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ORIGINAL ARTICLE

The role and the potential regulatory pathways of high expression of forkhead box C1 in promoting tumor growth and metastasis of basal-like breast cancer

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Summary

Purpose: *To investigate the role of high forkhead box C1 (FOXC1) expression in basal-like breast cancer (BLBC) in vitro and vivo and the underlying regulatory mechanism.*

Methods: *The lentivirus vector with green fluorescent protein (GFP) was used. MDA-MB-231 cells expressing consistently high levels of FOXC1 (FOXC1-MDA-MB-231) were established. The parental MDA-MB-231 cells served as controls. Western blot analysis was used to determine the FOXC1 expression. The invasion capability was tested using the Trans-well assay. The tumorigenicity and the pulmonary metastatic ability were determined in mice in vivo. Histopathology and microarray processing and analysis were performed, and the various pathways involved and the related genes were analyzed.*

Results: *The invasion ability of FOXC1-MDA-MB-231 cells was enhanced significantly ($p<0.01$). Pulmonary metastases were observed in vivo in 3 of 5 mice administered FOXC1-MDA-MB-231 cells through tail vein injection. However, no pulmonary metastatic lesions were observed with MDA-MB-231 cells. The average tumor volume was larger in the mice injected with FOXC1-MDA-MB-231 than in the control mice ($p<0.05$). The expression of Ki-67 in the FOXC1-MDA-MB-231 injected mice was higher than in the control mice. Ten of the most gene-enriched pathways and the critical genes (IL-6 and SNAI2) were found to be related to BLBC.*

Conclusion: *Elevated expression of FOXC1 enhanced the invasion ability of BLCB cells in vitro and promoted tumor growth and metastatic ability in vivo. This function may be regulated by many gene-enriched pathways and some critical genes.*

Key words: *basal-like breast cancer, forkhead box C1, invasion, Ki-67, metastasis*

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among females worldwide, with approximately 1,7 million cases and 521,900 deaths in 2012. Breast cancer alone accounts for approximately 25% of all cancer cases and 15% of all cancer-related deaths in females [1]. Great advances have been made in the early detection, diagnosis and treatment of breast cancer, but there is less optimism regarding long-term outcomes, especially in patients with recurrences and distant metastases. The different subtypes of breast cancer are closely related to clinical and biological implications [2]. Gene expression profiling can determine specific breast cancer molecular subtypes [3]. BLBC expresses genes in the basal cells of normal mammary gland [4]. This type of cancer is also known as triple-negative cancer. BLBCs have a high histologic grade, are aggressive tumors, and have a high rate of recurrence or metastasis [5,6]. However, there is no correlation between tumor size and metastasis in this type of cancer [7].

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The FOXC1 transcription factor was reported to play a key role in the regulation of ocular and vascular development [8]. This factor's mRNA is expressed exclusively in BLBC and correlates with an aggressive phenotype. Ectopic overexpression of FOXC1 in breast cancer cells can induce aggressive phenotypes, such as epithelial-mesenchymal transition (EMT) and increased cell proliferation, migration, and invasion *in vitro* [9]. A study revealed a novel EGFR-FOXC1 signaling axis critical for BLBC cell function, which might provide potential modalities for BLBC treatment. However, knockdown of FOXC1 using shRNA in breast cancer cells led to opposite results [10]. FOXC1 can also serve as a candidate predictive marker of microvascular invasion, and inhibition of FOXC1 expression partially reversed EMT [11].

Markers based on FOXC1 for the diagnosis and targeted therapy of breast cancer are promising and challenging. Recently, the application of a FOXC1-based assay was used to diagnose BLBC [12]. Currently, the specific effect of FOXC1 on BLBC cells *in vitro* and *in vivo* is not completely clear. Moreover, the high expression pattern of FOXC1 is less studied in BLBC. Therefore, this study was designed to determine the effects of elevated expression of FOXC1 on BLBC cells *in vitro* and *in vivo*.

