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Overexpression of Forkhead Box L1 (FOXL1) Inhibits the Proliferation and Invasion of Breast Cancer Cells

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Forkhead box L1 (FOXL1) is a member of the Forkhead box (FOX) superfamily and was reported to be dysregulated in various types of cancers. However, its expression pattern and underlying cellular function in breast cancer remain largely unexplored. Thus, the aim of this study was to detect FOXL1 expression in breast cancer and to analyze its role in the progression of breast cancer. Our results demonstrated that FOXL1 expression at both the mRNA and protein levels was downregulated in breast cancer tissues and cell lines. Ectopic FOXL1 suppressed breast cancer cell proliferation, migration, and invasion in vitro. Furthermore, overexpression of FOXL1 significantly attenuated tumor growth in breast xenograft models in vivo. Finally, overexpression of FOXL1 significantly downregulated the protein expression levels of β-catenin, c-Myc, and cyclin D1 in MDA-MB-231 cells. Taken together, the present study demonstrated that FOXL1 inhibited the proliferation, invasion, and migration of breast cancer in vitro and breast tumor growth in vivo through deactivating the Wnt/β-catenin signaling pathway. Thus, these findings suggest that FOXL1 may be a potential novel target for breast cancer therapy.

Key words: Forkhead box L1 (FOXL1); Breast cancer; Proliferation; Invasion

INTRODUCTION

Breast cancer is one of the most common malignancies in women in the world, and its incidence ranks first among malignant tumors in females[1]. Although massive efforts have been made to improve the diagnosis and treatment of breast cancer in the past several decades, the clinical outcome of patients with breast cancer remains unsatisfactory[2–4]. The high mortality rate is associated with the ability of breast cancer cells to metastasize to distant organs[5]. Thus, it is urgent to explore the mechanism in order to develop novel treatment strategies.

Forkhead box (FOX) proteins are a superfamily of transcription factors that share a highly conserved DNA-binding domain that regulate a broad spectrum of biological processes including cell proliferation, differentiation, DNA repair, apoptosis, drug resistance, and other cellular processes[6–8]. FOXL1 is a member of the FOX superfamily, which was initially discovered in the mesenchyme of the gastrointestinal tract[9]. Previous studies reported that FOXL1 is dysregulated in various types of cancers, including pancreatic ductal adenocarcinoma, clear cell renal cell carcinoma, gastric cancer, and osteosarcoma[10–13]. For example, Qin et al. confirmed that FOXL1 was significantly downregulated in gallbladder cancer (GBC) tissues and cell lines. Its higher expression is associated with better prognosis, and upregulation of FOXL1 greatly inhibited cell proliferation, migration, and invasion in vitro and tumorigenicity in nude mice[14]. However, its expression pattern and underlying cellular function in breast cancer remain largely unexplored. Thus, the aims of this study were to detect FOXL1 expression in breast cancer and to analyze its roles in the progression of breast cancer. This study provides evidences that FOXL1 is a tumor suppressor and plays an important role in regulating cell proliferation and metastasis in breast cancer.

MATERIALS AND METHODS

Tissue Collection

Human breast cancer tissues were obtained from patients with breast cancer who underwent tumor resection

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between 2013 and 2015 at the Department of Pathology, The First Affiliated Hospital of Xinxiang Medical University (P.R. China). The samples were immediately immersed in liquid nitrogen. Written informed consent was obtained from all patients. The study was approved by the medical ethics committee of the First Affiliated Hospital of Xinxiang Medical University.

Cell Culture

Three human breast cancer cell lines (MDA-MB-231, MCF-7, and BT-474) and the normal breast epithelial cell line (NBEC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin (Gibco), and 100 U/ml streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2.

