



# FOXO3a inhibits the EMT and metastasis of breast cancer by regulating TWIST-1 mediated miR-10b/CADM2 axis

Long Jin, Jun Zhang, Hui-Qun Fu, Xi Zhang, Yu-Liang Pan\*

Department of Oncology, Third Xiangya Hospital, Central South University, Changsha 410013, Hunan, China

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## ABSTRACT

**Background:** Breast cancer is the most common malignancy and has been considered as a leading cause of cancer death in women. Exploring the mechanism of breast cancer metastasis is extremely important for seeking novel therapeutic strategies and improving prognosis.

**Methods:** Clinical specimens and pathological characteristics were collected for evaluating the expression of forkhead box class O 3a (FOXO3a) and twist-related protein 1 (TWIST-1) in breast cancer tissues. CCK-8 assay was used to analyze cell proliferation. Cell invasion and migration were assessed by transwell assays. The expression of FOXO3a, TWIST-1, miR-10b, CADM2, FAK, phosphor-AKT and the epithelial–mesenchymal transition (EMT)-related protein (N-cadherin, E-cadherin and vimentin) were analyzed by RT-qPCR, immunohistochemical staining, immunofluorescence assay or western blot, respectively. Xenograft mouse models were used to analyze the role of the FOXO3a in breast cancer.

**Results:** FOXO3a was down-regulated and TWIST-1 was up-regulated in breast cancer tissues. Overexpression of FOXO3a or knockdown of TWIST-1 suppressed the proliferation, invasion, migration and EMT of breast cancer cells. Overexpression of TWIST-1 could reverse the effect of FOXO3a on the proliferation, invasion, migration and EMT of breast cancer. Moreover, FOXO3a suppressed the growth and metastasis of breast cancer by targeting TWIST1 *in vivo*.

**Conclusion:** FOXO3a inhibited the EMT and metastasis of breast cancer via TWIST-1/miR-10b/CADM2 axis.

## Introduction

Breast cancer is the most common malignancy among women and one of the most common cancers, along with lung and colon cancer worldwide, which affects millions of patients and has been considered as a leading cause of cancer death in women [1,2]. Although the incidence is relatively stable and even slightly decreased in several developed countries in recent years, it is still rapidly increased in many areas including Asia with a youth-oriented tendency [3,4]. Over the past three decades, great advances have been made in early diagnosis and comprehensive treatment of breast cancer, which greatly improve the long-term survival rate of patients [5]. However, the metastasis of breast cancer cells still leads to approximately 90% of cancer mortalities and is closely associated with the poor prognosis [6]. Therefore, eluci-

dating the mechanisms of the invasion and metastasis of breast cancer is extremely important for seeking novel therapeutic strategies and improving the long-term survival rate. The epithelial–mesenchymal transition (EMT), which was firstly described during embryonic development, causes the transition of epithelial cells to mesenchymal-like cells [7]. EMT also plays crucial roles in the invasion and metastasis of cancers and may represent the first step in the metastatic cascade [8]. Investigating the mechanisms of the EMT will greatly help us understand how breast cancer invades and metastasizes.

Forkhead box class O 3a (FOXO3a) belongs to the FOXO subclass of forkhead transcription factors and plays key roles in regulating various cellular processes such as proliferation, apoptosis, cell cycle and carcinogenesis [9]. Growing evidences show that FOXO3a serves as a tumor suppressor and is closely associated with cancer progression [9]. More-

**Abbreviations:** AKT, Protein kinase B; ATCC, American Type Culture Collection; CADM2, Cell Adhesion Molecule 2; CCK-8, Cell Counting Kit-8; DAPI, Diamidino-phenylindole; DMEM, Dulbecco's Modified Eagle's medium; ECL, Enhanced chemiluminescence; EMT, epithelial–mesenchymal transition; FAK, Focal adhesion kinase; FBS, Fetal bovine serum; FOXO3a, Forkhead box class O 3a; H&E, Haematoxylin and eosin; MiR-10b, MicroRNA 10b; miRNA, MicroRNA; PVDF, Polyvinylidene fluoride; RIPA, Radioimmunoprecipitation; RT-qPCR, Real-time quantitative reverse transcription-PCR; SD, Standard deviation; SPSS, Statistical Package for the Social Sciences; TWIST-1, Twist-related protein 1; WHO, World Health Organization.

\* Corresponding author.

E-mail address: [295105193@qq.com](mailto:295105193@qq.com) (Y.-L. Pan).

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**Table 1**  
Relationships between FOXO3a expression and clinico-pathologic parameters.

Variable	n = 48	FOXO3a expression		
		Low	High	p
<b>Age, years</b>				
≥60	33	15	8	0.3235
<60	15	7	8	
<b>Grade</b>				
Low	8	5	3	0.4548
High	40	18	22	
<b>Stage</b>				
I II	12	8	4	0.0011
III IV	36	9	17	
<b>Lymph node status</b>				
N0	39	10	29	0.0440
N1, N2	9	6	3	

over, FOXO3a overexpression could suppress the proliferation and invasiveness of cancer cells, while its silence could promote cancer progression [9]. FOXO3a has been identified as a potential biomarker for evaluating the prognosis of many cancers [10,11] and promising therapeutic target of chemotherapy drugs [12]. Twist-related protein 1 (TWIST-1) is a member of the basic helix-loop-helix transcription factor family and has been reported to facilitate cancer metastasis, including breast cancer, by acting as an EMT-inducing transcription factor [13,14]. TWIST-1 is commonly over-expressed in breast cancer and its overexpression indicates the poor prognosis of patients [15]. The signaling pathway regulated by TWIST-1 in breast cancer still remains largely unknown. MicroRNAs (miRNAs) generally function as pivotal post-transcriptional gene modulators to negatively regulate gene expression in many physiological processes [16]. Many researches have demonstrated that miRNAs could serve as oncogenic roles or tumor suppressors in breast cancer [17]. Growing evidences also indicated the role of miRNAs in regulating the metastasis of breast cancer cells by modulating EMT [18]. Although many advances have been made, the roles of miRNAs in the regulation of the EMT in breast cancer still need further investigations. MiR-10b is highly expressed in metastatic breast cancer cells and functionally contributes to cancer metastasis [19]. However, the mechanisms still remain largely unknown.

FOXO3a suppresses the invasion of the urothelial carcinoma by negatively regulating the expression of TWIST-1 [20]. TWIST-1 controls the expression of miR-10a/b in myelodysplastic syndromes [21]. MiR-10b directly targets cell adhesion molecule 2 (CADM2) to modulate EMT and promote cancer metastasis via the FAK/AKT signaling pathway in hepatocellular carcinoma [22]. However, the interaction of FOXO3a, TWIST-1 and miR-10b and their roles in the regulation of the EMT and metastasis of breast cancer are still unknown. Therefore, based on these observations, we proposed that FOXO3a might suppress the EMT and metastasis of breast cancer through regulating TWIST-1/miR-10b/CADM2 axis and activating FAK/AKT signaling pathway. In this study, we investigated the relationship between FOXO3a and TWIST-1 and explored their roles in the EMT and metastasis of breast cancer.