Methods

Lentiviral vector construction and packaging

The human FOXC1 gene (No.: NM_001453) was adopted. Lentiviral vectors that expressed elevated levels of FOXC1 with the puromycin antibody were constructed. A full-length human FOXC1 mRNA was stably transfected into MDA-MB-231 cells (FOXC1). A vector with elevated expression of FOXC1 (GV260, BamHI/AgeI enzyme digestion) was constructed by GeneChem (Shanghai, China). Polymerase chain reaction (PCR) fragments and the Ubi-MCS-IRES-Puromycin plasmid (GeneChem, Shanghai, China) were digested with BamHI/AgeI and subsequently ligated using Taq polymerase to generate Ubi-FOXC1-IRES-Puromycin. Gene sequencing and PCR restriction enzyme digestion were conducted to confirm that the sequence was correct. The primer sequences used for the identification were as follows: forward, 5'-GGGTCAATATGTAATTTTCAGTG-3'; reverse, 5'-TCTCGGTCTTGATGTCCTG-3'. The FOXC1 gene plasmid was then transfected into HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells were incubated and then placed in fresh medium with 10% fetal bovine serum (FBS). Lentiviral particles expressing FOXC1 (Lv-FOXC1) were collected and filtered through a 0.45 μm cellulose acetate filter. The lentivirus was then concentrated with a Plus-20 centrifugal ultrafiltration device (Millipore, Billerica, MA, USA) and stored at -80°C. The lentiviral titer was determined with enzyme-linked immunosorbent assay (ELISA) and a drug screening method with puromycin.

Cell culture, transfection and selection

MDA-MB-231 cells were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences. These cells were cultured in high-glucose DMEM medium containing 15% FBS (Gibco, Grand Island, NY, USA). The cells were transfected with the lentiviral vector and stable transfectants were selected. Stable cell lines were used for the experiments. Cells transfected with the empty lentiviral vector served as controls.

Western blotting analysis

FOXC1-MDA-MB-231 and parental cells were washed twice with PBS and were lysed with ice-cold lysis buffer. The sample was transferred into an Eppendorf (EP) tube and was lysed for 10-15 min on ice. Cytosolic extracts were resolved by SDS-PAGE using 12 or 10% gels. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes and were blocked with Tris-buffered saline with Tween (TBST) containing 5% defatted milk for 1 hr. The membranes were incubated with antibodies for 1 hr at 37°C, washed with TBST and incubated with secondary antibodies for 2 hrs and then the relative target proteins were detected by chemiluminescence. Anti-FOXC1 antibodies (goat polyclonal against FOXC1) were purchased from Santa Cruz Biotechnology.

Cell invasion assay

Cell invasion was evaluated *in vitro* using the BD Biocoat™ Matrigel™ Invasion Chamber (Corning, USA). The control MDA-MB-231 cells and the FOXC1-MDA-MB-231 cells were prepared and incubated overnight at 37°C in the assay medium. Cells (1×10^5) were placed onto the top chambers (100 μl of medium without FBS) of 24-well transwell plates, and the assay medium (600 μl) with 30% FBS was added to the bottom chambers. After 24-hr incubation, the non-migrated cells were removed, and the migrated cells were fixed and stained with Giemsa (400 μl) for 20 min. The stained cells indicated invasion. The cells were then dissolved with 10% acetic acid, and the optical density (OD) values were measured at a wavelength of 570 nm. Cells (1×10^4) were also added to 96-well plates. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA) was used. The invasive cell percentage was calculated by determining the OD570/MTT-OD490 ratio (with OD570 indicating the OD values of the stained cells and MTT-OD490 indicating the OD values of the cells).

In vivo tumorigenicity and in vivo metastasis

This study conformed to the relevant guidelines and regulations and was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Female athymic nude mice were maintained in laminar-flow cabinets under specific pathogen-free conditions. For subcutaneous tumors, 5×10^6 parental MDA-MB-231 and FOXC1-MDA-MB-231 cells were subcutaneously injected into the right front armpits of nude mice. Tumor sizes were measured with a digital caliper to calculate the tumor volume using the formula V=L×W×W/2 (V: volume; L: length; W: width). The average weight of the tumors was calculated.