Construction of Plasmids and Transfection

The full-length FOXL1 cDNA was amplified and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), whereas the empty vector pcDNA3.1 was used as the control. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

RNA Extraction and Quantitative Real-Time (qRT)-PCR

Total RNA was isolated from breast cancer tissues and cells using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. One microgram of total RNA was subjected to first-strand cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). RT-PCR was performed using SYBR Green PCR Mix (Invitrogen) and run on CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). FOXL1 was amplified using the sense primer 5′-TTATTTGGCGGACAGTGA CA-3′ and antisense primer 5′-ACACGGCATCAATCT TTTCC-3′. β-Actin was amplified as an endogenous control using the sense primer 5′-CCGTGAAAAGATGACC CAGATC-3′ and the antisense primer 5′-CACAGCCTG GATGGCTACGT-3′. The relative HOXL1 mRNA expression was calculated using the $2^{-\Delta\Delta}$Ct comparative method.

Western Blot Analysis

Total protein was extracted from breast cancer tissues and cells using RIPA lysis buffer (Invitrogen). Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equal amounts of total protein lysates (40 μg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Boston, MA, USA). The membranes were first blocked and then incubated with primary antibodies overnight at 4°C. The antibodies used were as follows: anti-FOXL1, anti-β-catenin, anti-c-Myc, anti-cyclin D1, and anti-GAPDH (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology) for 1 h. Blots were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA).

Cell Proliferation Assay

Cell proliferation was measured using the 3-(4.5-methylthiozol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Infected MDA-MB-231 cells (1×10^5 cells/well) were seeded onto 96-well plates and then cultured at 24-h intervals for 4 days. Then 20 μl of 5 mg/ml MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C, the culture medium was removed, and 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The absorbance values at 570 nm were measured using a microplate reader (Bio-Rad).

Migration and Invasion Assays

In vitro migration and invasion of the cells were assessed using 24-well inserts with 8-μm pores (BD Biosciences, Eugene, OR, USA). For invasion assays, the inserts were coated with 20 μg of Matrigel in 80 μl of serum-free media. Infected MDA-MB-231 cells (1×10^5 cells/well) were added to the upper chamber, and 750 μl of medium with 10% FBS to the lower chamber. The assays went for 16 h for migration and 24 h for invasion. The cells that had migrated or invaded through the inserts were fixed with 3.7% formaldehyde, stained with 1% crystal violet for 20 min, and counted in five random fields per well under a light microscope (magnification: 100×).

Tumorigenicity in Nude Mice

Four-week-old male BALB/c nude mice were obtained from the Center of Experimental Animal of Xinxiang Medical University (P.R. China) and bred under specific pathogen-free conditions. FOXL1-overexpressing cells (5×10^6) and control cells in a total volume of 100 μl of 1:1 (v/v) PBS were injected subcutaneously into flanks of BALB/c nude mice ($n=6$/group). Twenty days after inoculation, the animals were sacrificed, and the xenografts were isolated and observed; the weight (g) and size (mm^3) of the xenografts were then determined. All of the animal experiments were performed with the approval of the Animal Care and Use Committee of Xinxiang Medical University.

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The statistical significance of the difference was analyzed by ANOVA and post hoc Dunnett's test. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

FOXL1 Expression Was Reduced in Breast Cancer Tissues and Cell Lines

First, we used qRT-PCR to measure the FOXL1 mRNA levels in human breast cancer tissues and their adjacent noncancer tissues. FOXL1 mRNA levels were

