

## ORIGINAL ARTICLE

# The potential pathway of FOXC1 high expression in regulating the proliferation, migration, cell cycle and epithelial-mesenchymal transition of basal-like breast cancer and *in vivo* imaging

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## Summary

**Purpose:** To investigate the role of high forkhead box C1 (FOXC1) expression in basal-like breast cancer (BLBC) cells *in vitro* and *in vivo*, as well as its potential regulatory pathway.

**Methods:** Stable MDA-MB-231 cells, a type of BLBC cells, with high FOXC1 expression and luciferase (FOXC1) were established. The parental MDA-MB-231 cells with luciferase served as the control group. Proliferation, migratory capabilities and the cell cycle were evaluated. The tumorigenicity and the spontaneous pulmonary metastasis were measured in mice *in vivo*. *In vivo* imaging was also performed. Histopathology, immunohistochemical analysis and microarray processing were evaluated. Paired Student's t-test was used.

**Results:** The proliferation and migratory ability of FOXC1-MDA-MB-231 cells were enhanced significantly ( $p < 0.05$ ). Spontaneous pulmonary metastases were observed in 2 out of 5 mice, but no pulmonary metastases were observed in control animals. There were more FOXC1 cells in the G1 phase compared to the control ( $p < 0.05$ ), but there were

also significant reductions of cells in the S and G2 phases ( $p < 0.05$ ). The CD31 and endoglin (CD105) expression in the FOXC1 tumor was higher than in the control, especially CD105 ( $p < 0.05$ ). The total fluorescence expression quantity of FOXC1 was higher than in the control cells ( $p < 0.05$ ), and the apparent diffusion coefficient (ADC) values were lower compared with the control ( $p < 0.05$ ). One pathway with the most gene enrichment (p38 MAPK signalling) may play a key role in regulating BLBC cell proliferation, migration, cell cycle and epithelial-mesenchymal transition (EMT) through the interaction of related critical regulatory genes (IL-6 and FOXC1).

**Conclusion:** High FOXC1 enhanced the proliferation, migratory ability and EMT of BLBC cells. This function may be regulated by IL-6 and FOXC1 through the p38 MAPK signalling pathway.

**Key words:** basal-like breast cancer, forkhead box C1, proliferation, migration, cell cycle, epithelial-mesenchymal transition, MRI

## Introduction

Breast cancer is the most common and leading cause of mortality in females worldwide with approximately 1.7 million sufferers and 521,900 deaths in 2012. Breast cancer reaches approximately 25% of all the new cancer cases and 15% of all cancer-related deaths in females [1]. Different subtypes of breast cancer determine the clinical

and biological implications, and gene expression leads to breast-cancer-specific molecular subtypes, clinical features and survival outcomes [2,3]. BLBC is a special subtype of breast cancer that is also known as triple negative breast cancer (TNBC). The basal-like subtype accounts for approximately 15% of all breast cancer cases, is more aggres-

sive and has high incidence of recurrent lesions [4]. Due to frequent lack of endocrine receptors and human epidermal growth factor receptor-2 (HER2) amplification, this characteristic makes BLBC potentially susceptible to recurrence [5]. Notably, there is no correlation between tumor size and distant metastasis in this type breast cancer [6]. Therefore, patients diagnosed with BLBC have a poor prognosis and a short disease-free period and overall survival [7]. A better understanding of the biological characteristics, molecular basis and regulatory mechanisms of BLBC is necessary and worthwhile to investigate.

Forkhead box (FOX) family members were reportedly involved in many biological processes, including cell proliferation, differentiation, survival, and apoptosis [8]. Dysregulated expression of FOX proteins is closely related to the development and progression of cancers [9]. The FOXC1 transcription factor is an important member of the FOX family. The FOXC1 transcription factor has been shown to play an important role in the regulation of embryonic and ocular development. Mutations in this gene cause various glaucoma phenotypes, including primary congenital glaucoma, autosomal dominant iridogoniodysgenesis anomaly, and Axenfeld-Rieger anomaly [10,11]. In addition to its role in embryonic development, multiple studies revealed that FOXC1 plays a critical role in cancer occurrence and progression. Elevated FOXC1 expression indicates poor prognosis in many cancers, especially BLBC. It is involved in multiple steps of tumor progression, including cancer cell proliferation, invasion and metastasis [12,13]. Therefore, FOXC1 can serve as a biomarker for diagnosis and therapy for BLBC.

In recent years, many studies focused on the mechanism and regulatory pathways of FOXC1 in breast cancer, but the results have been varied. Therefore, the specific mechanism of FOXC1 regulating BLBC still remains unclear. Based on our previous study [13], we aimed to investigate the effects of FOXC1 high expression on the BLBC in cell proliferation, migration, the cell cycle, EMT and *in vivo* imaging. Importantly, we expected to find a potential regulatory pathway and critical regulatory genes and provide a therapeutic target for BLBC.

