

Forkhead box C1 promoter upstream transcript, a novel long non-coding RNA, regulates proliferation and migration in basal-like breast cancer

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Abstract. Recent studies have shown that long non-coding RNAs (lncRNAs) have crucial regulating roles in carcinogenesis. Forkhead box C1 (FOXC1) is an important cancer-associated gene in basal-like breast cancer (BLBC). In the present study, a novel lncRNA, FOXC1 promoter upstream transcript (FOXCUT) was investigated in BLBC patients using polymerase chain reaction analysis. The results showed that the expression of FOXCUT and FOXC1 were positively correlated. When the expression of FOXCUT was down-regulated by small interfering RNA, the expression of FOXC1 was similarly reduced. Furthermore, in MDA-MB-231 and MDA-MB-468 breast cancer cells, knockdown of FOXCUT markedly inhibited cell proliferation and migration *in vitro*. In conclusion, FOXCUT lncRNA may be functionally involved in the tumor progression of BLBCs through the regulation of its paired mRNA, FOXC1, demonstrating that FOXCUT may serve as a novel biomarker and therapeutic target in BLBCs.

Introduction

Basal-like breast cancers (BLBCs) are aggressive malignancies that express the gene signatures of basal/myoepithelial cells in mammary glands. In human breast cancers, the basal-like subtype has been identified as a distinct entity with a poor prognosis. Due to their underexpression of estrogen receptor (ER), progesterone receptor (PR) and HER2, BLBCs are unlikely to respond to the current targeted systemic therapy (1-3).

To overcome the challenges in the treatment of BLBCs, a novel avenue of investigation into the molecular basis for this disease is urgently required. Recently, a number of studies have demonstrated that long non-coding RNAs (lncRNAs) have pivotal roles in the origination and progression of certain types of cancer (4-6). While only a few studies on lncRNAs in BLBCs have been reported, targeting lncRNAs with critical regulating activities in BLBCs is a promising therapeutic strategy for the future.

lncRNAs are RNA transcripts with no protein-coding potential that are >200 bases in length, which have been identified as high level regulators with multiple molecular regulating mechanisms in gene networks (7-9). A recent study revealed that numerous lncRNAs functionally contact their adjacent mRNAs and take on the form of 'lncRNA-mRNA pairs' in the regulatory network (10).

Through preliminary bioinformatics analysis (<http://genome.ucsc.edu/>), the present study found the novel lncRNA TCONS_00011636, which is located at 6p25 and is transcribed from the upstream side of the FOXC1 promoter. Therefore, it was named as FOXC1 promoter upstream transcript (FOXCUT) by our group. FOXC1 is an important transcriptional factor regulating a variety of biological processes, including embryogenesis, tumorigenesis and epithelial-mesenchymal transition (EMT) (11-13). Several recent studies have shown that a high level of FOXC1 expression correlates with poor overall survival in BLBCs, and that FOXC1 is associated with aggressive phenotypes and increased cell proliferation

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Table I. Primers for real time polymerase chain reaction analysis.

Gene name	Forward	Reverse
β -actin	5'-CCACTGGCATCGTGATGGA-3'	5'-CGCTCGGTGAGGATCTTCAT-3'
FOXC1	5'-GGCGAGCAGAGCTACTACC-3'	5'-TGCGAGTACACGCTCATGG-3'
FOXCUT	5'-GTCGCACCGATGACTAACG-3'	5'-GCCCTGAAAGCCGAAGT-3'

Table II. Sequences for small interfering RNA analysis.

Gene name	Sense (5'-3')	Antisense (5'-3')
FOXCUT si1	5'-GAAUGGAGAACUAAGACAAUUAUUCT-3'	5'-AGAUAAUUGUCUUAGUUCUCCAUCGG-3'
FOXCUT si2	5'-CAGCCUCCUCCUGUGUGUCAGAG-3'	5'-CUCUGCACACACAGGAGGGAGGCUGCA-3'

and migration in breast cancer cells (14-16). In other types of malignant tumors, including pancreatic cancer, non-small cell lung cancer and hepatocellular carcinoma (12-14,17,18), overexpression of FOXC1 is strongly correlated with poor prognosis of the patients. FOXC1 is now recognized as an important cancer biomarker in BLBCs (15,16). However, the expression and function of FOXCUT lncRNA in BLBCs and its association with the adjacent mRNA FOXC1 remains to be determined.