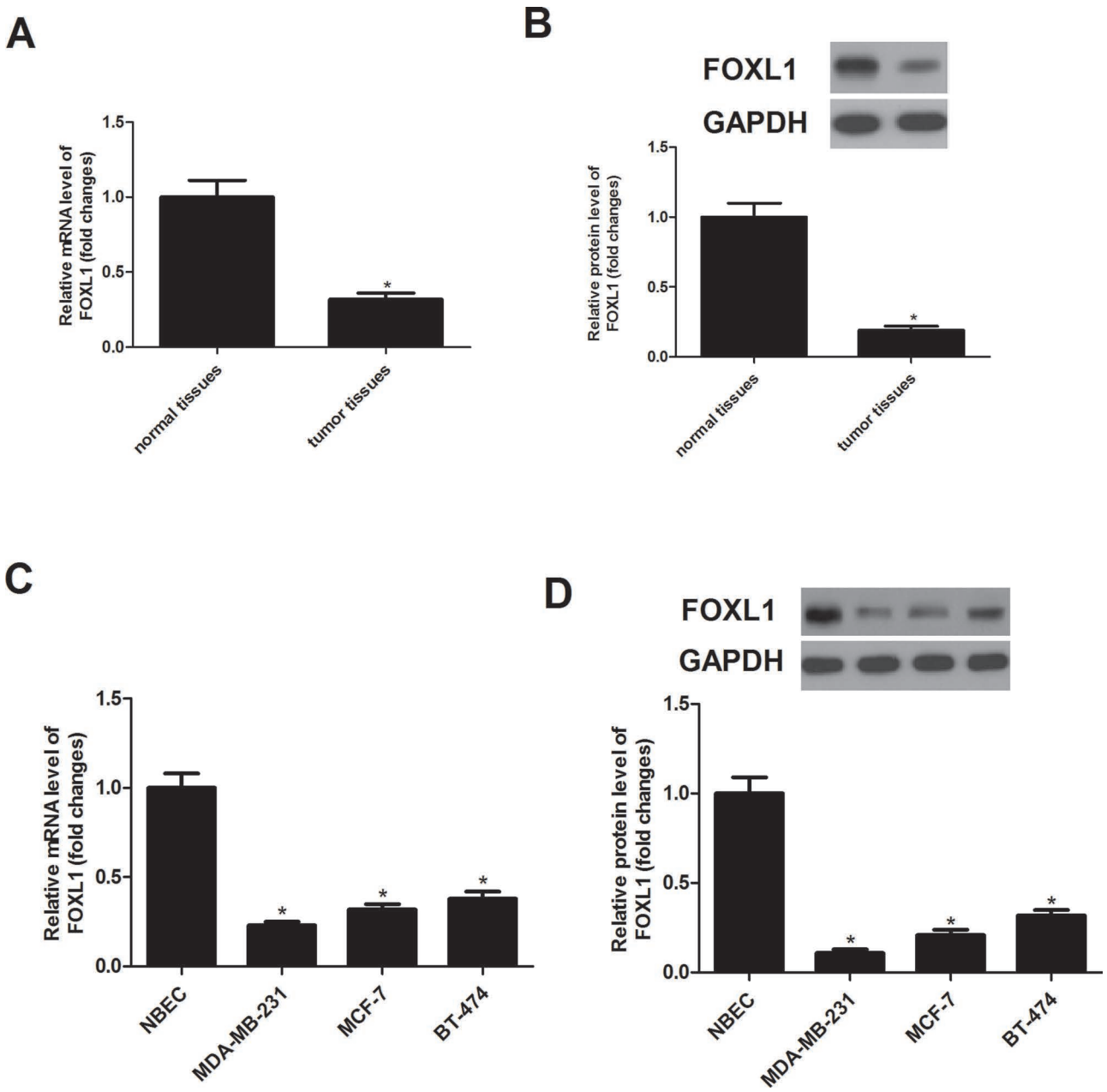

Figure 1. FOXL1 expression was reduced in breast cancer tissues and cell lines. (A) qRT-PCR was used to detect the level of FOXL1 mRNA expression in human breast cancer tissues. (B) Western blot was used to screen the level of FOXL1 protein expression in human breast cancer tissues. *$p < 0.05$ compared with normal tissues. (C, D) The expression of FOXL1 at both mRNA and protein levels was evaluated using qRT-PCR and Western blot in human breast cancer cell lines. *$p < 0.05$ compared with NBEC.

greatly downregulated in breast cancer tissues compared with noncancer tissues ($p<0.05$) (Fig. 1A). In addition, lower FOXL1 protein expression levels were observed in human breast cancer tissues compared with that in adjacent noncancer tissues (Fig. 1B). Similarly, we observed that the expression of FOXL1 at both mRNA (Fig. 1C) and protein levels (Fig. 1D) was significantly lower in breast cancer cell lines than in NBECs.