For tail vein injections, 1×10^5 cells were slowly and evenly injected into the nude mice (n=5/group). The animals were cared for and monitored for general health every week. After six weeks, the animals were euthanized, and the lungs were removed, fixed in 10% formalin and subsequently evaluated for pathology. Histological sections were cut at 75 μm intervals and were stained with H&E to evaluate the lung metastases.

H & E staining and immunohistochemistry (IHC) of tumor sections

H&E staining and IHC for Ki-67 were performed to examine the related protein expression in nude mice tumor tissues. For the analysis of Ki-67, three fields of view (400×) were randomly selected and photographed. The number of cells that were positive for Ki-67 was calculated with Image Pro-plus 6.0 software (Media Cybernetics, Rockville, USA).

Microarray processing and analysis

Total RNA was extracted from MDA-MB-231 cells infected with a lentivirus expressing elevated levels of FOXC1 (n=3) or the control lentivirus (n=3) using Trizol reagent. The RNA quality was subsequently tested with a NanoDrop 2000 (Waltham, USA) and an Agilent Bioanalyzer 2100 (Santa Clara, USA). The GeneChip Primeview Human Gene Expression Array supplied by Affymetrix (100 format) was used for determining and analyzing the gene expression profiles according the manufacturer's instructions. Briefly, reverse transcription and double-stranded DNA template conversion *in vitro* were performed with the GeneChip 3′ IVT PLUS reagent kit (Santa Clara, USA). The chips were hybridized, washed and stained with the GeneChip Hybridization Wash and Stain Kit (Santa Clara, USA)**.** The microarrays were then scanned using the GeneChip Scanner 3000 (Santa Clara, USA). Genes with significantly different expression between the control and FOXC1-MDA-MB-231 cells were chosen on the basis of the following criteria: p value <0.05 and fold change >1.5. Pathway enrichment was analyzed on the basis of KEGG pathway databases. A gene network diagram was constructed and analyzed.

Statistics

All the data in this study were expressed as means ± standard deviation. Analyses were performed using SPSS 19.0 software for Windows (IBM Corporation, Armonk, NY, USA). Student's t-test was applied throughout and a p level <0.05 was considered as statistically significant.

Results

Stable transfected MDA-MB-231 cell line establishment

The cells of the control and high FOXC1 expression groups showed good morphology (Figure 1A, B). High FOXC1 expression was verified by Western blot (Figure 1E).

Cell invasion assay

The mean invasive cell percentages of the high FOXC1 expression and control groups were 49.7±2.6 and 15.1±1.4, respectively (p<0.001). The results indicated that elevated FOXC1 expression plays an important role in enhancing the invasion ability of MDA-MB-231 cells (Figure 1C, D, F).

High FOXC1 expression promotes tumor growth and enhances pulmonary metastasis in vivo

In vivo, elevated FOXC1 expression promoted tumor growth. The average volumes of subcutaneous tumors in the FOXC1-MDA-MB-231 group were larger than in the control group at different points (p<0.05). The average weight of the tumors in the FOXC1-MDA-MB-231 and control groups was 2.02±0.6 g and 0.959±0.493 g, respectively (p<0.05) (Figure 2A-C). Regarding lung metastasis by tail vein injection, pulmonary metastatic lesions were observed in 3 of the 5 mice in the FOXC1-MDA-MB-231 group (Figure 2D). In contrast, no lung metastatic lesions were observed in the control group (Figure 2E).

High levels of FOXC1 expression enhance the expression of Ki-67

To evaluate the role of FOXC1 in tumor growth and metastasis, H & E staining (Figure 3A-B) and IHC were performed. Compared to the control, the high FOXC1 expression group had higher Ki-67 expression (Figure 3C-D). The average positive rate of the FOXC1 and control groups was 38.73±3.5 and 22.3±1.43, respectively (p<0.01). The results indicated that high FOXC1 expression may enhance Ki-67 expression.

