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Overexpression of Forkhead Box J2 Can Decrease The Migration of Breast Cancer Cells

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ABSTRACT

The prognosis of breast cancer patients with metastases is generally poor, so it is essential to elucidate related molecules mechanisms. Forkhead Box J2 (FOXJ2) is a member of Forkhead Box transcription factors, many of which have been reported to participate in tumor migration and invasion. In this study, we showed the expression of FOXJ2 was higher in primary breast cancer tissues without lymph nodes metastases than those with, and there was statistical significance between the expression of FXOJ2 and the clinical factors. Hence, we identified a novel function of metastasis, which was not previously known for FOXJ2. Overexpression of FOXJ2 decreased the motility property of highly migrative MDA-MB-231 cells in vitro by wound healing assays and trans-well migration assays, and it was concurrent with the increased expression of epithelial marker E-cadherin and the decreased expression of mesenchymal marker vimentin by Western blot analysis, reverse transcription PCR analysis, and immunofluorescence analysis. Consistent with these observations, the repression of FOXJ2 in weakly metastatic MCF-7 cells remarkably promoted cellular motility. Our study demonstrates that FOXJ2 can inhibit the metastasis of human breast cancer by regulating the EMT key markers E-cadherin and vimentin. J. Cell. Biochem. 113: 2729–2737, 2012.

KEY WORDS: FORKHEAD BOX J2 (FOXJ2); BREAST CANCER CELLS; METASTASES; E-CADHERIN

Tumor cells generally disseminate from the site of the primary tumors and establish secondary tumors in distant organs by metastasis, which is one of the six distinct hallmarks of cancer [Hanahan and Weinberg, 2000, 2011]. Clinically defined, metastasis is the major cause of lethality among human cancer patients, including those with breast cancer [Nicolini et al., 2006; Eccles and Welch, 2007]. Breast cancer is the leading type of cancer occurring in women. And it remains the second largest killer of women by cancer [Hinestrosa et al., 2007; Chan et al., 2008b]. Patients who cannot be cured are those in whom breast cancer has metastasized, which refers to the migration and invasion of breast cancer cells to other organs such as lung and bone [Xie et al., 2009]. The molecular mechanism underlying metastasis remains unclear. Hence, identifying functional metastasis genes and their molecular mechanisms underlying the metastatic process remains a top priority in the cancer research field.

Forkhead Box (Fox) family, of which there are more than 100 members [Coffer and Burgering, 2004], share a common Forkhead

Abbreviation used: FOXJ2, Forkhead Box J2; RT-PCR, reverse transcription polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Yingying Wang and Shuyun Yang contributed equally to this work.

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DNA binding domain [Granadino et al., 2000; Martin-de-Lara et al., 2008], which is a variant of the helix-turn-helix motif firstly identified in Drosophila [Weigel et al., 1989]. Most members have been identified in different organs and tissues, from yeast to humans, with the notably exception of green plants [Granadino et al., 2000]. These transcription factors have been proven important in many processes such as cell proliferation and differentiation [Birkenkamp and Coffer, 2003; Di Pietro et al., 2010], survival for embryonic development [Kalinichenko et al., 2003; Ustiyan et al., 2009], neural systems [Kalinichenko et al., 2003], or signal transduction [Kashii et al., 2000].

Fox genes have been causally linked to multiple cellular processes, which are often abnormal in cancer cells. Some examples are FOXA2 and FOXF1 in lung cancer [Malin et al., 2007; Tang et al., 2011], Foxp3 in epithelial ovarian cancer [Zhang and Sun, 2010], FOXO3a in urothelial cancer [Shiota et al., 2010], FOXQ1 in colorectal carcinoma [Kaneda et al., 2010], FOXA1 in prostate cancer, pancreatic cancer and breast cancer [Song et al., 2010; Mehta et al., 2011; Sahu et al., 2011], and FOXM1 in many kinds of cancers [Chan et al., 2008a; Zeng et al., 2009; Ahmad et al., 2010; Bao et al., 2011; Lok et al., 2011]. Focusing on breast cancer, one study displayed, after MDA-MB-231 cells stably transfected by FOXC1, the migration and invasion of the cells were repressed. Moreover, the tumorigenicity and the spontaneous metastatic capability regulated by FOXC1 were determined by using an orthotropic xenograft tumor model of athymic mice with the FOXC1-MDA-MB-231HM cells and the GFP-MDA-MB-231HM cells. And the results showed that FOXC1 in MDA-MB-231HM cells inhibited migration and invasion in vitro and reduced the pulmonary metastasis in vivo [Du et al., 2011]. Another report showed that overexpression of FOXC2 enhanced the metastatic ability of mouse mammary carcinoma cells. FOXC2 expression is induced in cells undergoing epithelial–mesenchymal transition (EMT) triggered by a number of signals, and the expression of FOXC2 is significantly correlated with the highly aggressive basal-like subtype of human breast cancer. These observations indicated that FOXC2 played a central role in promoting invasion and metastasis and that it proved to be a highly specific molecular marker for human basal-like breast cancers [Mani et al., 2007].