## Methods

### Clinical specimens

Breast cancer and normal mammary tissue specimens were collected from 48 patients who underwent surgery between Sep 2016 and Dec 2018 at the Department of General Surgery, the Third Xiangya Hospital of Central South University. All specimens were immediately frozen in liquid nitrogen or fixed in formaldehyde for subsequent application. Clinical pathology materials were also collected (Table 1). All cases were diagnosed, classified and graded according to the WHO Classification of Breast Tumors (2003). The axillary lymph node metastasis was

recorded. This study was performed with the approval of the Ethics and Research Committees of the Third Xiangya Hospital of Central South University. Written informed consent was obtained from all patients.

### Hematoxylin and eosin (H&E) and Immunohistochemical (IHC) staining

Normal mammary and carcinoma tissues were harvested from patients. Tumor tissues were collected from mice inoculated with MCF-7 or MDA-MB-231 cells. Tissues were fixed, dehydrated and embedded in paraffin. Then tissues were cut into 5 μm tissue sections. Sections were de-paraffinized and rehydrated. For H&E staining, sections stained with haematoxylin and eosin (H&E). For analyzing the expression of FOXO3a, CADM2 or TWIST-1, sections were antigen retrieved and incubated with anti-FOXO3a, CADM2 or TWIST-1 primary antibody (1:50, Cell signaling Technology, Boston, MA, USA). All sections were observed with an Olympus BX-60 microscope (Tokyo, Japan).

### Cell culture

Human mammary non-tumorigenic epithelial cell line MCF 10A, and breast cancer cell lines MCF-7 (ER+, PR+, HER2-, luminal A), MDA-MB-231 (ER-, PR-, HER2-, Triple negative A), SK-BR-3 (ER-, PR-, HER2 positive), T47D (ER+, PR+, HER2-, luminal A) and BT474 (ER+, PR+, HER2+, luminal A+) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in the Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator supplemented with 5% CO<sub>2</sub>. All reagents used in cell culture were ordered from ThermoFisher Scientific (Waltham, MA, USA).

### Cell transfection

MCF-7, MDA-MB-231 or SK-BR-3 cells were seeded in 6-well plates, grown to 80–90% confluency and transfected with FOXO3a overexpressing construct, control vector, siRNAs against TWIST-1 (si-TWIST1) or siRNA control (si-NC), respectively. For rescue experiments, cells were co-transfected with FOXO3a and TWIST-1 overexpressing constructs. 48 h later, cells were harvested for subsequent assays. siRNAs against FOXO3a (NM\_001455) and TWIST-1 (NM\_000474) were synthesized from Sigma-Aldrich (St. Louis, MO, USA), and FOXO3a overexpressing plasmids were synthesized by Shanghai Generay Company (Shanghai, China). MiR-10b mimics and mimics NC were obtained from Genepharma (Shanghai, China).

### Real-time quantitative reverse transcription-PCR (RT-qPCR)

Total RNA was extracted from cancer tissues from patients and mice inoculated with breast cancer cells including MCF-7, MDA-MB-231 and SK-BR-3 with TRIzol reagent (ThermoFisher Scientific), which were then reversely transcribed into cDNA. MiR-10b was reverse-transcribed with TaqMan microRNA reverse transcription kit (ThermoFisher Scientific). The relative expression of FOXO3a, TWIST-1 and miR-10b were analyzed using SYBR Green QPCR Master Mix (Toyobo, Osaka, Japan). Results were normalized to GAPDH or U6. The primers used in this study were in the Table 2.

### Cell Counting Kit-8 (CCK-8) assay

The cell proliferation was examined with CCK-8 kit (Sigma-Aldrich). MCF-7 and MDA-MB-231 cells were transfected with indicated constructs. 10 μl of CCK-8 was directly added into cells (100 μl/well) and incubated at 37 °C for 4 h. The absorbance at 490 nm (OD490) was measured with a microplate reader.

**Table 2**  
The primers used in this study.

FOXO3a	5'-CGGGCAGCCGAGGAAATGTT-3' 5'-TGTTGCTGTCGCCCTTATCCTT-3'
TWIST-1	5'-GGCTCAGCTACGCCCTTCTC-3' 5'-TCCTTCTCTGGAAACAATGACA-3'
CADM2	5'-TCTATTCCAACAAGTCAGAAAATAATG-3' 5'-CGCTTAGACTTGATTTGACGG-3'
GAPDH	5'-GGATTGGTCTGATTGGG-3' 5'-GGAAGATGGTGATGGGATT-3'
MiR-10b	5'-TACCCTGTAGAACCGAATTTGTG-3' 5'-CAGTCCGTCTGCTGGACT-3'
U6	5'-CTTCGGCAGCACATATACT-3' 5'-AAAATATGGAACGCTTACG-3'

### Cell migration and invasion assays

Cell migration ability was examined in a transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA). Cells were plated into the upper chamber. After 24 h, the migratory cells which penetrated to the lower surface were fixed and stained with crystal violet (Sigma-Aldrich). For invasion assay, a matrix gel was pre-coated on the poly-carbonate membranes of the upper chamber. Cells were observed with a BX-60 microscope (Olympus).

### Immunofluorescence assay

MCF-7 and MDA-MB-231 cells were transfected with indicated constructs and plated on chamber slides. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.15% Triton X-100 at room temperature. Then cells were washed, blocked with 5% BSA (in PBS) and incubated with anti-E-cadherin (1:500, Cell signaling Technology) and anti-N-cadherin (1:800, Cell signaling Technology) antibody at 4 °C for 16 h. Cells were then probed with Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibodies (ThermoFisher Scientific), stained with DAPI and mounted on microscopic slides. Cells were imaged using a Leica confocal system (Wetzlar, Germany).

### Western blot

Total protein was extracted from MCF-10A cells and breast cancer cells including MCF-7, MDA-MB-231, SK-BR-3, T47D and BT474 cells or mouse cancer tissues using RIPA lysis buffer (Abcam, Cambridge, UK). 50  $\mu$ g of cell lysates were electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA, USA). Membranes were then blocked and incubated with primary antibodies against TWIST-1 (1:1000), CADM2 (1:800), FAK (1:500), AKT (1:1000), phospho-AKT (1:500), E-cadherin (1:2000), N-cadherin (1:1000), FOXO3a (1:1000), Vimentin (1:500) and GAPDH (1:5000) at 4 °C overnight. Membranes were washed and probed with HRP-conjugated secondary antibodies. All the antibodies were purchased from Abcam (Jinan, China). ECL substrate was used to visualize the blots. The relative band intensity was analyzed with image J software (NIH).