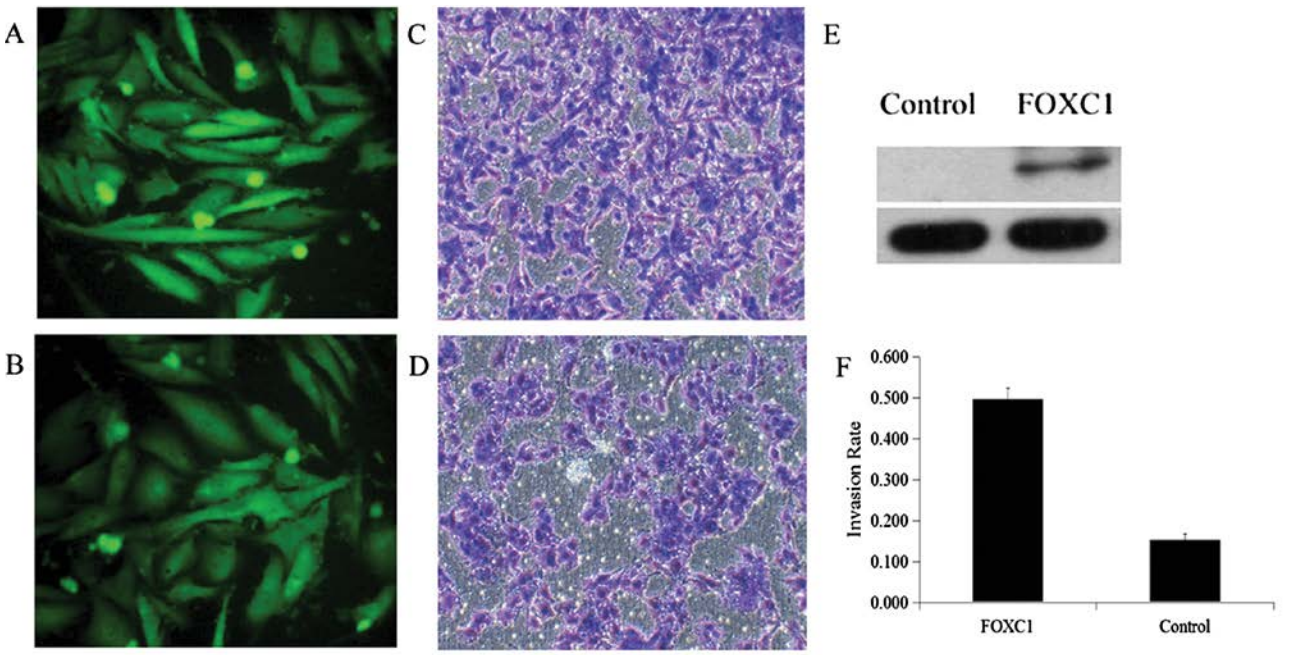

Figure 1. The cell state of the FOXC1-MDA-MB-231 cells **(A)** and control cells **(B)** (x200). The stable transfected MDA-MB-231 cells with high FOXC1 expression were established and determined by Western blot analysis **(E)**. High FOXC1 expression enhanced MDA-MB-231 cell invasion ability with trans-well chambers. After a 24-hr incubation, the invasive cells were fixed, stained, and photographed at a 100× magnification and studied under a microscope **(C,D).** The number of invasive cells in the FOXC1-MDA-MB-231 cells was higher than in the control ($p<0.05$) **(F).**