Ectopic FOXL1 Inhibited Cell Proliferation In Vitro

We then used the gain-of-expression approach to determine the effect of FOXL1 on breast cancer cell proliferation in vitro. The FOXL1 expression level was verified by qRT-PCR and Western blot (Fig. 2A and B). Furthermore, cellular proliferation of stably transfected cells was examined. The results of the MTT assay indicated that overexpression of FOXL1 significantly suppressed cellular proliferation of MDA-MB-231 cells, compared with pcDNA3.1 vector control (Fig. 2C).

Ectopic FOXL1 Suppressed Cell Migration and Invasion In Vitro

We studied the migration ability of MDA-MB-231 cells after performing FOXL1 ectopic transfection. Overexpression of FOXL1 significantly inhibited the migration of MDA-MB-231 cells, compared with the pcDNA3.1 vector control (Fig. 3A). Moreover, we examined the effect of FOXL1 on cell invasion using the Matrigel-coated Transwell assay. The results demonstrated that the number of invading cells was obviously decreased in FOXL1-overexpressing MDA-MB-231 cells, compared with the pcDNA3.1 vector control (Fig. 3B).

Ectopic FOXL1 Inhibited the Activation of the Wnt/β-Catenin Signaling Pathway in Breast Cancer Cells

To gain insight into the molecular mechanism underlying the growth attenuation caused by FOXL1 overexpression, we investigated the effect of FOXL1 on the Wnt/β-catenin signaling pathway in MDA-MB-231 cells using Western blot. A dramatic decrease in β-catenin expression was observed in FOXL1-transfected cells compared with vector cells (Fig. 4). In addition, the protein expression levels of c-Myc and cyclin D1 were downregulated in MDA-MB-231 cells infected with FOXL1.

Ectopic FOXL1 Attenuated the Growth of Tumor Xenografts in Nude Mice

Finally, we examined the effect of FOXL1 on tumor growth in vivo using a nude mouse xenograft assay. The volume of subcutaneous tumors derived from FOXL1-overexpressing MDA-MB-231 cells was dramatically reduced, compared with vector-transfected cells (Fig. 5A). On day 20 after implantation, total tumor weight was dramatically decreased in mice injected with FOXL1-overexpressing cells, compared with the vector control (Fig. 5B).