Forkhead Box J2 (FOXJ2) is a member of Forkhead Box family. It, formerly named as FHX [Perez-Sanchez et al., 2000], has been cloned and characterized in humans where it is expressed in all tissues assayed. Foxj2 was also expressed in the mouse embryo as early as the eight-cell stage and is present in both layers of the blastocyst [Granadino et al., 2000]. FOXJ2 appeared to be involved in positively regulating the progression of the cell cycle or aiding in tumorigenesis [Kehn et al., 2007]. However, in this article, we revealed that the expression of FOXJ2 was high in breast cancer tissues without lymph nodes metastases. In cell level, we found the expression of FOXJ2 was high in MCF-7 cells, and low in MDA-MB-231 cells. It was consisted with the expression of E-cadherin, while it was opposite to the expression of vimentin. Exogenous FOXJ2 changed the expression of E-cadherin and vimentin. Meanwhile, the motility of MDA-MB-231 reduced in vitro. These findings suggest a novel function for FOXJ2 in modulating cancer progression by decreasing tumor cell migration.

MATERIALS AND METHODS

TISSUE SAMPLES

One hundred ten breast cancer sections and eight breast cancer tissue samples from patients who underwent surgery between 2003 and 2006 at Department of Pathology, Nantong University Cancer Hospital were formalin-fixed and paraffin-embedded for histopathologic diagnosis and immunohistochemical study. The TNM system of tumor staging and histological grade were performed according to the World Health Organization guidelines [Hartmann et al., 1981]. Fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at −80°C until used for Western blot analysis. All human tissues were collected using protocols approved by the Ethics Committee of Nantong University Cancer Hospital. The clinical features of the patients, including age, histologic grade, tumor size, axillary lymph node status, and histology, were shown in Table I.

IMMUNOHISTOCHEMICAL STAINING

In brief, Sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 3% methanolic peroxide for 10 min. Then the slides were

TABLE I. Correlation Between FOXJ2 Expression and the Clinicopathologic Features of Breast Cancer

Criteria	No. of cases	FOXJ2 expression		*P*-value[a]	χ^2
		Low	High		
Age				0.809	0.058
<50	44	17	27		
≥50	66	24	42		
Tumor size				0.024*	5.072
≤2	71	21	50		
>2	39	20	19		
Histology				0.210	1.575
Ductal	20	5	15		
Others	90	36	54		
Grade				0.379	1.941
I	15	4	11		
II	50	17	33		
III	45	20	25		
Axillary lymph node status				0.010*	9.210
N0	31	6	25		
N1	47	17	30		
N2	32	18	14		
ER				0.038*	4.287
Negative	22	4	18		
Positive	88	37	51		
PR				0.616	0.251
Negative	49	17	32		
Positive	61	24	37		
HER-2				0.687	0.162
Negative (0–1+)	78	30	48		
Overexpressed (2–3+)	32	11	21		
E-cadherin				0.039*	4.27
Low	53	26	27		
High	57	17	40		

[a]Statistical analyses were performed by the Pearson χ^2 test.
*$P < 0.05$ is considered significant.

heated for 10 min at 105°C by autoclave in 0.1 M citrate buffer. Tissue sections were incubated with each primary antibody for 60 min at room temperature. After washed in phosphate-buffered saline (PBS), tissues were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig polymer as a second antibody (Envision kit, Dako) for 30 min at room temperature, according to the manufacturer's instructions. Finally, slides were counterstained with hematoxylin, dehydrated, and mounted in resin mount.

EVALUATION OF THE RESULTS OF IMMUNOHISTOCHEMICAL STAINING

The intensity of immunostaining in each tumor section was assessed as strong (3), moderate (2), weak (1), or negative (0); semiquantitatively using the following scale: <5% of cells (0), 5–25% (1), 26–50% (2), 50–75% (3), and >75% (4) of cells, and then combined these values. This resulted in an overall FOXJ2 immunohistochemical score ranging from 0 to 12. FOXJ2 expression was considered high when scores were >3, and low when scores were ≤3. E-cadherin antibody stained the membrane intensely and the cytoplasm of cancer cells weakly. E-cadherin expression was semi-quantitatively analyzed according to the percentage of cells showing membrane positivity: 0–10% (0), 10–30% (1), 30–70% (2), and >70% (3). E-cadherin expression was considered high when scores were >1, and low when scores were ≤1. A case with cytoplasmic staining only was determined as E-cadherin 0 [Kashiwagi et al., 2010]. Immunohistochemical evaluation for estrogen receptor (ER), progesterone receptor (PR), and HER-2 were finished by Department of Pathology, Nantong University Cancer Hospital.