## Methods

### *Lentiviral vector construction and package*

The human FOXC1 gene (No.: NM\_001453) was selected. Lentiviral vectors that express FOXC1 with the luciferase were constructed by Gene (Shanghai, China). A full-length human FOXC1 mRNA was stably trans-

fecting into MDA-MB-231 cells. The detailed procedures were the same as the protocols used in our previous study [13].

### *Cell culture, transfection and selection*

MDA-MB-231 cells were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose and mixed with 15% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The cells were transfected with the lentiviral vectors, and stable cell lines that expressed high FOXC1 expression were selected with a luciferase antibody. Cells transfected with the empty lentiviral vectors served as controls.

### *Western blotting analysis*

The process of Western blotting was completed as follows: 1: cells were washed and lysed with ice-cold lysis buffer; 2: the samples were lysed for 10-15 min on ice; 3: cytosolic extracts were resolved with SDS-PAGE; 4: the proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with tris-buffered saline with Tween (TBST) for 1 hr; 5: the membranes were incubated with primary and secondary antibodies; and 6: the target proteins were detected by chemiluminescence. The FOXC1 antibody was purchased from Santa Cruz Biotechnology(CA, USA) [13].

### *Cell proliferation assay by MTT*

After cells were trypsinized in the logarithmic growth phase, cell suspension was made. The cell density (2500 cells/well) was determined by cell counting. A total of 5 plates with 96 wells each were continuously tested over 5 days. The cells in the 96-well plates were incubated in an incubator. From the second day after cells were plated, 20  $\mu$ L of MTT (5 mg/ml) were dripped into the wells before 4 hrs of cell culture termination. After 4 hrs, the DMEM medium mixed with MTT was removed and 150  $\mu$ L of DMSO was dripped into the wells to dissolve the formazan particles. Finally, optical density (OD) 490 values were measured with an enzyme-labelled instrument (Biotek Elx800, USA).

### *Cell migration assay*

Cell migration test was performed *in vitro* with Transwell assay (Corning, USA). The control and the FOXC1 high expression MDA-MB-231 cells were prepared and incubated in the DMEM medium with 15% FBS. The wells with 100  $\mu$ L of serum-free medium were placed in an incubator for 2 hrs. A cell suspension was prepared and cell counting was performed with a counting chamber. After 2 hrs, the serum-free medium in the wells was removed and 600  $\mu$ L of DMEM medium with 30% FBS was added to the lower chambers. Next, a 100  $\mu$ L cell suspension with  $1 \times 10^5$  cells was added to the wells. Then, the wells were transferred to the lower chambers and incubated for 24 hrs. After 24 hrs, the DMEM medium with 30% FBS and the nonmigratory cells were removed. The wells were immersed in the Giemsa staining fluid for 20 min. The migratory cells

were stained and photographed under a microscope. Then, all the stained cells were dissolved in 10% acetic acid and the optical density (OD) 570 values were measured. Cells (5000) were placed into 96-well plates. The migration cell rate was calculated by determining the OD570/MTT-OD490 ratio with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA) (OD570 indicates the OD values of the stained cells and MTT-OD490, which indicates the OD values of the unstained cells).

#### *Cell cycle assay*

A cell suspension containing  $10^6$  cells was collected in a centrifuge tube (5 ml). Three parallel wells were tested. The suspension was centrifuged for 5 min (1500 rpm) and washed with pre-cooled D-Hanks (4°C) once. The cells were fixed with pre-cooling 75% ethyl alcohol (4°C) for 1 hr after centrifugation (1500 rpm, 5 min). Then, the cells were washed with pre-cooled D-Hanks (4°C) one time again and were stained with 1 ml mixed dye solution (VPI:RNaseA:D-Hanks = 25:10:1000). Finally, all the cells were tested with flow cytometry (FCM) and the data were analysed.

#### *The tumorigenicity and spontaneous metastasis in vivo*

This study complied with the relevant guidelines and regulations for animal testing, and the animal experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of North Sichuan Medical College. Female athymic nude mice were fed in pathogen-free and aseptic conditions. For the subcutaneous tumors experiment,  $5 \times 10^6$  parental MDA-MB-231 and FOXC1-MDA-MB-231 cells were injected into the right front armpits with adjacent subcutaneous tissues of nude mice. The mice were cared for and observed for general health every week and recorded. The pulmonary metastatic lesions were observed to evaluate the spontaneous metastatic ability. After six weeks, the animals were euthanized, and the lungs were removed, fixed in 10% formalin and subsequently stained with H&E to evaluate the spontaneous lung metastases.