### Dual-luciferase reporter assay

TWIST-1 or pre-miR-10b promoter was inserted into the pGL3-Basic vector (Promega, Madison, WI, USA). MCF-7 and MDA-MB-231 cells were co-transfected with TWIST-1 reporter and FOXO3a overexpressing construct or vector control. SK-BR-3 cells were co-transfected with TWIST-1 reporter and siFOXO3a or siNC. The wild-type or mutated binding site of miR-10b in the 3'UTR of CADM2 was cloned into the pmirGLO vector (Promega). MCF-7 and MDA-MB-231 cells were co-transfected with miR-10b reporter and siTWIST-1 or siNC. MCF-7 and MDA-MB-231 cells were co-transfected with CADM2 reporter and miR-10b mimics

or mimics NC. 48 h post transfection, luciferase activity was examined using the Dual-Glo Luciferase Assay System (Promega). The firefly luciferase activity was normalized to renilla luciferase activity.

### Chromatin Immunoprecipitation (ChIP) assay

SK-BR-3 and MDA-MB-231 cells with indicated transfection were crosslinked using 1% formaldehyde and lysed. DNA fragments were obtained by sonication. Subsequently, the lysates were subjected to immunoprecipitation with a FOXO3a antibody. A normal IgG isotype was used as a control. The recovered DNA was amplified with PCR and electrophoresed or used for qPCR to examine its enrichment.

### Mouse models of breast cancer

Female nude mice (8–10 weeks) were randomly divided into 10 groups (7 mice per group): MCF-7-Control, MCF-7-Vector, MCF-7-FOXO3a, MCF-7-TWIST-1, MCF-7-FOXO3a+TWIST-1, MDA-MB-231-Control, MDA-MB-231-Vector, MDA-MB-231-FOXO3a, MDA-MB-231-TWIST-1 and MDA-MB-231-FOXO3a+TWIST-1. MCF-7 and MDA-MB-231 FOXO3a/TWIST-1 groups were inoculated with MCF-7 or MDA-MB-231 cells with stably overexpressed FOXO3a/TWIST-1. MCF-7 and MDA-MB-231 FOXO3a+TWIST-1 groups were inoculated with MCF-7 or MDA-MB-231 cells with stably overexpressed FOXO3a and TWIST-1. MCF-7 and MDA-MB-231 Vector groups were inoculated with MCF-7 or MDA-MB-231 cells transfected with adenovirus control construct.  $1 \times 10^6$  exponentially growing cells were implanted into the right chest mammary fat pad area. The tumor formation rate was 100%. The volume of tumor was monitored every 4 days. 4 weeks after implantation, tumor tissues were collected for subsequent analysis following animal sacrifice. For metastasis analysis,  $1 \times 10^6$  aforementioned MCF-7 or MDA-MB-231 cells were suspended and injected to mice via the tail vein. Eight weeks post injection, mice were sacrificed and the lungs were excised for imaging and HE and IHC staining. The metastatic nodules were counted. Procedures were approved by the Institutional Animal Care and Use Committee of the Third Xiangya Hospital of Central South University and conducted in accordance with the National Institutes of Health guidelines.

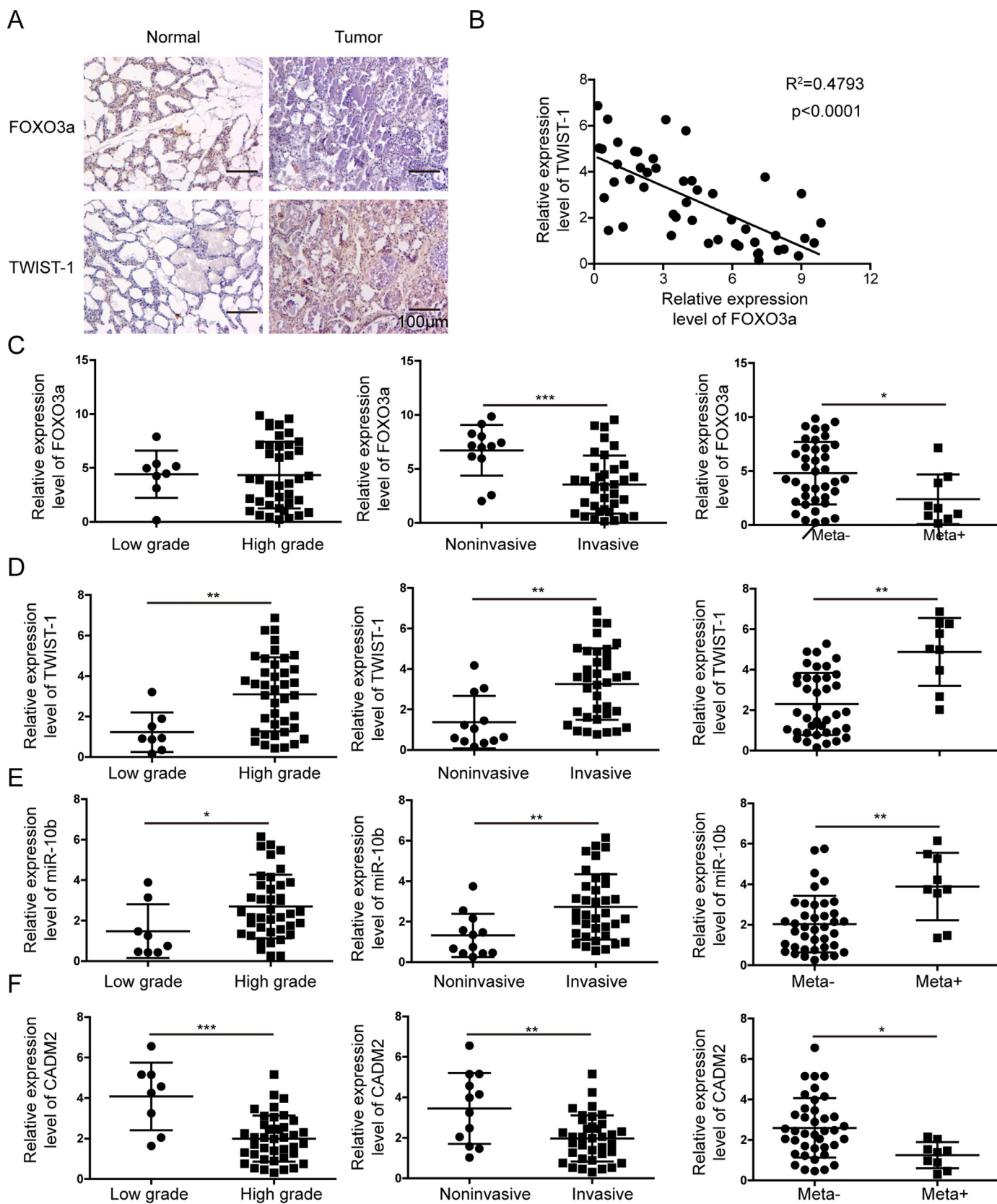
### Statistical analysis

All data in this study was analyzed with SPSS 22.0 software (IBM, Armonk, NY, USA) and presented as mean  $\pm$  standard deviation (SD) of at least three independent experiments. The Student's *t*-test for comparing paired independent groups, the  $\chi^2$  test for enumeration data and Pearson correlation analysis for correlation test were performed for statistical analysis.  $p < 0.05$  was considered statistically significant.