CELL CULTURES

The human breast cancer cell lines: HBL-100, MDA-MB-453, MDA-MB-435, MDA-MB-231, and MCF-7 (which were gifted from Department of Oncology, Affiliated Cancer Hospital of Fudan University were used in this study) were maintained in Dulbecco's modified Eagle's medium (DMEM) (GibCo BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin mixture (GibCo BRL) at 37°C and 5% CO_2.

WESTERN BLOT ANALYSIS AND ANTIBODIES

Prior to immunoblotting analysis, cells were washed with ice-cold PBS, resuspensed in 2× lysis buffer (50 mM Tris–HCl, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na_3VO_4, and protease inhibitor mixture), and incubated for 20 min at 4°C while rocking. Lysates were cleared by centrifugation (10 min × 12,000 rpm, 4°C) and 50 μg total protein was resolved by SDS–PAGE and transferred on to a polyvinylidene fluoride (PVDF) membrane (Immbilon; Millipore). The membranes were first blocked and then incubated with the primary antibodies described below for 2 h at room temperature. The secondary antibodies were visualized using LumiGLO Regent and Peroxide (Cell Signaling). The optical density on the film was measured with a computer imaging system (Imaging Technology, Ontario, Canada).

The antibodies used in this study included: anti-FOXJ2 (anti-goat, 1:500, Santa Cruz Biotechnology), anti-E-cadherin (anti-mouse, 1:1,000, Santa Cruz Biotechnology), anti-vimentin (Abcam), anti-β-actin (anti-mouse, 1:1,000, Sigma), and anti-GAPDH (anti-rabbit, 1:1,000, Sigma).

IMMUNOFLUORESCENCE ANALYSIS

Cells were fixed with 4% paraformaldehyde-PBS for 30 min at room temperature, washed with PBS, and then permeabilized with 0.2% TritonX-3% bovine serum albumin–PBS for 5 min at room temperature. Cells were stained with primary antibodies for 2 h at room temperature. After being washed with PBS, the cells were incubated with AlexFluor-conjugated secondary antibodies (Molecular Probe, Inc.), counterstained with hochest, and observed with a fluorescence microscope (Leica CTR 5000).

RNA ISOLATION AND REVERSE TRANSCRIPTASE PCR (RT-PCR) ANALYSIS

Total RNA was isolated using the Trizol reagent (Invitrogen) following manufacturer's instructions. One microgram RNA was used for cDNA synthesis using a reverse transcriptase reaction kit (Promega). The primers were 5′-TACGACAGGCAGAGCAGA-3′ and 5′-AGTCGAAGTCATCAGGGATC-3′ for FOXJ2, 5′-AGAGGTGGGTGACTACAAA-3′ and 5′-CTCCGAAGAAACAGCAA-G-3′ for E-cadherin, 5′-GCCAGGCAAAGCAGGAGT-3′ and 5′-TGGGTATCAAC-CAGAGGGA-3′ for vimentin, 5′-GTTTACCTTCCAGCAGCCCTAC-3′ and 5′-GC-CTTTCCCACTGTCCTCAT-3′ for Snail, 5′-CTCCTCCATCTGACACCTCCTCC-AA-3′ and, 5′-TGGGTCCGAATATGCATCTTCAGGG-3′ for Slug, 5²CGACGAC-AGCCTGAGCAACA-3′ and 5′-CCACAGCCCGCAGACTTCTT-3′ for TWIST, 5′-ATAAAGAACGCCAGGCCAAACAAC-3′ and 5′-CCCCAGTCGTTCAGGTA-ATCATAGT-3′ for N-cadherin, 5′-AACAGCATCCGCCACAACCT-3′ and 5′-CC-ACCTTCTTCTCGGCCTCC-3′ for FOXC2, and 5′-TGATGACATCAAGAAGGT-GGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′ for GAPDH. PCR amplification was carried out with an initial denaturing step at 94°C for 5 min, then 30 cycles at 94°C for 45 s, 56°C for 45 s and 72°C for 45 s, followed with a further extension at 72°C for 7 min. The PCR products were electrophoresed through a 1% agarose gel, and visualized by ethidium bromide staining.

PLASMIDS AND shRNA

FOXJ2 expression vector contained a full-length FOXJ2 cDNA cloned into pEGFP-N3 plasmid (Invitrogen) that had been described previously [Erickson et al., 2003]. The FOXJ2 promoter was amplified using primers 5′-TTCTCGAGTTATGG-CTTCTGACCTAGAGAGTAGC-3′ and 5′-TAGGATCCAGTGATCAAGTCCCA-GTCGAAGTC-3′ from genomic DNA extracted from MCF-7 cells.