#### *In vivo imaging by Caliper IVIS Lumina II and MRI*

Before euthanasia, all the mice were subjected in *in vivo* imaging with Caliper IVIS Lumina II (Berthold Technologies, UK) and 3.0T Verio MRI (Siemens, Germany).

For live fluorescence imaging of small animals, before imaging, 10  $\mu$ l/g D-Luciferin was injected into the abdominal cavity. After 15 min, all the mice were anaesthetized with 0.7% pentobarbital sodium by intraperitoneal injection. During anaesthesia, the mice were placed into the imager and imaging was performed *in vivo*.

For MRI, T2WI TSE and diffusion-weighted imaging (DWI) sequences were used. TSE main parameters: TR: 700.0 ms, TE: 15.0 ms, number of echoes: 1, slice thickness: 0.7 mm, slice gap: 0, number of slices: 12, NEX: 4.0, FOV: 62, voxel size:  $0.2 \times 0.2 \times 0.7$  mm. DWI main parameters: TR: 3800.0 ms, TE: 86 ms, number of echoes: 1, slice thickness: 1 mm, slice gap: 0, number of slices: 13, NEX: 4.0, FOV: 93, voxel size:  $1.2 \times 1.2 \times 1.0$  mm.

B values: 0 and 600 s/mm<sup>2</sup>. After anaesthesia, the mice were fixed in the small animal coil to make sure the tumors were located in the centre of the coil. All the images and data were processed at a workstation (ADW 4.4). The ADC map was generated automatically and ADC values were measured on ADC maps by drawing the region of interest (ROI) on the centre of the largest slice.

#### *H & E staining and immunohistochemistry (IHC) of tumor tissues*

H&E staining and IHC for CD31 and endoglin (CD105) were performed to observe the metastatic lesions and examine the related protein expression in tumor tissues. For analysis of CD31 and CD105, three fields of view (200 $\times$ ) were selected and photographed. The microvessel density (MVD) counting method was used to analyse the potential invasive and metastatic ability of the cells with Image-Pro-plus 6.0 software (Media Cybernetics, Rockville, USA).

#### *Microarray processing and analysis*

The detailed procedures and description were the same as the protocols used in our previous study [13].

#### *Statistics*

The quantitative data were expressed as means  $\pm$  standard deviation. SPSS 19.0 statistical software for Windows (IBM Corporation, Armonk, NY, USA) was used. A paired Student's t-test was used and a p value <0.05 was considered to be statistically significant.

## **Results**

#### *Western blotting and cell proliferation by MTT*

FOXC1 expression was verified by Western blotting. The MDA-MB-231 and FOXC1-MDA-MB-231 cells grew in good condition and good shape. There was no significant difference between the control and FOXC1 cells before 3 days ( $p > 0.05$ ), but the FOXC1 cell proliferation increased compared to the control and enhanced remarkably on the 5<sup>th</sup> day ( $p < 0.05$ ; Figure 1 A-D).