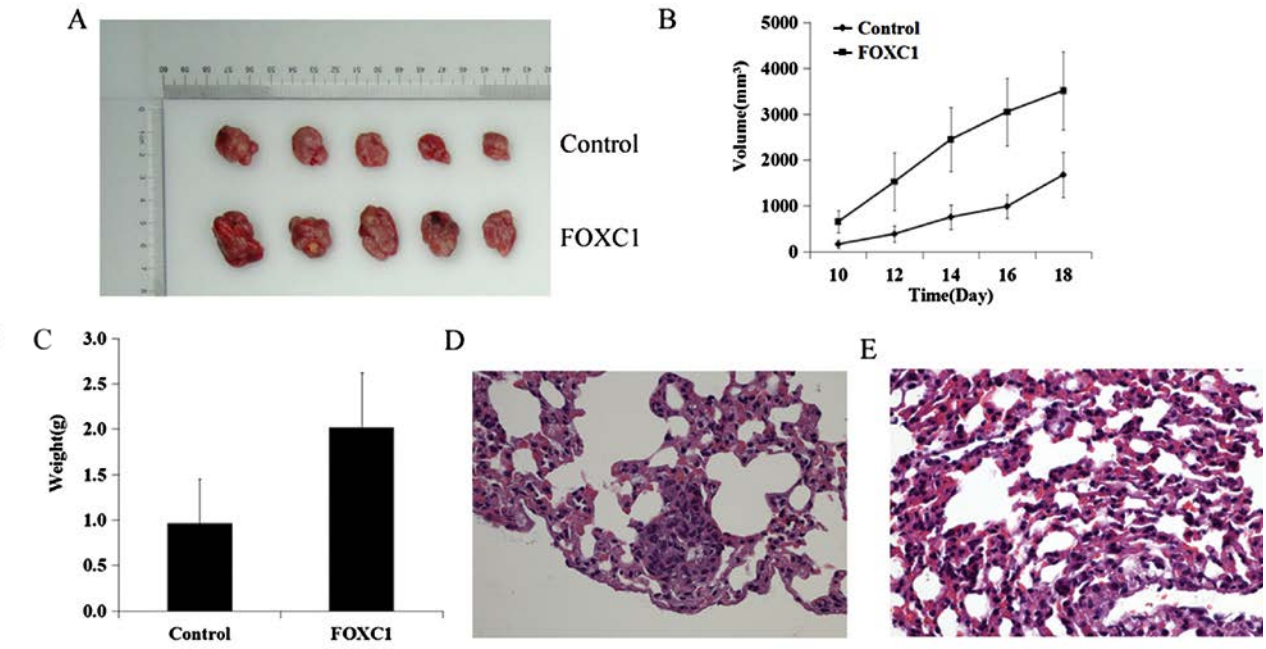

Figure 2. *In vivo* tumor evaluation and the staining of lung samples. **A:** Subcutaneous tumors samples. **B:** The average tumor volume in the FOXC1-MDA-MB-231 groups was higher than in the control ($p<0.001$). **C:** The tumors in the FOXC1-MDA-MB-231 groups had increased weights compared to the control groups ($p<0.05$). **D:** Photomicrograph of pulmonary metastatic lesion in lung sections of athymic mice injected with FOXC1-MDA-MB-231 cells for 6 weeks (H&E, x200). **E:** In lung section of control group no metastatic lesions were observed (H&E, x200).

Canonical pathways and genes involved in cancer cell invasion and tumor development and metastasis in vivo with elevated FOXC1 expression

The above experiments indicated that FOXC1 is critical for tumor progression in the MDA-MB-231 human BLBC cell line.