Oligonucleotides containing the shRNA target sequences were synthesized, annealed and ligated into the *pSilencer4.1*-CMV-neo vector (Ambion Applied Biosystems, USA) pre-cut with *Bam*HI and *Hin*dIII. The target sequences for FOXJ2 gene was 5′-AAGCTTGGAAGTGCCTCCCAG-3′, 5′-AATGCTGGCATTGGTTG-GAAG-3′, and 5′-AACCATGACTTTAAATTCTCC-3′, respectively.

WOUND HEALING ASSAYS

Cells were seeded to nearly complete confluence in a monolayer in 6-well plates. After transfected 48 h, cell were serum starved for

12 h. Then scratching the monolayer with a 10 μl pipette tip, cells were washed with PBS, cultured in 5% FBS-DMEM at 5% CO_2 and 37°C, and photographed under 20× objective lens every 3 h by inverted Leica phase-contrast microscope (Leica DFC 300 FX).

TRANS-WELL MIGRATION ASSAYS

Cells which pretreated with FOXJ2 plasmid or shRNAs were starved overnight in DMEM media with 0.1% FBS, then were trypsinized and resuspended into DMEM containing 0.1% bovine serum albumin. Cells (1×10^5) were added to the top chambers of 24-well transwell plates (Corning, 8 μm pore size), and DMEM with 10% FBS was added to the bottom chambers. After overnight incubation, top (nonmigrated) cells were removed, and bottom (migrated) cells were fixed and stained with crystal violet to visualize nuclei. The number of migrating cells in five fields was counted under 200× magnification, and the means for each chamber were determined. All experiments were conducted in triplicate and repeated twice.

STATISTICAL ANALYSIS

Data were presented as (mean ± SD). Statistiscal analysis was performed using SPSS 13.0 statistical software (SPSS, Inc. Chicago, IL). $P < 0.05$ was considered significant. Expression of FOXJ2 in a subset of human breast cancer samples was analyzed by using the cumulative hypergeometric distribution and χ^2 test.

RESULTS

CORRELATION BETWEEN FOXJ2 AND E-CADHERIN EXPRESSION IN PRIMARY BREAST TUMOR SECTIONS

To reveal the role of FOXJ2 in breast cancer, immunohistochemistry was performed to measure FOXJ2 and E-cadherin expression in breast cancer tissues (Fig. 1). One hundred ten tumor-banked specimens were available for review between the specified time period 2003 and 2006. Median age at cancer diagnosis in the corresponding patients was 52.8 years (range 28–90). The presence of FOXJ2 in different grade, histological types, lymph node status, and ER status were summarized in Table I. In each case in which FOXJ2 immunoreactivity was observed, the immunoreactivity was localized to the nucelus of tumor cells. FOXJ2 immunoreactivity was identified in 94 cases (85.5%) of breast cancer, while there was no detectable FOXJ2 immunoreactivity in 16 cases (14.5%). There is no statistical relationship between the presence of FOXJ2 and age, the histological type of the tumor, tumor grade, PR, or HER-2 status. But there is statistically significant between ER, tumor size and the presence or absence of lymph nodes metastases ($P < 0.05$). Moreover, the expression of FOXJ2 was high in carcinoma cells that expressed membrane-associated E-cadherin (Fig. 1).

DOWN-REGULATED EXPRESSION OF FOXJ2 IN HIGHLY METASTATIC BREAST CANCER TISSUES AND CELLS

Because FOXJ2 expression was correlated with lymph nodes metastases and tumor size in breast cancer sections, we presumed that FOXJ2 may be associated with the migration of breast cancer. An earlier study by F. Martin-de-Lara found out FOXJ2 could bind to the promoter region of the genes coding for Connexin-43 and E-Cadherin, and transactivate their transcription [Martin-de-Lara et al., 2008]. We were interested in the expression status of FOXJ2 in breast cancer fresh tissues, thus Western blotting analysis was performed to measure FOXJ2 protein level. In Figure 2A, the expression of FOXJ2 was high in tissues without lymph nodes metastases, and low in tissues with lymph nodes metastases. Next we took an interest in investing the function of FOXJ2 in cell level. E-cadherin and vimentin are the markers of epithelial cells and mesenchymal cells, respectively [Thiery and Sleeman, 2006]. Breast cancer cell lines range from epithelial-like, with low invasiveness, to mesenchymal-like, exhibiting high invasive capacity [Kalluri and

