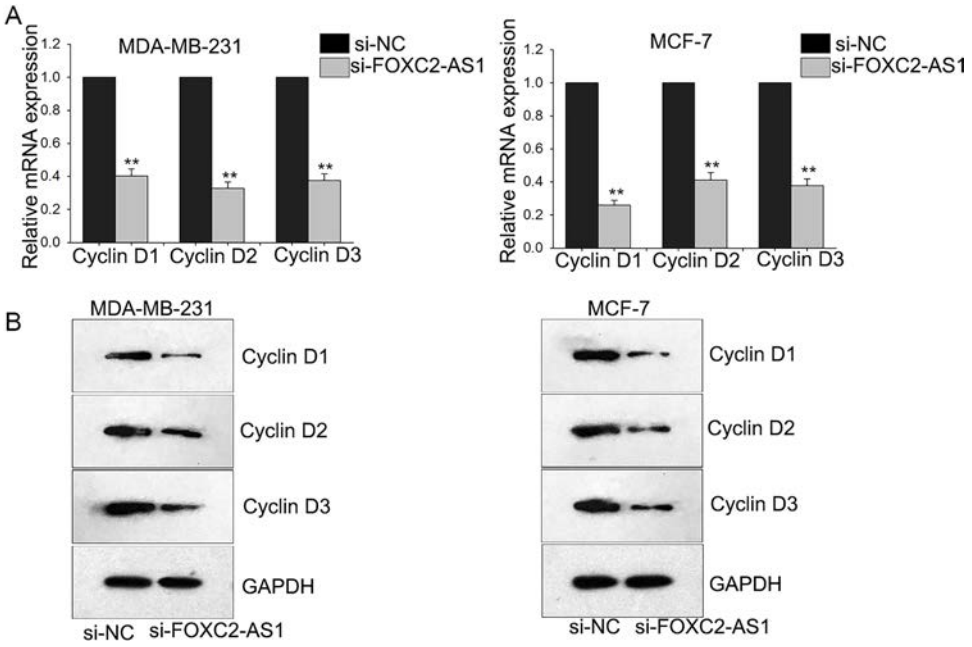

Figure 5. Knockdown of FOXC2-AS1 negatively modulated proteins related with the cell cycle in BC cells. (A) The high mRNA level of cyclin D1, cyclin D2, and cyclin D3 was weakened by transfecting si-FOXC2-AS1. (B) The protein level of cyclin D1, cyclin D2, and cyclin D3 was largely reduced by si-FOXC2-AS1 by comparison with si-NC. Mean±SD shown. $^{**}p<0.01$.

In our present study, we first identified that the FOXC2-AS1 transcript was detected in human tissues, revealing a high expression level in BC tissues compared with the adjacent tissues, and a high level of FOXC2-AS1 was associated with poor prognosis and could act as an independent prognostic predictor. Then after using specific siRNA to silence the endogenous levels of FOXC2-AS1, cell proliferation was markedly suppressed. Knockdown of FOXC2-AS1 also led to BC cell arrest at the G_1 phase and induced cell apoptosis. We have uncovered the biological effect of FOXC2-AS1 in BC cells, but the underlying mechanism has not yet been investigated. Currently, multiple lncRNA regulation mechanisms have been proposed, such as functioning as competing endogenous RNAs (ceRNAs) regulating target genes at the posttranscriptional level[29,30] or acting as a scaffold to recruit molecules like EZH2, LSD1, etc., to the target genes' promoter and regulating the genes at the transcriptional level[31–33]. In our future study, we will concentrate our focus on the regulatory mechanism of FOXC2-AS1 in BC.

In summary, we have shown that FOXC2-AS1 was upregulated in BC cell lines and tissues, and this correlated with poor prognosis in BC patients. Cellular experiments demonstrated that FOXC2-AS1 was involved in the progression of BC. FOXC2-AS1 is also able to regulate proteins that are related to the cell cycle. Our results indicate that FOXC2-AS1 might be a candidate prognostic biomarker and a target for BC treatment.

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