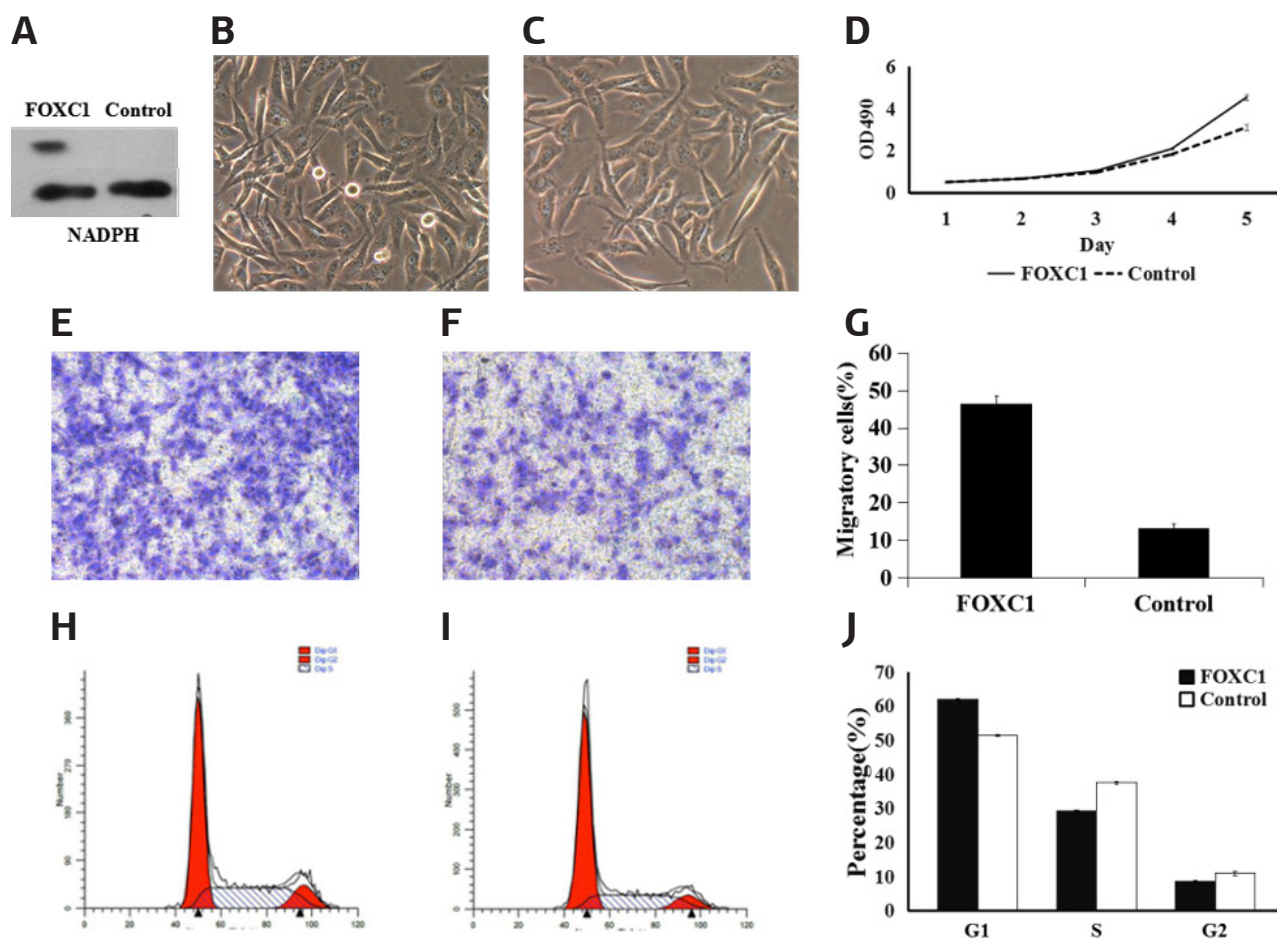
#### *Cell migration assay*

The migratory rates of the FOXC1 and control cells were  $46.3 \pm 2.1\%$  and  $13.2 \pm 1\%$ , respectively ( $p < 0.05$ ). The results suggested that high FOXC1 expression might enhance the migratory abilities of the MDA-MB-231 cells (Figure 1 E-G).