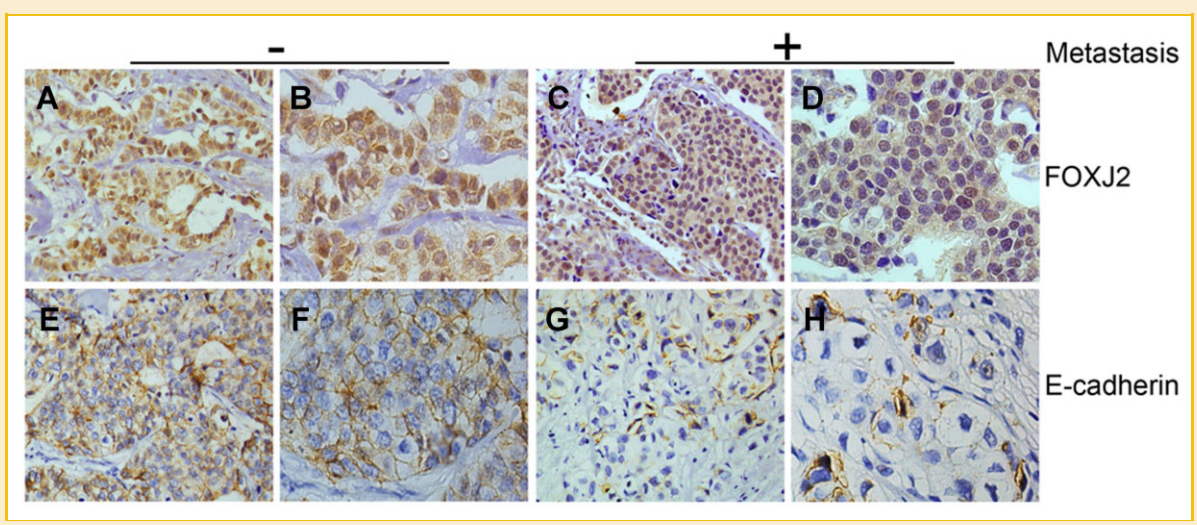

Fig. 1. Immunohistochemical staining reveales FOXJ2 and E-cadherin expression in paraffin-emedded cancerous breast tissues. A,B,E,F: Cancer tissues with no lymph node metastasis showed high FOXJ2 and E-cadherin expression (200× and 400×). C,D,G,H: Cancer tissues with lymph nodes metastases showed low FOXJ2 and E-cadherin expression (200× and 400×). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

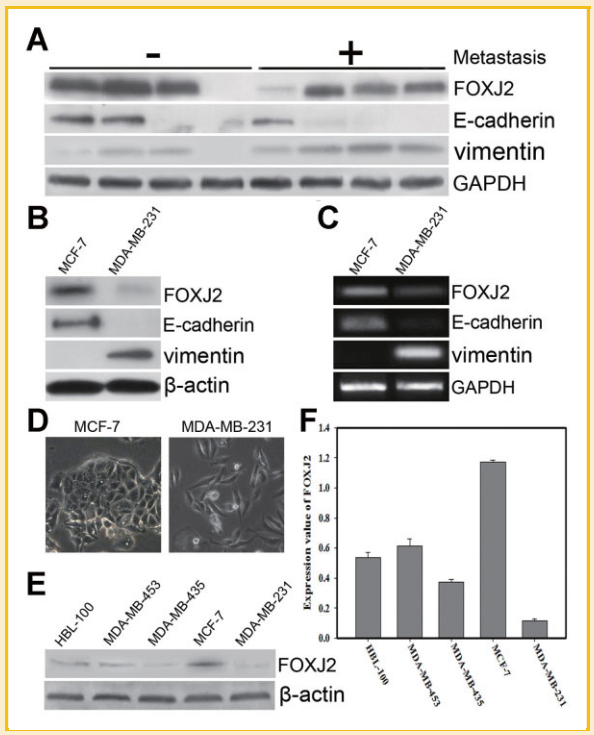

Fig. 2. The expression of FOXJ2 is high in breast cancers with no lymph node metastasis. A: FOXJ2 protein levels in human breast tissues (n = 8 patients) determined by Western blot analysis. B: FOXJ2, E-cadherin, vimetin, and β-actin protein in MCF-7 and MDA-MB-231 breast cancer cell lines analyzed by Western blot analysis. C: FOXJ2, E-cadherin, vimentin, and GAPDH mRNA in the different breast cancer cell lines analyzed by RT-PCR analysis. D: Phase-contrast micrographs of two breast cancer cell lines (400×). E: FOXJ2 and β-actin protein in the different breast cancer cell lines analyzed by Western blot analysis. F: The relative differences of the expression between cell lines were normalized with β-actin. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