### Results

#### FOXO3a, TWIST-1, miR-10b and CADM2 were associated with the progression of breast cancer

To investigate the roles of FOXO3a and TWIST-1 in breast cancer, we collected normal mammary and tumor tissues from patients who were diagnosed with breast cancer. All cases were diagnosed, classified and graded according to the WHO Classification of Breast Tumors (2003). Clinical pathology materials and the axillary lymph node metastasis were also recorded. As shown in Table 1, the expression of FOXO3a was correlated with tumor stage and lymph node metastasis of breast cancer, but not age and tumor grade. FOXO3a was highly expressed in normal mammary tissues but downregulated in tumor tissues. On the contrary, TWIST-1 was suppressed in normal mammary tissues but up-regulated in tumor tissues (Fig. 1A). Then, we examined the expression of FOXO3a and TWIST-1 and analyzed their relationship. We found that



**Fig. 1.** FOXO3a, TWIST-1, miR-10b and CADM2 were associated with the progression of breast cancer. (A) Immunohistochemical staining of FOXO3a and TWIST-1 in normal mammary and breast cancer tissues from patients. (B) Correlation analysis of the expression of FOXO3a and TWIST-1 in patients ( $n = 48$ ). The relationship between the expression of FOXO3a (C), TWIST-1 (D), miR-10b (E) or CADM2 (F) and the grade (low and high), invasive (noninvasive and invasive) or metastasis (Meta- and Meta+) of breast cancer ( $n = 48$ ). All data were from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to controls. Error bars indicate the mean  $\pm$  standard deviation (SD).

the expression of FOXO3a was negatively correlated with the expression of TWIST-1 in patients (Fig. 1B). The expression of FOXO3a and CADM2 were negatively correlated with the grade, invasive potential and metastasis of breast cancer (Fig. 1C and F). Conversely, the expression of TWIST-1 and miR-10b were positively correlated with the grade, invasive potential and metastasis (Fig. 1D and E). These observations suggested that FOXO3a, TWIST-1, miR-10b and CADM2 might be associated with the progression of breast cancer.

#### *Overexpression of FOXO3a suppressed the EMT and metastasis of breast cancer*

To further study the role of FOXO3a in breast cancer, we analyzed the expression of FOXO3a in non-tumorigenic mammary epithelial cell MCF 10A and breast cancer cells including MCF-7, MDA-MB-231, SK-BR-3, T47D and BT474. Compared with MCF 10A cells, all breast cancer cells showed decreased expression of FOXO3a (Fig. 2A). MCF-7 and MDA-MB-231 cells were selected for subsequent analysis due to the lowest expression of FOXO3a. We overexpressed FOXO3a in MCF-7 and MDA-MB-231 cells and found that the expression of FOXO3a was significantly upregulated and TWIST-1 and miR-10b were significantly inhibited in FOXO3a overexpressing cells (Fig. 2B). Cell proliferation was suppressed by FOXO3a overexpression (Fig. 2C). In addition, overexpression of FOXO3a also inhibited the invasive and migration potential of MCF-7 and MDA-MB-231 cells (Fig. 2D), indicating that FOXO3a might contribute to attenuating the invasion and metastasis of breast cancer. Then we examined the expression of the hallmark of the EMT, E-cadherin to N-cadherin switch. E-cadherin was downregulated and N-cadherin was up-regulated in MCF-7 and MDA-MB-231 cells transfected with vectors, while overexpression of FOXO3a completely reversed this expression patterns (Fig. 2E). Moreover, the protein expression of N-cadherin and vimentin were decreased, and E-cadherin was increased upon FOXO3a overexpression (Fig. 2F). FOXO3a overexpressing MCF-7 and MDA-MB-231 cells showed reduced expression of TWIST-1 and enhanced expression of CADM2 (Fig. 2F). As the FAK/AKT signal pathway plays an important role in the EMT of cancers [23,24], we analyzed and found that FOXO3a overexpression could enhance the expression of FAK and phosphorylation level of AKT (Fig. 2F). In addition, we knocked-down FOXO3a in SK-BR-3 cells with high expression of FOXO3a. We found that, FOXO3a silence increased the expression of TWIST-1, N-cadherin, Vimentin and miR-10b and reduced the expression of FOXO3a, CADM2, FAK, p-AKT and E-cadherin in SK-BR-3 cells (Fig. S1A-B). These results raised the possibility that FOXO3a might regulate the EMT and metastasis of breast cancer through the TWIST-1/miR-10b/CADM2/FAK/AKT axis.

#### *Knockdown of TWIST-1 inhibited the EMT and metastasis of breast cancer*

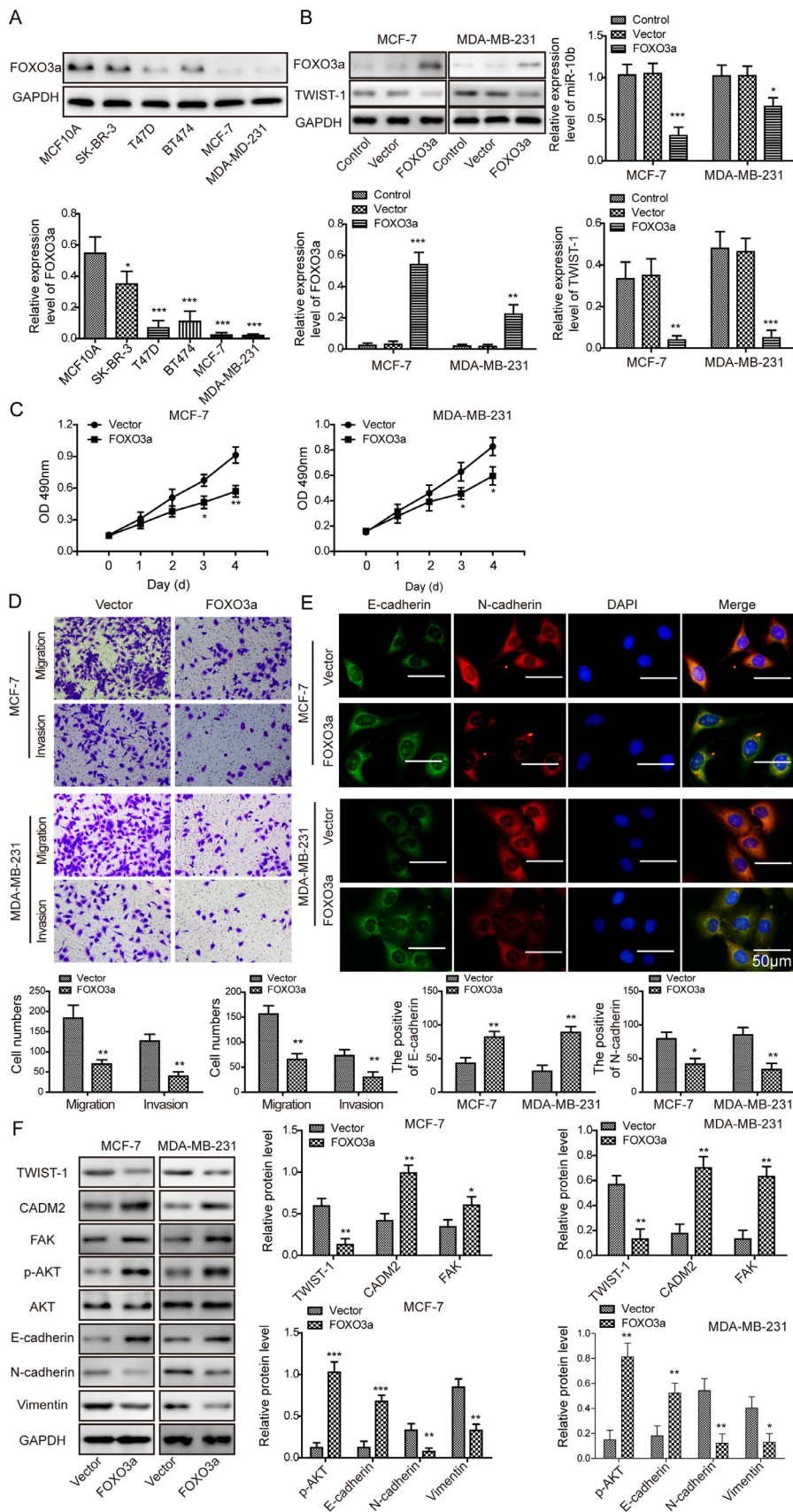
As TWIST-1 might be the mediator of FOXO3a to exert its role in the regulation of the metastasis in breast cancer, TWIST-1 was knocked-down efficiently in MCF-7 and MDA-MB-231 cells (Fig. 3A). Knockdown of TWIST-1 decreased the expression of miR-10b (Fig. 3A), which has been previously reported to play important roles in the metastasis of breast cancer [19]. The proliferation, invasive and migration potential of MCF-7 and MDA-MB-231 cells were significantly suppressed by knockdown of TWIST-1 (Fig. 3B-C). In addition, knockdown of TWIST-1 attenuated the EMT process of MCF-7 and MDA-MB-231 cells (Fig. 3D). The protein expression of CADM2, FAK, p-AKT and E-cadherin were increased, and Vimentin and N-cadherin were downregulated in MCF-7 and MDA-MB-231 cells with knockdown of TWIST-1 (Fig. 3E). These data demonstrated that TWIST-1 might promote the EMT and metastasis of breast cancer through miR-10b/CADM2/FAK/AKT signal pathway.