The aim of the present study was to investigate the expression profile of FOXCUT lncRNA in breast cancer tissues and the functional role of FOXCUT in MDA-MB-231 and MDA-MB-468 human BLBC cells *in vitro*.

Materials and methods

Patient samples. A total of 55 specimens were collected from 55 patients previously diagnosed with primary breast cancers at the PLA General Hospital (Beijing, China) between 2007 and 2013. Clinical classification was performed by immunohistochemical studies for ER, PR, HER2, cytokeratin 5/6 and EGFR (19,20). The series included examples from each of the molecular subtypes based on their immunohistochemical surrogate: 16 luminal A-like (ER+/PR+/HER2-), 8 luminal B-like (ER+/PR+/HER2+), 6 HER2-enriched (ER-/PR-/HER2+) and 25 basal-like (ER-/PR-/HER2+/CK5/6+ or EGFR+). The utilization of tumor material for research was approved by the ethical committee of PLA General Hospital and written informed consent was obtained from the patients or their families.

Cell line and cell culture. The human BLBC cell lines MDA-MB-231 and MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA, USA). The MDA-MB-231 cells were incubated in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), and MDA-MB-468 cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% FBS at 37°C with 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from breast cancer tumor tissues, matched adjacent normal tissues and breast cancer

cells using the TRIzol Total RNA reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The primers were obtained from Sheng Gong (Shanghai, China) and the sequences are presented in Table I. RT-qPCR was performed using the SYBR PrimeScript RT-PCR kit (Takara, Ohtsu, Japan) in an Applied Biosystems 7500 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated at 95°C for 30 sec, followed by 40 amplification cycles of 95°C for 5 sec and 60°C for 34 sec. The comparative Ct method was used to quantify relative expression of mRNA and lncRNA. Expression levels of housekeeping gene β -actin were used to normalize gene-of-interest expression. The expression levels of a target gene in a patient were calculated as the ratio of the target expression levels in tumor tissue to the target expression levels in non-tumorous tissue (T/N).

Transfection of siRNA. The siRNA sequences were obtained from GenePharma (Shanghai, China), including one negative control siRNA (NC siRNA) sequence and two FOXCUT siRNA sequences. The target sequences are presented in Table II. siRNA transfection was performed with X-tremeGENE transfection reagent (Roche, Mannheim, Germany). In brief, ~5% cells were plated in each well of 12-well plates at least 24 h prior to transfection to achieve 30-50% confluency. siRNA transfection was then performed with X-tremeGENE transfection reagent (Roche) according to the manufacturer's instructions. Two days post-transfection, RNA isolation, cell proliferation assay, scratch wound healing assay and matrigel invasion assays were performed.

Cell proliferation assay. Following transfection, cell proliferation was assessed by a CellTiter 96® Aqueous 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (Promega) according to the manufacturer's instructions. MDA-MB-231 and MDA-MB-468 cells (2,000 cells per well) in each group were plated in 96-well plates. MTS reagent (20 μ l) was added to each well containing 100 μ l culture medium. The plate was incubated for 2 h at 37°C in a humidified, 5% CO₂ atmosphere. The plate was read at a wavelength of 490 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

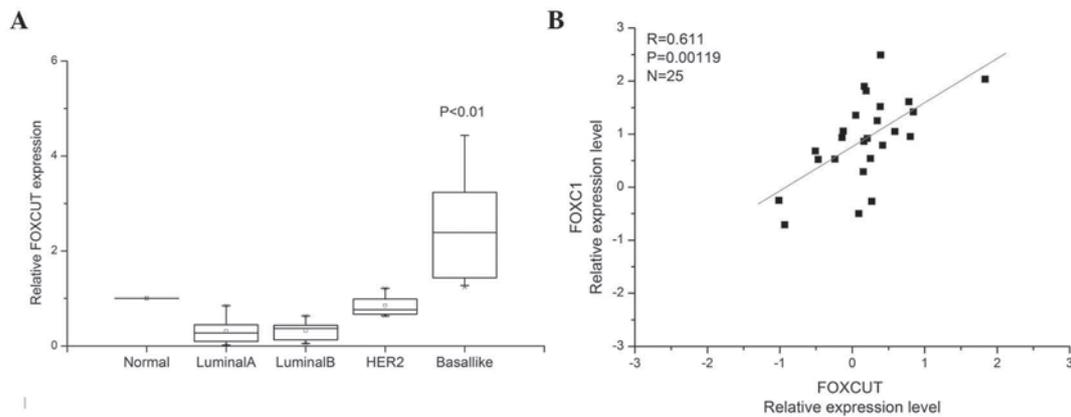


Figure 1. FOXCUT long non-coding RNA expression levels were analyzed by RT-qPCR in 55 breast cancer tissue samples. (A) Expression levels of FOXC1 in breast cancer were significantly higher than those in adjacent normal tissues by RT-qPCR. (B) The expression levels of FOXC1 were significantly correlated with those of FOXCUT lncRNA ($R=0.611$ $P<0.01$). RT-qPCR, reverse transcription quantitative polymerase chain reaction; FOXCUT, forkhead box C1 promoter upstream transcript.