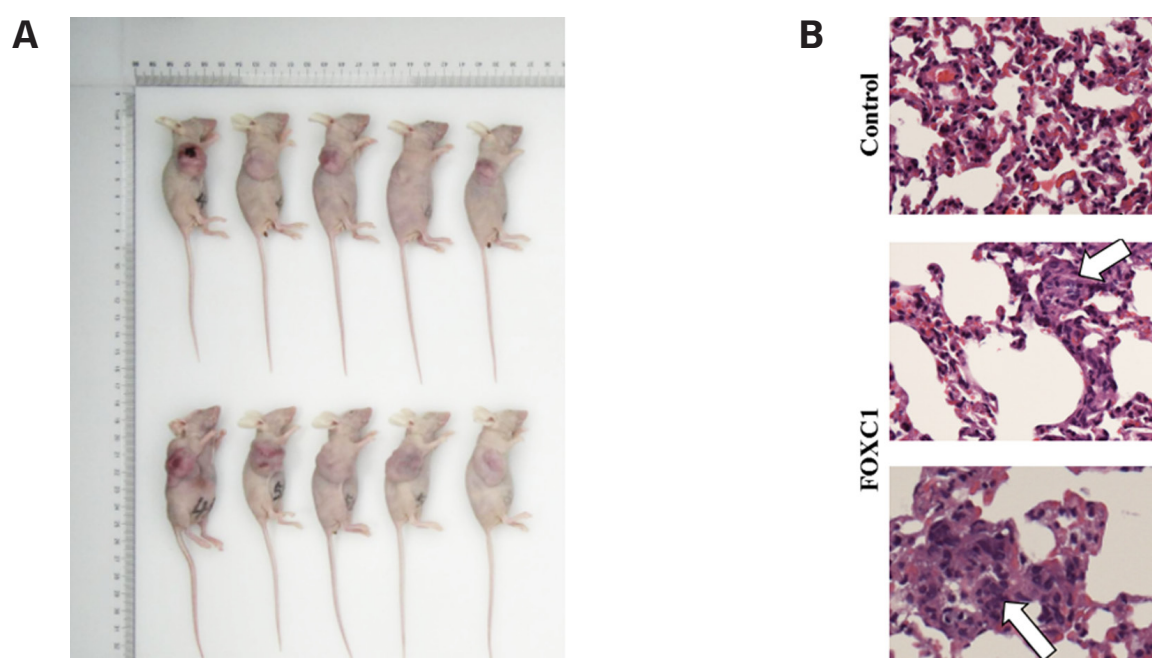
#### *Cell cycle assay*

In the cell cycle test, there was a higher amount of FOXC1-expressing cells in the G1 phase compared to the control ( $p < 0.05$ ), but there were fewer cells in the S and G2 phases compared to the control ( $p < 0.05$ ; Figure 1 H-J).





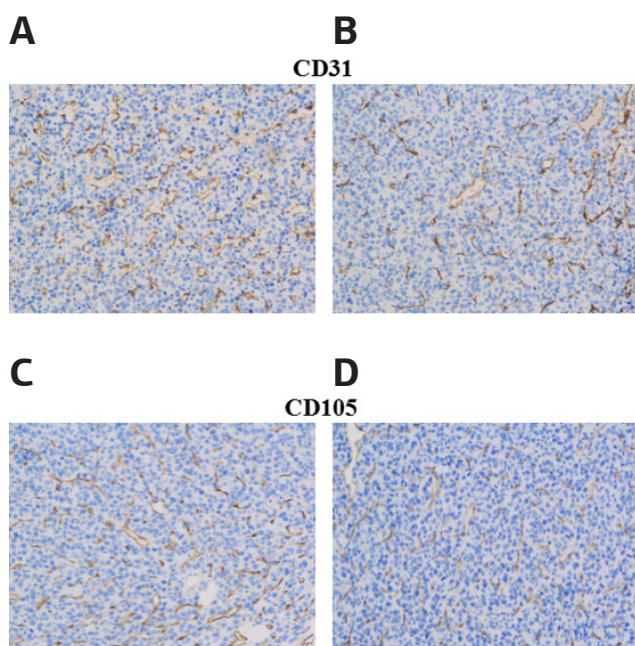
**Figure 1.** High FOXCl expression was determined by Western blotting (A). The cells were in a good state (B: FOXCl-MDA-MB-231, C: Control cells, 200 $\times$ ). No difference was found before the first three days, but a significant difference could be observed on the fifth day between FOXCl and the control ( $p<0.05$ ) (D). The migratory ability of FOXCl high expression cells was increased by comparison with the control ( $p<0.05$ ; E, F, G). In the cell cycle test, there was a higher amount of FOXCl-expressing cells in the G1 phase compared to the control ( $p<0.05$ ), but there were fewer cells in the S and G2 phases compared to the control ( $p<0.05$ ; H, I, J).



**Figure 2.** The mouse models of BLBC (A) and pulmonary metastases (B) (200 $\times$ ). The metastases (white arrows) were observed on 2 FOXCl high expressing mice, but one metastasis was observed in the control mice.

### High FOXC1 expression may enhance BLBC spontaneous metastatic ability *in vivo*

*In vivo*, elevated FOXC1 expression promoted tumor growth and lung metastasis by tail vein injection, as reported in our previous study [13]. In this study, we expected to observe spontaneous metastasis of BLBC *in vivo*. The tumor formation rate in 5 mice was 100% (Figure 2A). Regarding spontaneous metastasis, lung metastases were observed in 2 out of 5 mice in the FOXC1 group (Figure 2B). In contrast, no metastasis was observed in the control group (Figure 2B).



**Figure 3.** The CD31 and CD105 immunohistochemistry of the FOXC1 high expression tumor sections (A, C) and control tumor sections (B, D) (200×). The mean CD31 MVD values of the FOXC1 high expression were higher than the control, but without statistical difference ( $p > 0.05$ ). The mean CD105 MVD values of the FOXC1 high expression were higher than the control ( $p < 0.05$ ).

### FOXC1 high expression enhances CD31 and CD105 expression

The CD31 mean MVD of FOXC1 and control slices was  $329.78 \pm 19.69$  and  $279.70 \pm 37.61$ , respectively. The CD105 mean MVD of FOXC1 and control slices was  $285.36 \pm 25.52$  and  $195.83 \pm 20.12$ , respectively. For CD31, compared to the control, the mean MVD values were higher, but without significant difference ( $p > 0.05$ ; Figure 3A-B). For CD105, the mean MVD increased markedly ( $p < 0.05$ ; Figure 3C-D).