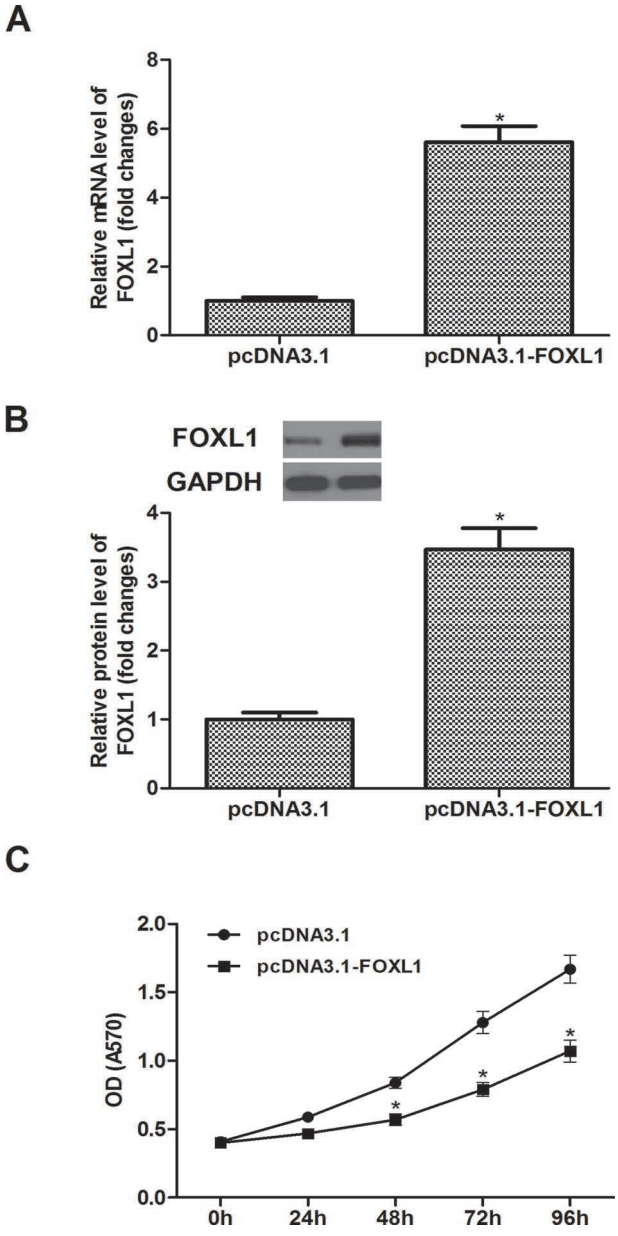

Figure 2. Ectopic FOXL1 inhibited cell proliferation in vitro. MDA-MB-231 cells were transfected with pcDNA3.1-FOXL1 or empty vector pcDNA3.1 for 48 h, respectively. (A, B) The transfection efficiency was confirmed by qRT-PCR and Western blot. (C) Cellular proliferation of stably transfected cells was examined using the MTT assay. *$p<0.05$ compared with pcDNA3.1.

DISCUSSION

To the best of our knowledge, in this study we provided the first evidence that FOXL1 expression at both the mRNA and protein levels was downregulated in breast cancer tissues and cell lines. Ectopic FOXL1 suppressed breast cancer cell proliferation, migration, and

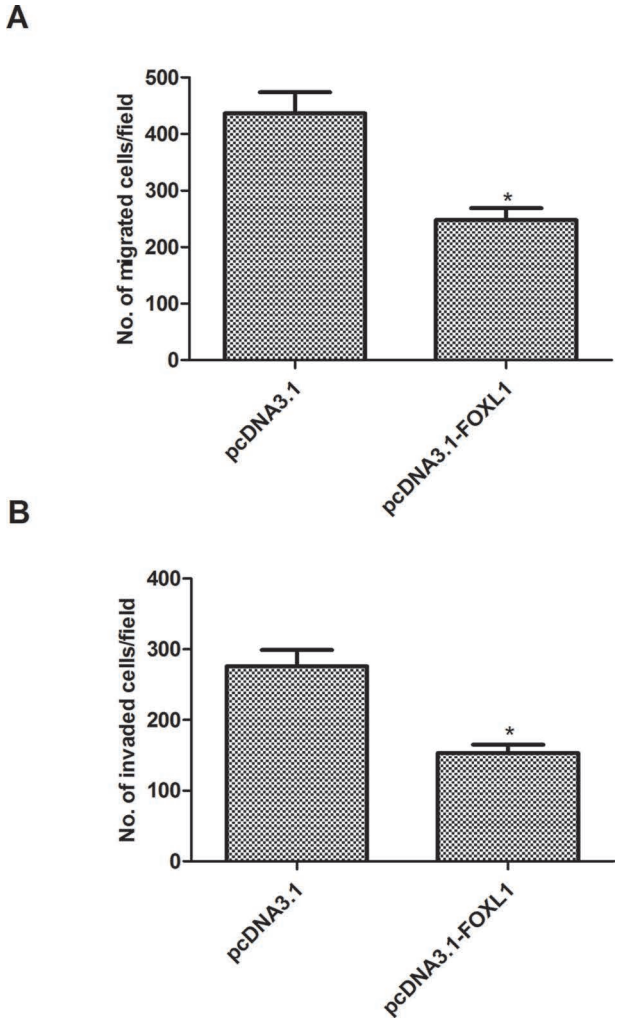

Figure 3. Ectopic FOXL1 suppressed cell migration and invasion in vitro. MDA-MB-231 cells were transfected with pcDNA3.1-FOXL1 or empty vector pcDNA3.1 for 48 h, respectively. (A) Cellular migration of stably transfected cells was examined using the Transwell migration assay. (B) Cellular invasion of stably transfected cells was examined using the Matrigel-coated Transwell assay. *$p<0.05$ compared with pcDNA3.1.