#### *FOXO3a suppressed the EMT and metastasis of breast cancer via TWIST-1/miR-10b/CADM2 axis*

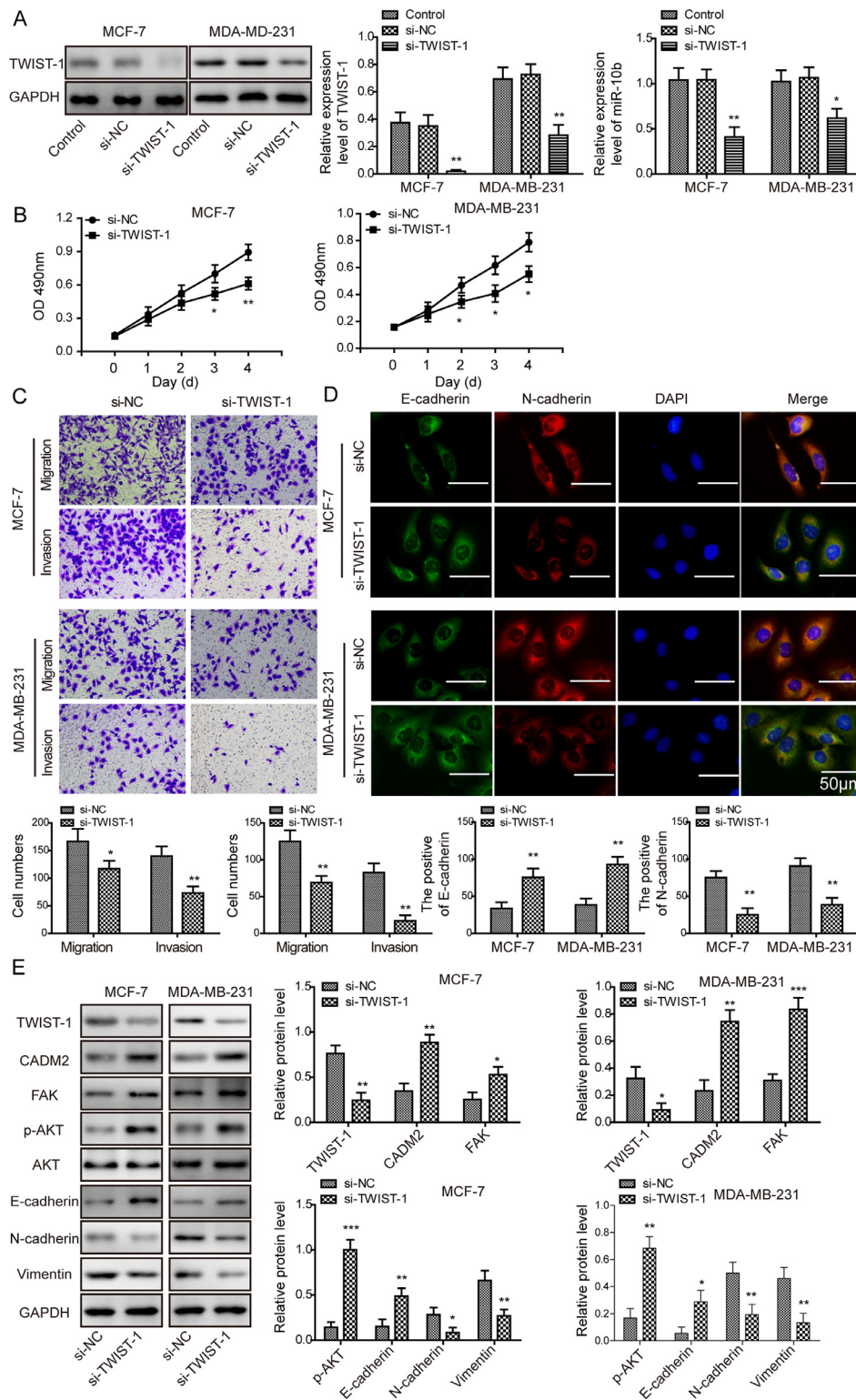
To investigate whether FOXO3a exerted its role through regulating the TWIST-1/miR-10b/CADM2 axis, we overexpressed FOXO3a and TWIST-1 in MCF-7 and MDA-MB-231 cells. In MCF-7 and MDA-MB-231 cells with FOXO3a overexpression, the expression of TWIST-1 and miR-10b were obviously decreased and the expression of CADM2 was promoted (Fig. 4A). However, overexpression of TWIST-1 restored the expression of TWIST-1, miR-10b and CADM2 to normal levels in MCF-7 and MDA-MB-231 cells with FOXO3a overexpression (Fig. 4A). The suppressive effects mediated by FOXO3a overexpression on the proliferation, invasion and migration of MCF-7 and MDA-MB-231 cells were reversed by overexpression of TWIST-1 (Fig. 4B,C). Besides, FOXO3a overexpression-mediated suppressive EMT, increased protein expression of CADM2 and FAK and phosphorylation levels of AKT were all reversed by overexpression of TWIST-1 (Fig. 4D). To investigate the mechanism by which FOXO3a regulated TWIST-1, luciferase assays showed that FOXO3a overexpression inhibited the luciferase activity of TWIST-1 reporter in MCF-7 and MDA-MB-231 cells, and FOXO3a knockdown enhanced the luciferase activity of TWIST-1 reporter in SK-BR-3 cells (Fig. S1C). These results suggested FOXO3a directly bound to the promoter of TWIST-1 to inhibit its expression in breast cancer cells. Besides, we performed a ChIP assay using a FOXO3a antibody. In FOXO3a knockdown SK-BR-3 cells, the recruitment of FOXO3a to the promoter of TWIST-1 was significantly reduced compared to that in shNC transfected cells (Fig. S2A-B). In addition, in MDA-MB-231 cells with FOXO3a overexpression, the recruitment of FOXO3a to the promoter of TWIST-1 was dramatically higher than in vector transfected cells (Fig. S2A-B). Furthermore, we found that TWIST-1 knockdown impaired the luciferase activity of wild-type pre-miR-10b promoter reporter, but not that of mutated one (Fig. S1D). In addition, the luciferase activity was significantly reduced in cells co-transfected with miR-10b mimics and wild-type CADM2 3'UTR construct but unaffected with mutated one, which demonstrated that miR-10b directly targeted the 3'-UTR of CADM2 (Fig. S1E). TWIST-1 was silenced alone or in combination with overexpression of miR-10b in MDA-MB-231 cells with low expression of FOXO3a. Knockdown of TWIST-1 inhibited the expression of miR-10b, N-cadherin and Vimentin and promoted the expression of CADM2, FAK, p-AKT, AKT and E-cadherin, which were all reversed by simultaneous overexpression of miR-10b (Fig. S2C-D). Additionally, TWIST-1 was overexpressed or/and miR-10b was silenced in SK-BR-3 cells with high expression of FOXO3a. Overexpression of TWIST-1 promoted the expression of miR-10b, N-cadherin and Vimentin and suppressed the expression of CADM2, FAK, p-AKT, AKT and E-cadherin, and simultaneous knockdown of miR-10b abrogated these effects (Fig. S2C-D). These data implied that FOXO3a might inhibit the EMT and metastasis of breast cancer by regulating TWIST-1/miR-10b/CADM2 axis and activating the FAK/AKT signaling pathway.