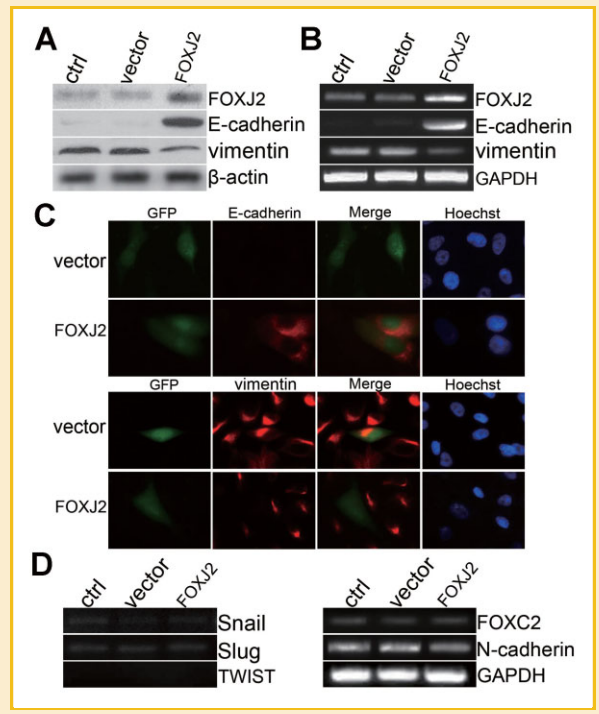

Fig. 3. Cells with exogenous expression of FOXJ2 expresses epithelial markers and showed loss of mesenchymal markers. A: Western blot analysis of FOXJ2, E-cadherin, vimentin, and β-actin in control, pEGFP-N3-vector, and pEGFP-N3-FOXJ2 cells. B: RT-PCR analysis of FOXJ2, E-cadherin, vimentin, and GAPDH in control, pEGFP-N3-vector, and pEGFP-N3-FOXJ2 cells. C: pEGFP-N3-vector and pEGFP-N3-FOXJ2 cells were fixed and processed for immunofluorescence with antibodies recognizing E-cadherin and vimentin. D: RT-PCR analysis of Snail, Slug, TWIST, N-cadherin, FOXC2, and GAPDH in control, pEGFP-N3-vector, and pEGFP-N3-FOXJ2 cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Weinberg, 2009; Katz et al., 2011]. Since it had been hypothesized that FOXJ2 was involved in tumor metastasis, we wanted to see if FOXJ2 expression level was correlated with cellular phenotype. We detected the expression of FOXJ2 in five breast cancer cell lines by Western blot analysis (Fig. 2E). The relative differences of the expression between cell lines were normalized with β-actin (Fig. 2F). The results showed that the expression level of FOXJ2 was the highest in MCF-7 cells, while it was lowest in MDA-MB-231 cells. Two breast cancer cell lines were selected: one weakly invasive and epithelial-like (MCF-7) and one highly invasive and mesenchymal-like (MDA-MB-231; Fig. 2D). FOXJ2 expression was correlated perfectly with cellular phenotype: the noninvasive cell line had high level of FOXJ2 protein and mRNA, whereas in the invasive, it was nearly undetectable (Fig. 2B,C). The expression was coincided with E-cadherin and opposite to vimentin.

HIGH EXPRESSION OF FOXJ2 DECREASES MDA-MB-231 CELLS MOTILITY

A series of Forkhead Box transcription factors have showed they could affect the migration and invasion of tumors. Downregulation of FOXM1 could lead to the inhibion of invision of U2OS osteosarcoma cells and pancreatic cancer cells [Wang et al., 2008; Bao et al., 2011]. FOXO3a promoted tumor cell invasion through the induction of MMPs [Storz et al., 2009]. Up-regulation of FOXP3 inhibited cell migration and invasion in epithelial ovarian cancer [Tallon and Bhawan, 2010; Zhang and Sun, 2010]. To further address the above-mentioned hypothesis, a FOXJ2 expression plasmid was transient transfected into MDA-MB-231 cells to examine the role of FOXJ2. The alteration of FOXJ2 was accompanied by the up-regulation of epithelial marker E-cadherin and downregulation of mesenchymal marker vimentin (Fig. 3A,B). This data was confirmed with an immunofluorescence analysis using the same cell lines (Fig. 3C). A number of EMT pathways converge on the transcription factors Snail, Slug, Twist, and FOXC2 to inhibit E-cadherin transcription and the epithelial phenotype [Shi et al., 2008]. We asked whether other EMT markers were regulated after up-regulating FOXJ2 expression. N-cadherin expression, at the mRNA level, was slightly decreased in pEGFP-N3-FOXJ2 cells (Fig. 3D), suggesting that N-cadherin was a part of the pathway underlying FOXJ2-induced MET. But such correlations were not