invasion in vitro. Furthermore, overexpression of FOXL1 significantly attenuated tumor growth in breast xenograft models in vivo. Finally, overexpression of FOXL1 significantly downregulated the protein expression levels of β-catenin, c-Myc, and cyclin D1 in MDA-MB-231 cells.

Downregulation of FOXL1 was observed in several cancers and has been reported to be associated with the progression of tumors. Ertao et al. reported that FOXL1 expression was downregulated in gastric cancer tissues, and FOXL1 expression was significantly correlated with tumor stage, lymph node metastasis, and distant metastasis[12]. Another study confirmed that the expression of FOXL1 was significantly downregulated in osteosarcoma tissues and cell lines, and overexpression of FOXL1 substantially inhibited osteosarcoma cell proliferation in vitro and in vivo[13]. In agreement with the results of previous studies, we observed that FOXL1 expression at both the mRNA and protein levels was downregulated in breast cancer tissues and cell lines. In addition, we found that ectopic FOXL1 suppressed breast cancer cell proliferation in vitro and attenuated tumor growth in breast xenograft models in vivo. These data obtained from both in vivo and in vitro experiments suggest that FOXL1 may act as a tumor suppressor in the development and progression of breast cancer.

Metastasis is the most important biological characteristic of breast cancer[15]. Yang et al. confirmed that overexpression of FOXL1 inhibited the migration and invasion in renal cancer cells[11]. Similarly, we found that ectopic FOXL1 expression suppressed breast cancer cell migration and invasion in vitro.

The Wnt/β-catenin signaling cascade plays an important role in human carcinogenesis[16]. It is frequently

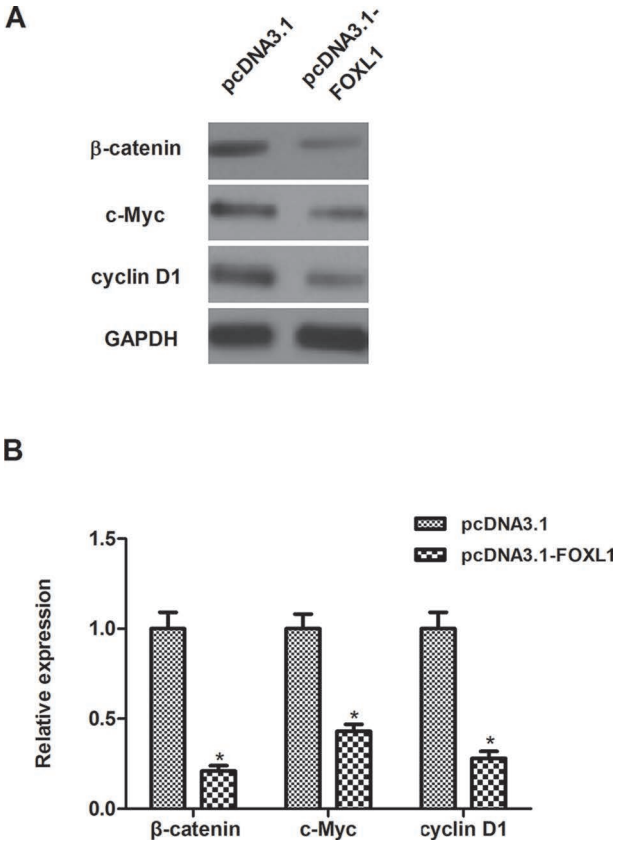

Figure 4. Ectopic FOXL1 inhibited the activation of the Wnt/β-catenin signaling pathway in breast cancer cells. MDA-MB-231 cells were transfected with pcDNA3.1-FOXL1 or empty vector pcDNA3.1 for 48 h, respectively. (A) Western blot was used to screen the protein expression levels of β-catenin, c-Myc, and cyclin D1 in MDA-MB-231 cells. (B) Quantification analysis was performed using the Gel-Pro Analyzer version 4.0 software. *$p<0.05$ compared with pcDNA3.1.