#### *FOXO3a suppressed the growth and metastasis of breast cancer by targeting TWIST-1 in vivo*

To evaluate whether FOXO3a and TWIST-1 affect tumorigenesis *in vivo*, we constructed MCF-7 and MDA-MB-231 cells with stable expression of FOXO3a, TWIST-1 or both and injected them into mammary pads of female nude mice. Our results showed that mammary tumor growth was obviously reduced by overexpression of FOXO3a but significantly accelerated by TWIST-1 overexpression (Fig. 5A). Intriguingly, FOXO3a-mediated suppression of tumor growth was abrogated by simultaneous overexpression of TWIST-1 (Fig. 5A). In control and vector groups, most cancer cells were ovoid with obvious nucleolus and mitosis, and no obvious necrosis was observed (Fig. 5B). In addition to these features, increased cancer cells were observed in TWIST-1 groups (Fig. 5B). However, cancer cells in the FOXO3a groups showed fuzzy nucleolus morphology, obvious karyopyknosis and large necrotic ar-



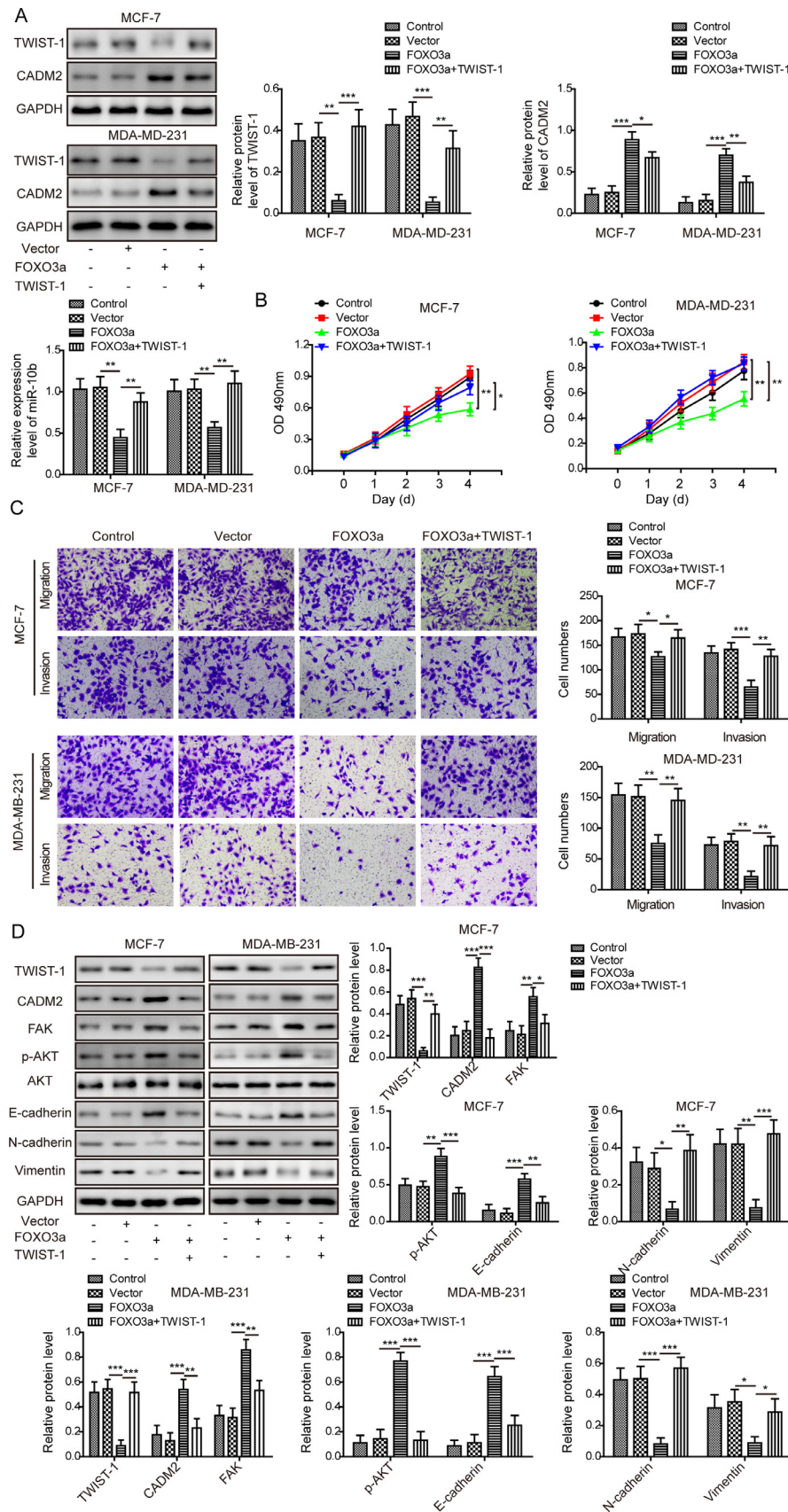
**Fig. 2.** FOXO3a overexpression inhibited the EMT and metastasis of MCF-7 and MDA-MB-231 cells. (A) Western blot analysis of FOXO3a in MCF10A, MCF-7, MDA-MB-231, SK-BR-3, T47D and BT474 cells ( $n = 3$ ). (B) Western blot analysis of FOXO3a and TWIST-1 and RT-qPCR analysis of miR-10b in MCF-7 and MDA-MB-231 cells transfected with FOXO3a overexpressing construct or control vector ( $n = 3$ ). (C) Proliferation analysis of MCF-7 and MDA-MB-231 cells transfected with FOXO3a overexpressing construct or control vector using the CCK-8 assay ( $n = 3$ ). (D) Transwell analysis of MCF-7 and MDA-MB-231 cells transfected with FOXO3a overexpressing construct or control vector ( $n = 3$ ). (E) Immunofluorescence staining of E-cadherin (green) and N-cadherin (red) in MCF-7 and MDA-MB-231 cells transfected with FOXO3a overexpressing construct or control vector ( $n = 3$ ). DAPI was used for nuclear staining (blue). Scale bar, 50  $\mu\text{m}$ . (F) Western blot analysis of TWIST-1, CADM2, FAK, AKT, phosphorylated-AKT (p-AKT), E-cadherin, N-cadherin and Vimentin in MCF-7 and MDA-MB-231 cells transfected with FOXO3a overexpressing construct or control vector ( $n = 3$ ). All data were from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  respectively, compared to controls. Error bars indicate the mean  $\pm$  standard deviation (SD).