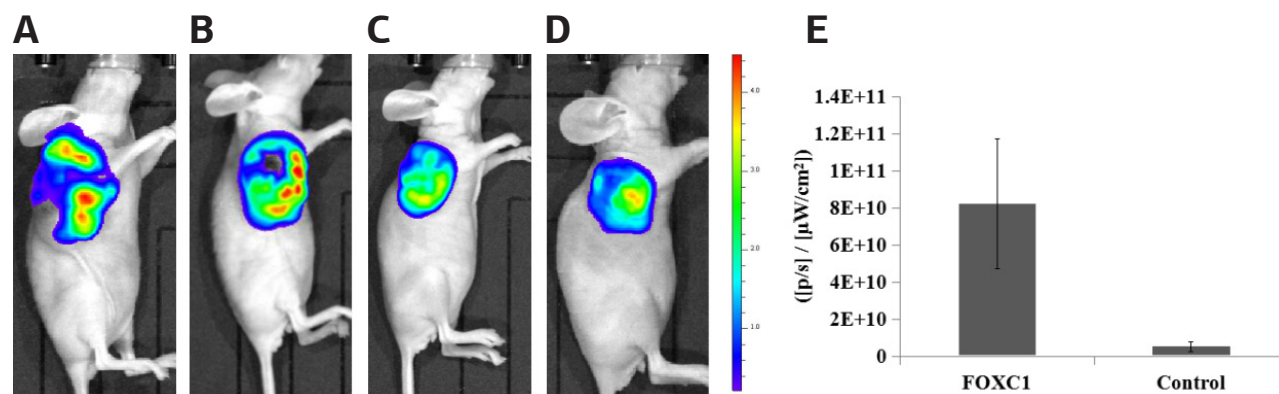
### *In vivo* imaging with Caliper IVIS Lumina II and MRI

Based on *in vivo* fluorescence imaging, the total fluorescence expression quantity in the average area of the tumor of FOXC1 and control were  $8.24 \pm 3.49 \times 10^{10}$  and  $3.42 \pm 0.81 \times 10^9$  [p/s] / [ $\mu\text{W}/\text{cm}^2$ ] ( $p < 0.05$ ; Figure 4A-E).

In T2WI TSE, the tumor showed slight heterogeneous hyperintensity (Figure 5A, D). In DWI, the tumors of the FOXC1 and control groups showed hyperintensity (Figure 5B, E). The ADC values of the FOXC1 and control groups were  $1.03 \pm 0.17 \times 10^{-3} \text{ mm}^2/\text{s}$  and  $1.29 \pm 0.11 \times 10^{-3} \text{ mm}^2/\text{s}$  ( $p < 0.05$ ; Figure 5C, F).

### Potential regulatory pathways and enriched genes regulating cancer cell proliferation, migration, cycle and EMT related to metastasis with high FOXC1 expression *in vivo*

A clustering figure shows the gathering of all the samples and differential genes in expressed values level. In the tree structure of these two samples, two adjacent samples or genes had higher similarity (Figure 6A). The above results indicated that FOXC1 plays an important role in regulating the biobehavioral characteristics of the MDA-MB-231 human BLBC cell line. By gene chip analysis, we found that an important role may be



**Figure 4.** Fluorescent imaging *in vivo* (A-D). The data (E) showed that the total fluorescence expression quantity in the average area of the tumor with FOXC1 high expression (A-B) was much higher compared to the control (C-D) ( $p < 0.05$ ).



regulated by p38 MAPK signalling, which involves many regulatory genes. In this signalling pathway, IL-6 and FOXC1 were involved in cell proliferation, migration, invasion and tumor metastasis. CDKN2A may be related to the cell cycle of BLBCs (Figure 6B).

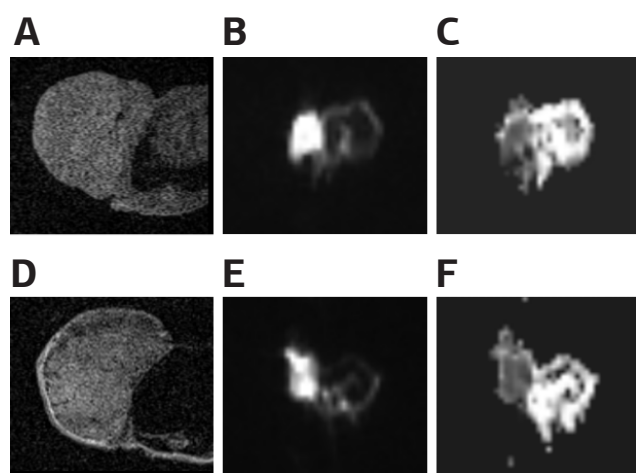
## Discussion

In this research, we found FOXC1 high expression can promote cell proliferation, migration and may regulate the cell cycle *in vitro*, as well as