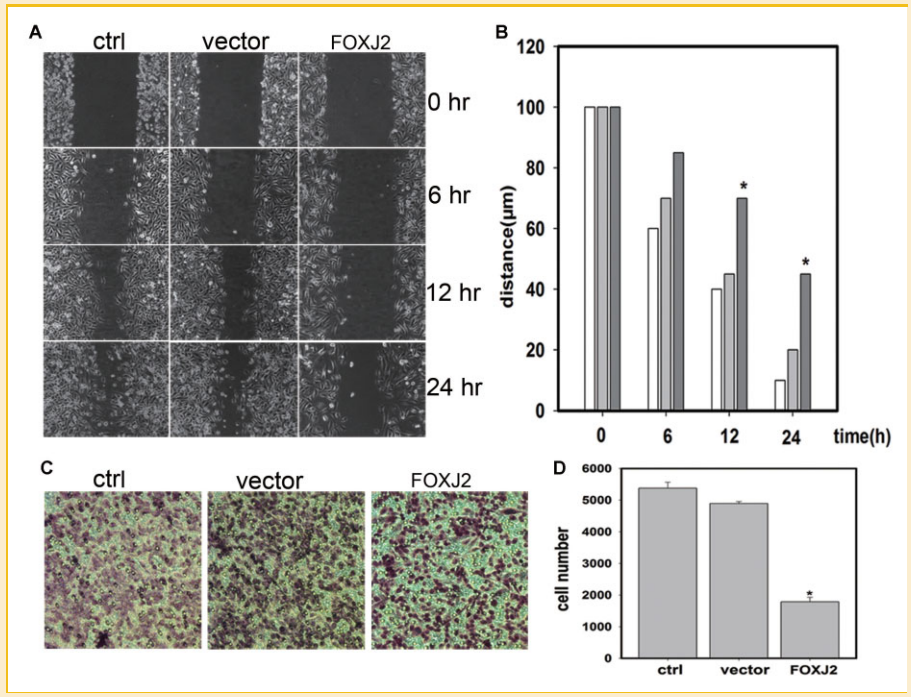

Fig. 4. Exogenously expressed FOXJ2 protein inhibits breast cancer cells migration ability. A,B: Wound healing assays with pEGFP-N3-vector and pEGFP-N3-FOXJ2 cells. Migration of the cells to the wound was visualized at 0, 6, 12, and 24 h with an inverted Leica phase-contrast microscope (200× magnification). $^{*}P < 0.05$. C: FOXJ2 expression inhibited cell migration by trans-well assays. pEGFP-N3-vector cells showed higher penetration rate through the membrane compared with pEGFP-N3-FOXJ2 cells. D: Number of cells that invaded through the member was counted in 10 fields under 20× objective lens. Bars, SD. $^{*}P < 0.01$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

observed with Snail, Slug, Twist, or FOXC2. Hence, we speculate that FOXJ2 may regulate the process of breast cancer metastasis through other pathways.

Cell motility is indispensable for cancer metastasis. Wound healing assays and trans-well assays were performed to determine the potential for FOXJ2 to induce cell migrate. The wound healing assays was conducted at different confluence levels of control, pEGFP-N3-vector, and pEGFP-N3-FOXJ2 cells. Figure 4A shows representative photo-micrographs taken at 0, 6, 12, and 24 h after the cell surfaces are scratched for the wound healing assays. The results showed that the overexpression of FOXJ2 could decrease the wound healing process by creating the slower closure of a "wound" scratched into a confluent epithelial monolayer (Fig. 4A,B). Meanwhile, the overexpression of FOXJ2 inhibited cell migration to the bottom chambers compared to control (Fig. 4C,D). These observations indicate that FOXJ2 does inhibit cell motility.

INHIBITING FOXJ2 EXPRESSION PROMOTES MCF-7 MOTILITY

To do so, we undertook to suppress FOXJ2 expression in MCF-7 cells by constructing a series of short hairpin RNA (shRNA) oligonucleotides targeting FOXJ2 mRNA. These shRNAs were transient expressed in MCF-7 cells by using *pSilencer4.1*-vector. Of these, the FOXJ2 shRNA3 reduced the level of FOXJ2 protein expression by >60% (Fig. 5A). We also found that the downregulation of FOXJ2 caused a decrease in the expression of epithelial marker E-cadherin and an increase in the expression of mesenchymal marker vimentin (Fig. 5B). In addition, shRNA3 promoted the migration of MCF-7 cells by wound healing assays (Fig. 5C). In summary, these results confirm that the downregulation of FOXJ2 increases the motility of MCF-7 cells.

DISCUSSION

Breast cancer, a common malignant disease in women, is prone to invade into adjacent regions and to metastasize to lymph nodes and distant organs [Guo et al., 2011]. To develop novel treatments and cures, it is imperative to address the factors underlying tumorigenesis, invasion and metastasis. In this study, we identify and functionally characterize FOXJ2 as an important player in breast cancer progression. The current studies first illustrate the expression of FOXJ2 in primary breast cancer tissues, followed by demonstrating the association between the FOXJ2 expression and clinicopathologic parameters and finally addressing the role of FOXJ2 in breast cancer prognosis in a large series of 110 samples. Differential expression of FOXJ2 has been detected between primary cancer tissues with or without lymph nodes metastases. FOXJ2 expression is significantly higher in breast cancer tissues with no lymph nodes metastases compared with the tissues with lymph nodes metastases (Fig. 1).