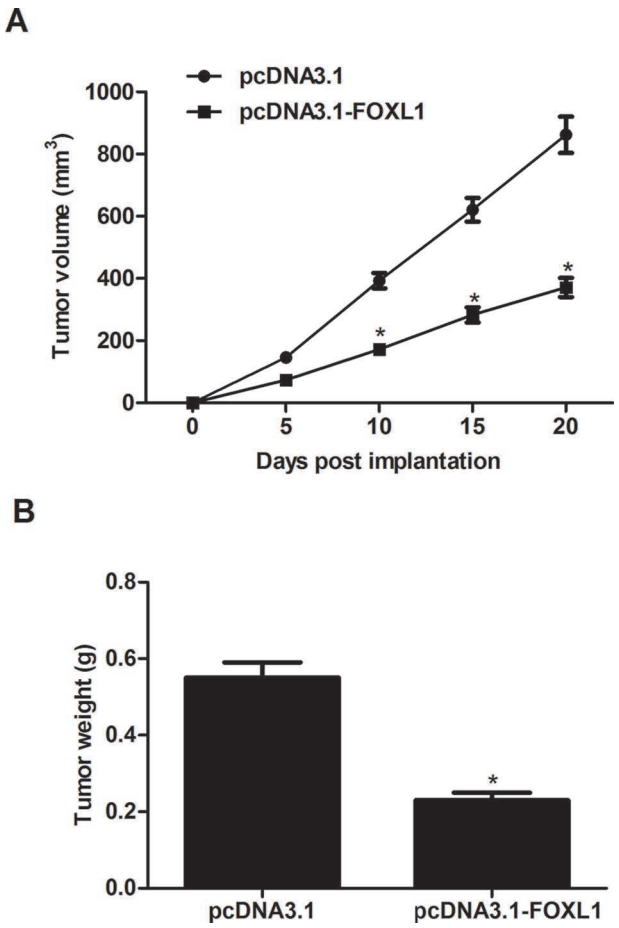

Figure 5. Ectopic FOXL1 attenuated the growth of tumor xenografts in nude mice. FOXL1-overexpressing cells (5×10^6) and control cells in a total volume of 100 µl of 1:1 (v/v) PBS were injected subcutaneously into flanks of BALB/c nude mice ($n=6$/group). (A) The volume of subcutaneous tumors was monitored every 5 days. (B) On day 20 after implantation, the tumor was weighed. *$p<0.05$ compared with pcDNA3.1.

activated in a variety of tumors, including breast cancer[17–19]. β-Catenin is a main downstream effector of the canonical Wnt signaling pathway. Following activation by Wnt ligand–receptor binding, β-catenin escapes proteosomal degradation and translocates into the nucleus, where it binds its target genes, such as cyclin D1, c-Myc, and MMPs, which are implicated in cell differentiation, proliferation, migration, and invasion[20,21]. A growing body of evidence demonstrates that aberrant activation of Wnt/β-catenin signaling promotes cell proliferation and survival and enhances characteristics of the malignant phenotype in breast cancer and is associated with poor prognosis of breast cancer patients[22–24]. In the current study, we observed that overexpression of FOXL1 significantly downregulated the protein expression levels of β-catenin, c-Myc, and cyclin D1 in MDA-MB-231 cells. These data strongly suggest that FOXL1 inhibited the proliferation, invasion, and migration of breast cancer in vitro and breast tumor growth in vivo through deactivating the Wnt/β-catenin signaling pathway.

In conclusion, the present study demonstrated that FOXL1 may play an important role in the proliferation, migration, and tumorigenesis of breast cancer. Thus, these findings suggest that FOXL1 may be a potential novel target for breast cancer therapy.

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