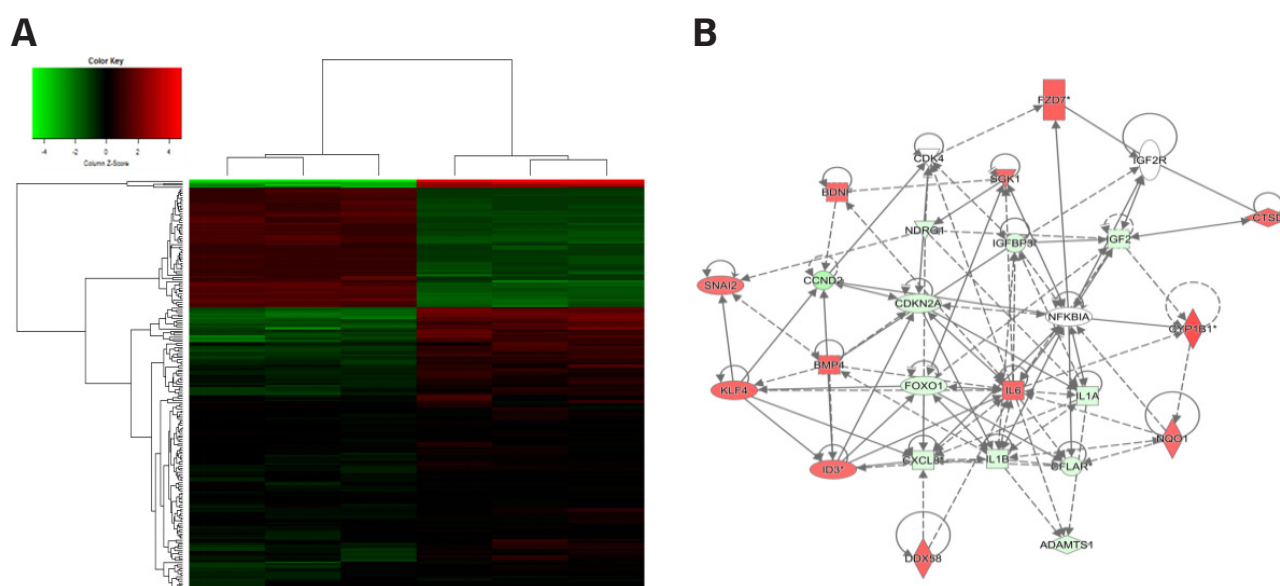
EMT, which is closely related with metastasis *in vivo*. The results were supplemented and verified by fluorescence imaging and DWI. In addition, the mechanism may be regulated by p38 MAPK signalling, which involves many enriched genes. Among the involved genes, IL-6 and FOXC1 may play a key role in cell proliferation, migration and EMT. In addition, CDKN2A may be related to cell cycle regulation. *In vivo* fluorescence imaging and DWI can also indicate a key role of FOXC1 in BLBC. This finding provides useful information and lays the foundation for BLBC targeted therapy in the future.

In many previous studies, FOXC1 was proven to be an important factor and marker for breast cancer. Ray et al. reported that FOXC1 may be a potential prognostic biomarker of BLBC [14]. Elevated FOXC1 expression indicates poor overall survival in BLBC, is independent of other factors and may be related to a higher risk of brain metastasis. Ectopic overexpression of FOXC1 in breast cancer cells contributed to cell proliferation, migration, and invasion [14]. Additionally, BLBC defined by a triple-negative phenotype (TNP) plus FOXC1 demonstrated a superior prognostic approach compared to BLBC defined by TNP or TNP plus basal cytokeratins, and the predictive utility of FOXC1 in BLBC management and clinical trial design is expected [15]. In contrast to BLBC, FOXC1 knock-down inhibited cell proliferation and migration *in vitro* [16]. Our results for this study were consistent with previous findings.

FOXC1 has high expression that is also closely related with metastasis [17], and this finding may



**Figure 5.** The T2WI TSE sequence imaging (A, D), DWI (B, E) and ADC imaging (C, F). The tumors of FOXC1 high expression and the control showed heterogeneous hyperintensity on T2WI TSE and DWI, but the ADC values were reduced significantly in a FOXC1 high expression tumor ( $p < 0.05$ ).



**Figure 6.** Clustering figure showing all the samples and differential genes according to their expression values. In the tree structure, two adjacent samples or genes had higher similarity (A). The associated genes were analysed using the STRING database. The solid lines with arrows indicate specific regulation and the dotted lines with arrows indicate predicted regulation (B).

be related to EMT. EMT is a process in which epithelial cells lose their inherent characteristics and generate cancer cells with migratory, invasive and stem-cell-like characteristics [18]. FOXC1 was proved to be an inducer of the EMT process [19]. Elevated FOXC1 expression participates directly in EMT and many steps of the invasion-metastasis [20,21]. 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 [16]. Notably, the EMT process can not only be induced and occur *in vivo* but also be induced *in vitro* by elevated FOXC1 expression [22]. All the previous studies and our findings demonstrate that powerfully FOXC1 high expression play a crucial role in BLBC.