Scratch wound healing assay. Prior to transfection, uniform wounds were scraped into MDA-MB-231 and MDA-MB-468 cells grown on plastic six-well plates using a pipette tip. The initial gap length (0 h) and the residual gap length 48 h after wounding were calculated from photomicrographs. Images were captured using a Olympus BX51 Clone fluorescence microscope (Olympus Corp., Tokyo, Japan).

Matrigel invasion assays. A cell invasion assay was performed using modified Boyden Chambers consisting of Transwell-precoated matrigel membrane filter inserts with 8-mm pores in 24-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA). Culturing medium containing 10% FBS in the lower chamber served as the chemoattractant. Cells that had migrated through the filter were stained and counted. The average migration rate was calculated as the increasing radius of the entire cell population over time.

Statistical analysis. Differences between groups were analyzed using a Student's *t*-test. Correlation between gene expression levels were studied using Pearson's correlation. Statistical analyses were performed using SPSS version 18.0 (International Business Machines, Armonk, NY, USA). For all statistical analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

FOXCUT is overexpressed in BLBC tissue specimens. The FOXCUT lncRNA expression levels were assessed in a panel of paired specimens obtained from 55 patients with breast cancer using RT-qPCR. The results revealed that the FOXCUT expression levels in BLBC tumor tissues were significantly higher than those in matched non-tumorous tissues. The expression levels of FOXCUT were significantly higher in BLBCs than those in non-basal like breast cancer subtypes (Fig. 1A, $P<0.01$). In addition, the relative expression of FOXCUT was positively correlated with that of FOXC1 in the BLBC tissue samples (Fig. 1B; $R=0.611$, $P<0.01$).

FOXC1 mRNA expression is suppressed by FOXCUT siRNA in BLBC cell lines. In MDA-MB-231 and MDA-MB-468 cells, RNA interference analysis was conducted to further clarify the correlation between the expression of FOXCUT lncRNA and FOXC1 mRNA. RT-qPCR was performed to evaluate the expression levels of FOXC1 mRNA and FOXCUT lncRNA. The results showed that FOXCUT expression was efficiently knocked down by FOXC1 siRNA (Fig. 2, $P<0.05$). In addition, FOXC1 expression was downregulated in the FOXCUT siRNA1 group compared with that of the NC siRNA group (Fig. 2, $P<0.05$). This indicated that the expression of FOXC1 mRNA may be modulated by FOXCUT lncRNA in BLBCs.

Knockdown of FOXCUT inhibits the cell proliferation ability of MDA-MB-231 and MDA-MB-468 cells. To investigate the effects of FOXCUT knockdown on the *in vitro* growth characteristics of the MDA-MB-231 and MDA-MB-468 BLBC cell lines, an MTS assay was performed to assess the cell proliferation ability. The results showed that cell growth was inhibited in FOXCUT siRNA groups compared with that in the NC siRNA group (Fig. 3, $P<0.05$).

Knockdown of FOXCUT suppresses the migration ability of MDA-MB-231 and MDA-MB-468 cells. To further identify the function of FOXCUT, a scratch wound-healing assay and matrigel invasion assay were performed following siRNA transfection. The results showed that the migration capacity of the MDA-MB-231 and MDA-MB-468 cells was markedly inhibited by FOXCUT siRNA (Fig. 4, $P<0.05$). The results of the FOXCUT knockdown concurred with the effects of FOXC1 knockdown on the *in vitro* growth characteristics of MDA-MB-231 and MDA-MB-468 as shown above.

Discussion

Advances in high-throughput technologies have resulted in the biological classification of breast cancer into subtypes with distinct gene expression profiles, and BLBC is the most aggressive subtype, with a unique gene-expression pattern of basal/myoepithelial cells characteristics (1-3).