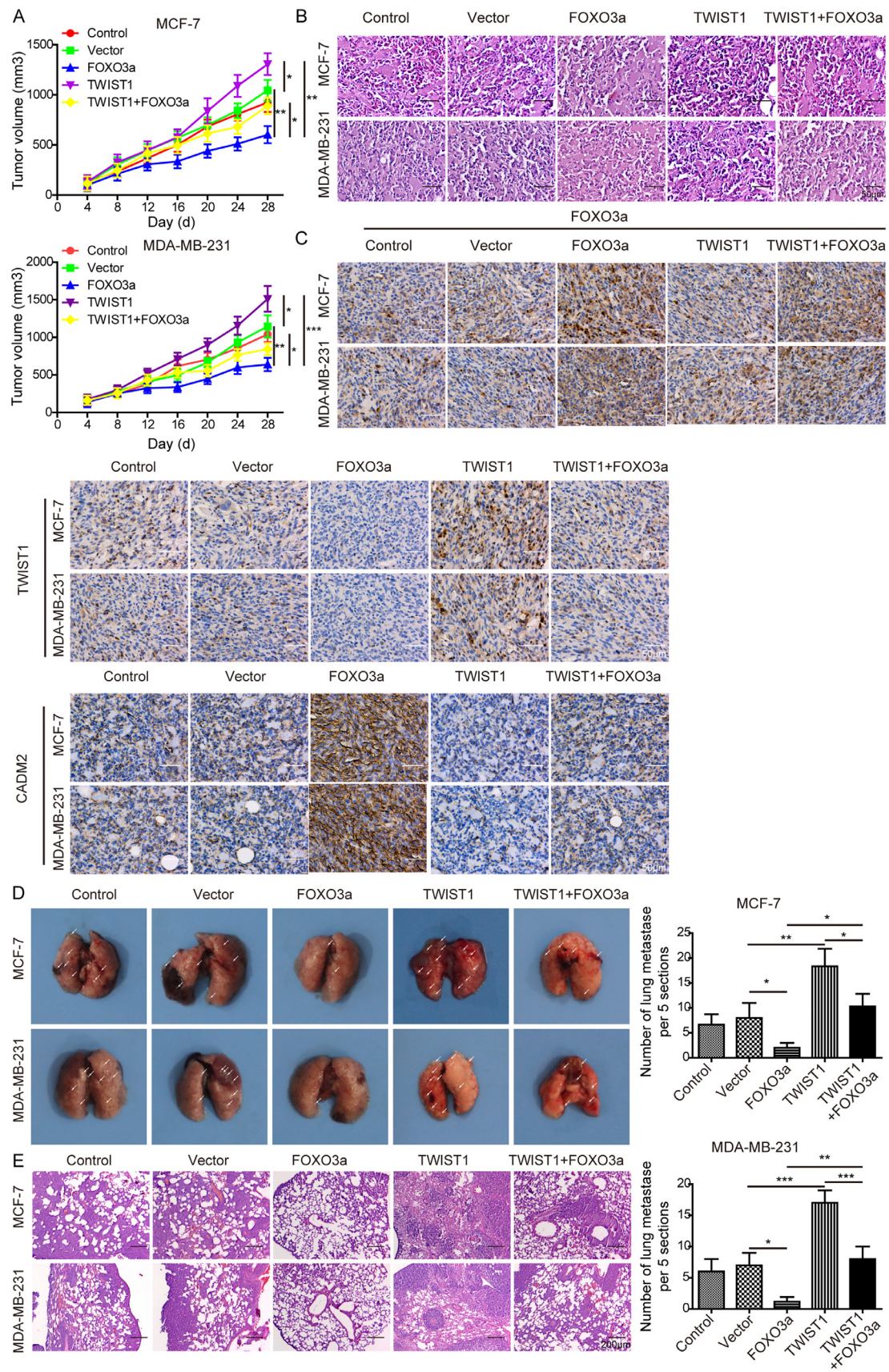
**Fig. 3.** Knockdown of TWIST-1 suppressed the EMT and metastasis of MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were transfected with siRNAs against TWIST-1 or control siRNA. (A) Western blot analysis of TWIST-1 and RT-qPCR analysis of miR-10b in MCF-7 and MDA-MB-231 cells ( $n = 3$ ). (B) Proliferation analysis of MCF-7 and MDA-MB-231 cells using the CCK-8 assay ( $n = 3$ ). (C) Transwell analysis of MCF-7 and MDA-MB-231 cells ( $n = 3$ ). (D) Immunofluorescence staining of E-cadherin (green) and N-cadherin (red) in MCF-7 and MDA-MB-231 cells ( $n = 3$ ). DAPI was used for nuclear staining (blue). Scale bar, 50  $\mu$ m. (E) Western blot analysis of TWIST-1, CADM2, FAK, AKT, phospho-AKT, E-cadherin, N-cadherin and Vimentin in MCF-7 and MDA-MB-231 cells ( $n = 3$ ). All data were from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  respectively, compared to controls. Error bars indicate the mean  $\pm$  standard deviation (SD).