To verify the *in vitro* findings, the mouse models of breast cancer were established. The pulmonary metastasis by tail vein injection was tested in our previous study [13]. The spontaneous metastasis experiments were performed *in vivo*. The tumor formation rate in 5 mice was 100%. In the FOXC1 high expression group, the pulmonary metastases were observed in 2 out of 5 mice; however, no metastases were observed in the control group. The results indicated that high FOXC1 expression may enhance BLBC metastatic ability *in vivo*. Tkocz et al. reported that high FOXC1 expression led to drug resistance in BLBC patients and the occurrence of a subtype related to BLBC progression. Meanwhile, the results suggested FOXC1 is an important regulator of BLBC invasion characteristics and plays a key role in the proliferation and survival of breast cancer cells [23].

The immunohistochemical analysis indicated that high FOXC1 expression can increase CD31 and CD105 expression, which is essential for the tumor growth, invasion and metastasis [24,25]. The CD105+ subpopulation from breast cancer MDA-MB-231 cells was shown to possess "mesenchymal stem cell-like" characteristics, and its high migratory ability might be associated with EMT [25]. Therefore, CD105 can serve as a new marker for the identification of mesenchymal stem cells and might provide new evidence for the recurrence and metastasis of breast cancer.

So far, many regulatory pathways and regulators of FOXC1 in breast cancer have been studied, but the results were different or even contradictory. Some pathways may be involved in the occurrence and development of BLBC, including MEK/PI3K [26], integrin [27], the Notch pathways [28] and NF- $\kappa$ B [29]. A recent study reported that FOXC1-regulated BLBC tumor stem cells function through the Smoothened (SMO)-independent Hedgehog (Hh) pathway [30]. The novelty of this study was that

it revealed that FOXC1 activates the smoothened-independent Hedgehog signalling pathway in regulating the characteristics of the tumor stem cells in the BLBC through direct interaction with Gli2. In our study, the p38 MAPK signalling pathway may be critical for regulating BLBC. The final results were the same as those of the previous studies.

Many regulatory factors may be involved in cell proliferation, migration, invasion and metastasis, such as the matrix metalloproteinase (MMP) family. MMP2, MMP7 and MMP9 may be related to tumor invasion, metastasis and progression by degrading the extracellular matrix [14,31]. MMP2, MMP7 and MMP9 may serve as the downstream targets. FOXC1 may endow BLBC with an aggressive phenotype that plays a crucial role in BLBC [32]. In our previous study, IL-6 was shown to be an important factor for promoting tumor invasion and metastasis [13]. An elevated IL-6 level can enhance the cell proliferation and migration [33,34], and the level of IL-6 increased with elevated FOXC1 expression. Another downregulated gene, FOXC1, was observed. The normal level of FOXC1 can inhibit breast cancer cell proliferation, migration and invasion, but with the FOXC1 downregulation, the opposite effect occurs [35]. In the p38 MAPK signalling pathway, the regulator cyclin-dependent kinase inhibitor 2A (CDKN2A) may be related to BLBC cycle regulation [36]. The multiple regulatory functions of IL-6 were proven and confirmed again in this study.

Through *in vivo* imaging, we found that the total fluorescence expression quantity for FOXC1 in the average region of the FOXC1 high expressed tumor was higher than in the control group, which implied that FOXC1 can enhance BLBC proliferation ability and tumor metabolism *in vivo* [37]. On ADC maps, the ADC values of FOXC1 were lower compared to the control. ADC is a sensitive imaging marker for the identification of benign or malignant tumors or the tumor malignant grade. The ADC values correlated negatively with tumor malignancy [38] and the data indicated the BLBC proliferation and malignancy increased with elevated FOXC1 expression.

Some limitations existed in this study, including lack of orthotopic implantation, no evaluation of metastasis in other organ and a lack of another breast cancer cell line as a control. We will do more research to improve our study.

Overall, high FOXC1 expression plays a vital role in enhancing cell proliferation, migration and promoting tumor metastasis *in vivo* through the p38 MAPK signalling pathway and some critical genes (i.e., IL-6, FOXC1 and CDKN2A). *In vivo* imaging was a good supplement to the findings. The aim

of this study was to provide some useful information for targeted therapy and a better understanding of the mechanism underlying BLBC.

## Conflict of interests

The authors declare no conflict of interests.

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