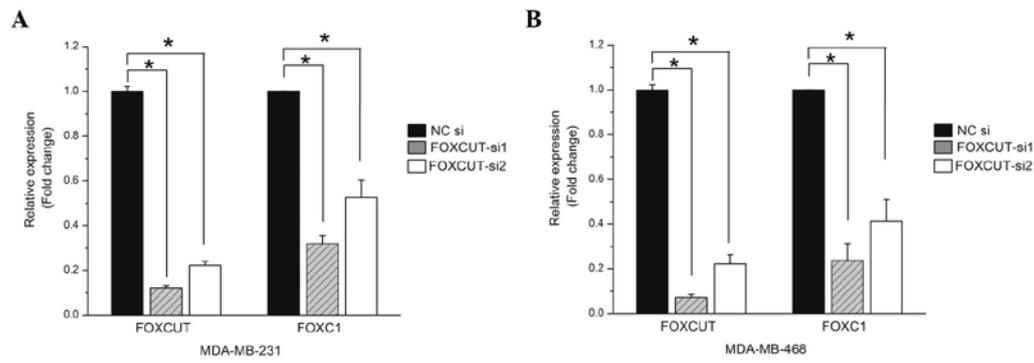


Figure 2. The expression levels of FOXC1 mRNA and FOXCUT long non-coding RNA in (A) MDA-MB-231 and (B) MDA-MB-468 cells after siRNA transfection. The results showed that the expression levels of both FOXCUT and FOXC1 were significantly knocked down in FOXCUT siRNA groups. * $P < 0.05$ compared with the control. FOXCUT, forkhead box C1 promoter upstream transcript; siRNA, small interfering RNA; NC, negative control.

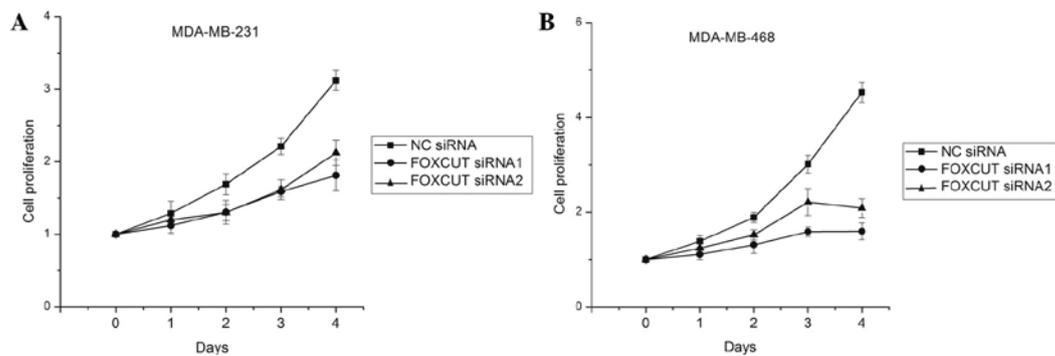


Figure 3. Effects of FOXCUT knockdown on the proliferation of MDA-MB-231 and MDA-MB-468 cells. FOXCUT siRNAs evidently reduced the growth of (A) MDA-MB-231 and (B) MDA-MB-468 compared with NC siRNA in an MTS assay (* $P < 0.05$). FOXCUT, forkhead box C1 promoter upstream transcript; NC, negative control; siRNA, small interfering RNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