neas, and overexpression of TWIST-1 reversed FOXO3a-mediated alleviative pathological features (Fig. 5B). Compared with control and vector groups, FOXO3a groups showed very high expression of FOXO3a, decreased expression of TWIST-1 and increased expression of CADM2 (Fig. 5C). Conversely, FOXO3a and CADM2 were downregulated and TWIST-1 was upregulated in TWIST-1 groups (Fig. 5C). Overexpression of TWIST-1 reversed FOXO3a-mediated regulation of the expression of TWIST-1 and CADM2 (Fig. 5C). Furthermore, we investigated

whether FOXO3a and/or TWIST-1 overexpression regulated pulmonary metastasis of MCF-7 and MDA-MB-231 cells by intravenously injecting control, vector, FOXO3a, TWIST-1 or FOXO3a+TWIST-1 transfected cells into nude mice. Overexpression of TWIST-1 enhanced pulmonary metastasis (Fig. 5D). However, FOXO3a overexpression obviously reduced the number of tumor nodules in the lung, which was reversed by TWIST-1 overexpression (Fig. 5D), indicating that FOXO3a inhibited breast cancer cell metastasis by targeting TWIST-1 *in vivo*. H&E staining







**Fig. 5.** FOXO3a suppressed the growth and metastasis of breast cancer *in vivo*. MCF-7 and MDA-MB-231 cells transfected with vector, FOXO3a, TWIST-1 or FOXO3a in combination with TWIST-1 or untransfected cells were injected into mammary pads of female nude mice. (A) Tumor volume ( $n = 7$  per group). (B) H&E staining of mammary tumor sections. (C) Immunohistochemical staining of FOXO3a, TWIST-1 and CADM2 in mammary tumor tissues from nude mice. (D) Pulmonary metastasis and its quantification ( $n = 7$  per group). (E) H&E staining of lung sections. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  respectively, compared to controls. Error bars indicate the mean  $\pm$  standard deviation (SD).

showed decreased lung metastases in FOXO3a groups but increased lung metastases in TWIST-1 groups (Fig. 5E). FOXO3a-mediated suppressive effect on metastasis was abolished by overexpression of TWIST-1. Taken together, these observations suggested that FOXO3a suppressed the growth and metastasis of breast cancer by targeting TWIST-1 *in vivo*.

## Discussion

Breast cancer affects millions of patients all over the world and has attracted a great deal of attention in recent years. Growing advances have been made recently. For example, primary associated risk factors, including age, race, family history, obesity, alcohol use and hormone level, have been identified [25]. Along with the advances in early diagnosis and systemic therapy, most of patients with early non-metastatic breast cancer is considered potentially curable [1,26]. However, metastatic breast cancer is still considered incurable with high mortality [27]. Consequently, exploring the mechanisms of the metastasis is key to develop more effective therapies against breast cancer. EMT is the first step of the metastasis cascade to facilitate the escape of cancer cells from the primary tumor [28]. In this study, we demonstrated that FOXO3a suppressed the EMT and metastasis of breast cancer potentially by down-regulating the expression of TWIST-1 and controlling the expression of miR-10b and CADM2 for the first time. In addition, we also firstly found that the FAK/AKT signal pathway, which serves key roles in cancer metastasis [29,30], might be associated with FOXO3a/TWIST-1/miR-10b/CADM2 axis-mediated regulation of the EMT of breast cancer. Coming with other studies, our data revealed vital roles of the FOXO3a/TWIST-1/miR-10b/CADM2 axis in the EMT and metastasis of breast cancer and indicated FOXO3a/TWIST-1/miR-10b/CADM2 axis and FAK/AKT signal pathway could be considered as potential molecular targets for developing better treatment against metastatic breast cancer.

As important regulators in the EMT and metastasis of cancers, FOXO3a and TWIST-1 have attracted much attention from scientists recently. Knockdown of FOXO3a could facilitate the EMT and metastasis of pancreatic ductal adenocarcinoma [31]. TWIST-1 promotes the EMT and metastasis in several cancers including breast cancer [32,33]. Besides, FOXO3a suppresses the invasion of urothelial cancer by negatively regulating the expression of TWIST-1 [20]. Even so, the relationship between FOXO3a and TWIST-1 and their role in the regulation of the EMT of breast cancer are still unclear. In our study, the aberrant expression of FOXO3a in patients and clinicopathological characteristics indicated the association of FOXO3a with the metastasis of breast cancer. For the first time, we reported that both overexpression of FOXO3a and knockdown of TWIST-1 inhibited the EMT and metastasis of breast cancer. FOXO3a might negatively regulate TWIST-1 expression to suppress the metastasis and EMT of breast cancer. Emerging evidences have suggested that EMT is not always a binary process, and cells rarely undergo a full EMT and may exhibit a hybrid epithelial/mesenchymal phenotype [34,35]. Therefore, although we observed that most cells showed uniform change of the expression of E-cadherin and N-cadherin, more single-cell experiments need to be performed to quantify the extent of EMT at the single-cell level in our further studies.

The expression level of miR-10b correlates not only with the metastatic potential of breast cancer [19], but also with the expression of TWIST-1, which activates the transcription of miR-10b [36]. Previous studies implied that miR-10b might be the mediator of FOXO3a/TWIST-1 axis to exert functions in the metastasis and EMT of breast cancer. Indeed, both overexpression of FOXO3a and knockdown of TWIST-1 could inhibit the expression of miR-10b. Overexpression of TWIST-1 could promote the expression of miR-10b. CADM2, a recently identified target of miR-10b, was up-regulated by overexpression of FOXO3a or knockdown of TWIST-1. Based on these results, we firstly raised up the possibility that FOXO3a suppressed the EMT and metastasis of breast cancer by regulating TWIST-1-mediated miR-10b/CADM2 axis. The FAK/AKT signaling pathway might contribute to the regulation of FOXO3a in EMT

and metastasis of breast cancer as we observed the change of their phosphorylation levels. However, it still needs further investigation.

In summary, in this study, we demonstrated that FOXO3a suppressed the EMT and metastasis of breast cancer by controlling TWIST-1 mediated miR-10b/CADM2/FAK/AKT axis for the first time. We not only reveal novel regulatory mechanism of the metastasis of breast cancer, but also provide potential targets for developing novel strategies for treating breast cancer. Based on our data, Targeting FOXO3a/TWIST-1/miR-10b/CADM2 axis could be a new strategy to treat breast cancer. In future studies, it will be interesting to explore potential avenues to target FOXO3a/TWIST-1/miR-10b/CADM2 axis for therapeutic gains.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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## Ethics statement

All cases were diagnosed, classified and graded according to the WHO Classification of Breast Tumors (2003). This study was performed with the approval of the Ethics and Research Committees of the Third Xiangya Hospital of Central South University.

Procedures were approved by the Institutional Animal Care and Use Committee of the Third Xiangya Hospital of Central South University and conducted in accordance with the National Institutes of Health guidelines.

## Author Contributions Statement

Long Jin: experimental studies, data acquisition, data analysis, preparation; Jun Zhang: editing; Hui-Qun Fu: editing; Xi Zhang: design; Yu-Liang Pan: concepts, review, supervision;

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101096.

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