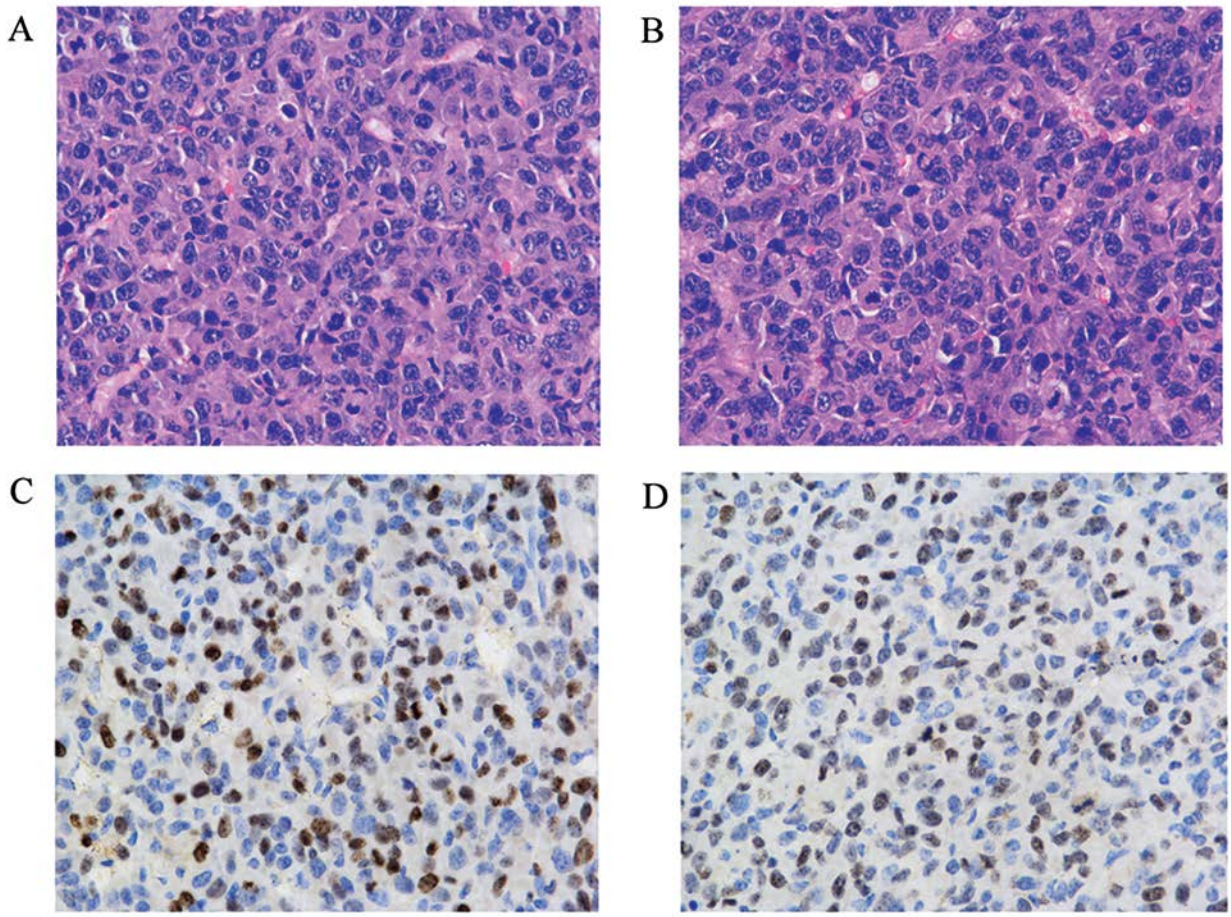

Figure 3. H&E staining of FOXC1 high expression tumor section **(A)** and control tumor section **(B)** (x200) and Ki-67 immunohistochemistry. The number of Ki-67-positive cells was higher in tumor sections with high FOXC1 expression **(C)** than in the control (x400) **(D)**.

Ten pathways in which these differential genes were significantly enriched were identified based on a canonical pathway analysis with a $p<0.05$ threshold. These pathways included aryl hydrocarbon receptor signaling, p53 signaling, molecular mechanisms of cancer, regulation of the EMT pathway, PI3K/AKT signaling, adipogenesis pathway, cell cycle: G1/S checkpoint regulation, glioblastoma multiforme signaling, role of p14/p19ARF in tumor suppression and Wnt/β-catenin signaling. A functional linked network diagram showed the related genes were closely related with each other (Figure 4A, B). These results indicated that FOXC1 might regulate the expression levels of IL-6 and SNAI2 to promote BLBC progression and metastasis.

Discussion

In this study, our findings indicated that the elevated expression of FOXC1 enhanced cell invasion ability *in vitro* and promoted tumor growth and metastasis *in vivo* through multiple functional gene-enriched pathways and the regulation of several related genes. We expect that hopefully our findings might provide useful information for targeted therapy for BLBC in the future.