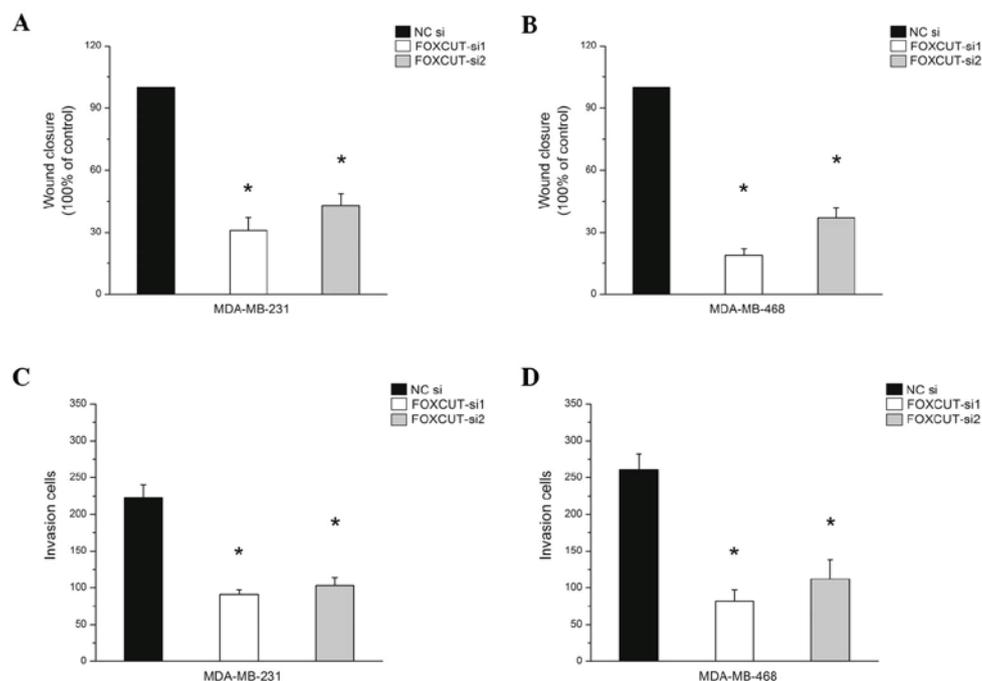


Figure 4. Effects of FOXCUT knockdown on the cell migration ability in MDA-MB-231 and MDA-MB-468. FOXCUT siRNAs evidently reduced the (A) MDA-MB-231 and (B) MDA-MB-468 cell migration compared with that of the NC siRNA control in the scratch wound healing assay. The capacity of cell invasion in the FOXCUT siRNA (C) MDA-MB-231 and (D) MDA-MB-468 cells was markedly impaired. * $P < 0.05$ compared with that of the NC siRNA control; FOXCUT, forkhead box C1 promoter upstream transcript; NC, negative control; siRNA, small interfering RNA.

Previous studies regarding BLBC-associated genes primarily focused on protein-coding genes. In recent years, numerous studies have demonstrated the involvement of lncRNAs in the development and progression of a number of types of malignant tumors (4-6). In breast cancers, several lncRNAs have been identified as novel biomarkers and therapeutic targets, including HOTAIR, BC200 and CCAT2 (21-23). However, the expression patterns and functional roles of cancer-associated lncRNAs in BLBCs remain to be determined.

The present study reported a novel lncRNA, FOXCUT, which may be a cancer-promoting gene responsible for the aggressive phenotype in BLBCs. Through RT-qPCR investigation, it was determined that the expression levels of lncRNA-FOXCUT were remarkably elevated in BLBCs, yet not in other non-basal like breast cancer subtypes. The expression levels of FOXCUT in BLBCs were significantly higher than those in non-basal like breast cancer subtypes, suggesting that lncRNA-FOXCUT may serve as a novel specific biomarker in BLBCs.

Furthermore, in BLBC cell lines, it was revealed that the knockdown of lncRNA-FOXCUT markedly inhibited cell proliferation and migration. This indicated that FOXCUT is not only a pure diagnostic marker in BLBCs, but also an important functional regulator in the cell aggressiveness, similar to the well-known lncRNA HOTAIR in breast cancer (21).

Cancer-associated lncRNAs may exert their regulating activities through diverse mechanisms. Certain lncRNAs may perform their functional roles by directly regulating their neighboring protein coding genes, such as lncRNA PVT1 and protein coding gene c-MYC (24,25). FOXC1 (FOXCUT) is an adjacent lncRNA upstream of the FOXC1 promoter. These ncRNAs are called promoter upstream transcripts (PROMPTs) and are often functionally associated with the adjacent protein-coding transcripts (26-28). Given that the function of the protein-coding gene FOXC1 and FOXCUT lncRNA are involved in the progression of BLBCs by affecting the cell proliferation and cell migration (14-16), it is therefore speculated that FOXCUT lncRNA and FOXC1 mRNA may be another functional lncRNA-mRNA pair that interact with each other in BLBC. RT-qPCR results showed that the expression of FOXCUT lncRNA was positively correlated with FOXC1 mRNA. Through RNA interference analysis, it was determined that the knockdown of lncRNA-FOXCUT clearly reduced the levels of FOXC1 mRNA expression, which was in line with the inhibited cell growth rate and migration ability. This indicated that FOXCUT lncRNA may promote the aggressiveness of BLBC cells partly by regulating the expression of protein-coding gene FOXC1. However, additional studies are required for complete elucidation of the underlying mechanisms.

In conclusion, the present study was the first to identify the expression and functional role of a novel lncRNA, FOXCUT, and its association with the adjacent FOXC1 mRNA in BLBCs. The results indicated that FOXCUT may be a potential diagnostic marker and therapeutic target in the future.

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