The FOXC1 protein belongs to the forkhead box (FOX) family of transcription factors, and FOXC1 plays an important role in the development of cancer by regulating target genes. Based on a gene expression analysis of publicly available human breast cancer microarray data sets, the overall survival rate of patients with basal-like subgroup breast cancer was significantly reduced in those with elevated FOXC1 mRNA levels [13]. Many studies have demonstrated that several genes could directly regulate FOXC1 expression. In BLBC, FOXC1 knockdown reduced cell proliferation and migration [10]. Previously, FOXC1 expression was reported to be significantly higher in the basal-like subgroup than in other subtypes, consistent with reports that 60-90% of triple-negative breast cancers are of the basal-like subtype [9,13,14]. Ray et al. demonstrated that FOXC1 may be a crucial prognostic biomarker of

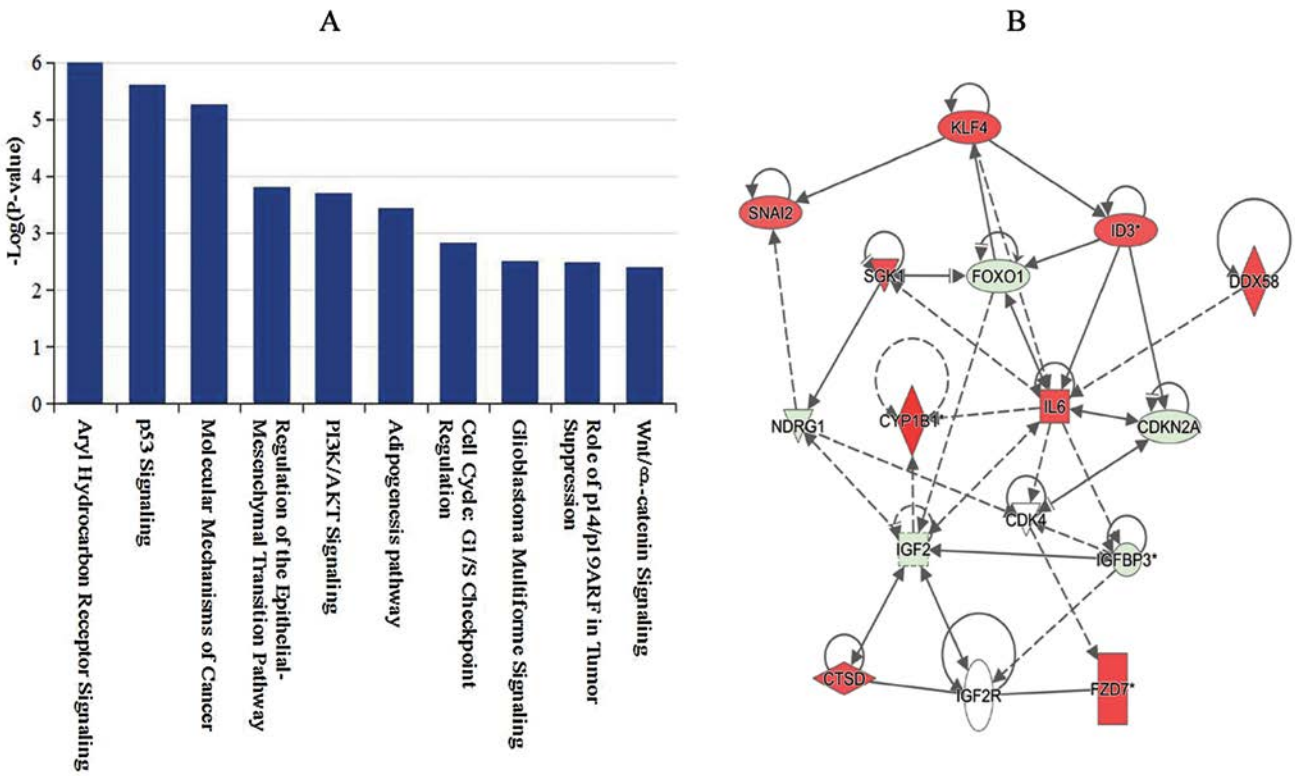

Figure 4. Multiple involved functional pathways **(A)** and functional association network of FOXC1-regulated target genes **(B)**. The associated genes were analyzed using the STRING database. The solid lines and arrows indicate confirmed regulatory relationships, and the dotted lines and arrows indicate predicted regulatory relationships.

BLBC [13]. The results of this study verified the findings mentioned above.

FOXC1 plays pivotal roles in brain development, brain angiogenesis and tumorigenesis, which might explain why triple-negative tumors preferentially metastasize to the brain [15,16]. Ray et al. illustrated this point by testing the function of FOXC1 in MDA-MB-231 BLBC cells *in vitro* [9,13]. Taube et al. have shown that FOXC1 is an inducer of the EMT. This finding correlates strongly with the poor survival of breast cancer patients, who exhibit stem cell characteristics and metastatic potential [17]. FOXC1 also participates in EMT and is involved in many of the steps in the invasion-metastasis cascade. The role of changes in cellular architecture and polarity in the behavior of cancers has gained increasing and extensive attention [18]. EMT is a process by which epithelial cells lose their adherent epithelial characteristics and acquire mesenchymal properties, including fibroblastoid morphology, characteristic gene expression changes, increased potential for motility and, with respect to cancer cells, increased invasion, metastasis, and resistance to chemotherapy [16,19]. EMT generates cancer cells that are invasive, migratory, and exhibit stem cell characteristics, all of which are hallmarks of cells with the potential to generate metastases by transmigration through the extracellular matrix (ECM) as a single cell. EMT has now been linked to the metastatic progression of cancer [19,20]. Importantly, EMT is considered a pivotal behavior for cancer cell migration by endowing an individual cell with increased migration and invasion potential through the barrier matrix. Notably, the EMT process can be induced *in vitro* by FOXC1 [21].

In vivo, high FOXC1 expression could promote tumor growth and enhance metastatic ability. Huang et al. found that FOXC1 overexpression in hepatocellular cancer cells increased the number of metastases that formed in mice [22]. Therefore, FOXC1 expression may increase the invasive and metastatic abilities of HCC cells. Our findings were consistent with the findings of Huang et al. In sample sections, we found that the Ki-67 index was higher in sections with high FOXC1 expression than in the control sections. Ki-67 is a type of nucleoprotein that is related to tumor differentiation, invasion, metastasis and prognosis [23]. This result indicated that high FOXC1 expression may affect cell invasion and metastatic potential.

The microarray analysis revealed many significant differentially expressed genes. Related gene signatures with multiple functional pathways involved in breast cancer cells and cancer development were further analyzed. The most highly enriched pathways in samples expressing elevated levels of FOXC1 included pathways that are essential for cancer cell proliferation, migration, invasion, cell cycle and tumor metastasis and related genes (IL-6 and SNAI2) play a pivotal role in cancer cell proliferation, migration, invasion, cell apoptosis, cancer progression and metastasis [24,30]. Among these genes, IL-6 and SNAI2 were significantly regulated by high FOXC1 expression. IL-6 was associated with cancer cell invasion, induced an EMT phenotype in human breast cancer cells and might promote mammary stem cell-like properties in a BLBC-specific manner [31,32]. The SNAI2 transcription factor has been shown to be related to tumor invasion, metastasis and poor prognosis [25]. In addition, SNAI2 was associated with stem cell properties [26-30]. In primary breast cancers, SNAI2 expression was positively correlated with metastasis [33]. Therefore, high FOXC1 expression may exert its effects through significantly related and differentially expressed genes involved in multiple enriched pathways.

The lack of an *in vivo* orthotopic implantation model is a limitation of this study. Additionally, metastases to other organs were not evaluated. In light of these deficiencies, more research should be conducted to obtain more detailed findings.

In summary, high FOXC1 expression plays an important role in enhancing cell invasion and promoting tumor growth and metastasis by regulating multiple functional gene-enriched pathways and several critical genes. Our findings may provide useful information for targeted therapy and better understanding of the pathways and mechanisms underlying basal breast cancer metastasis.

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Conflict of interests

The authors declare no confict of interests.

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