Otherwise, we demonstrate that E-cadherin expression is positive correlated with FOXJ2 protein level in breast cancer (Table I). Immunohistochemistry results show that high expression of FOXJ2 is correlated with high E-cadherin, while low expression of FOXJ2 is

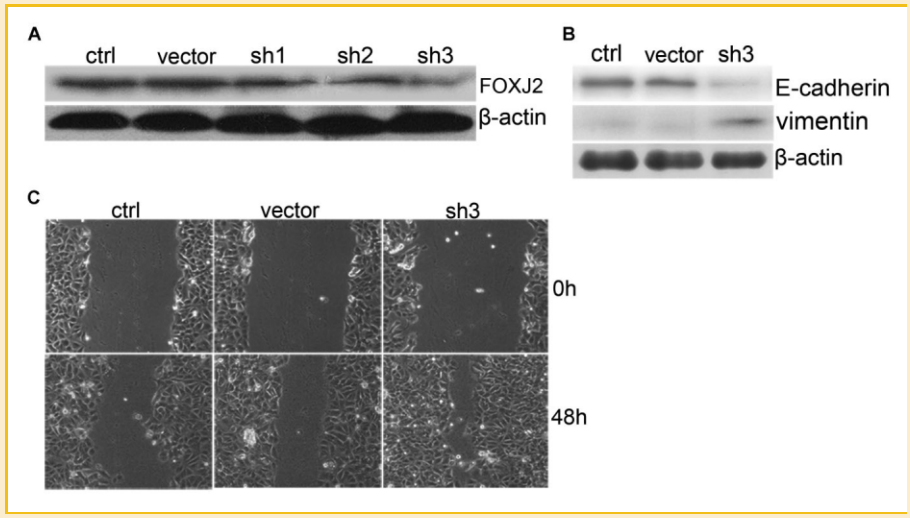

Fig. 5. Inhibiting FOXJ2 expression promotes the migration of MCF-7 cells. A: Western blot analysis of FOXJ2 in control, vector, sh1, sh2, and sh3. B: Western blot analysis of E-cadherin, vimentin, and β-actin in control. C: Wound healing assays with vector and sh3 cells (200× magnification).

correlated with low E-cadherin in the same breast cancer specimen (Fig. 2A). Western blot analysis and RT-PCR analysis present the similar results, as shown in Figure 2B,C, FOXJ2 prefer to express in weakly invasion breast cancer cells lines (MCF-7), compared with highly invasion breast cancer cell lines (MDA-MB-231; Fig. 2D). As a result, we suppose that FOXJ2 is involved in tumor migration.

FOXJ2 belongs to the human Forkhead Box (Fox) gene family, which consists of at least 100 members [Coffer and Burgering, 2004]. Deregulation of the Fox family genes, caused by various mechanisms such as mutation, amplification, and gene fusion, leads to congenital disorders, diabetes mellitus, or carcinogenesis [Katoh, 2004]. Many Fox family genes play important roles in tumor cells dissemination [Malin et al., 2007; Mani et al., 2007; Zeng et al., 2009; Song et al., 2010; Du et al., 2011]. The present study extended those findings by investigating FOXJ2 expression in breast cancerous cells. In this research, we identify and functionally characterize FOXJ2 as an important player in breast cancer metastasis (Figs. 4 and 5).

Several prognostic gene setting for breast cancer have been reported in the literature, and they are functionally important for tumor progression. Jeanette Nilsson et al. showed transcription factor nuclear factor 1-C2 (NF1-C2) was lost during mammary tumor progression, and was almost invariably absent from lymph nodes metastases. Changing NF1-C2 level indicated it counteracted EMT, motility, and invasiveness. In addition, NF1-C2S, constitutive activation of NF-1C, controlled tumor progression through directly suppressing FOXF1 [Nilsson et al., 2010]. Hence, they added new insights into the mechanism underlying EMT and show that NF1-C2 and FOXF1 are two novel potential therapeutic targets against breast cancer invasion and metastasis [Lo et al., 2010]. FOXF1 is a potential tumor suppressor gene that is epigenetically silenced in breast cancer. Meantime, it has been reported that BRCA1 is the downstream target of FOXF1 signaling. BRCA1 is a well-known tumor suppressor gene that plays multiple roles in transcription and DNA repair [Rosen et al., 2006; Coleman and Greenberg, 2011]. So up-regulation of DNA repair genes BRCA1 by FOXF1 further supported the role of FOXF1 in maintaining genomic stability. BRCA1 is inactivated through genetic mutation or aberrant downregulation of its mRNA expression. It is tempting to speculate that epigenetic inactivation of FOXF1 is one of mechanisms leading to the downregulation of BRCA1 expression in breast cancer [Lo et al., 2010]. Moreover, BRCA1 can bind to the consensus sequence in the promoter region of FOXJ2. Based on the implication of FOXJ2 in the oncogenic process, the researcher speculate that BRCA1 may aid in suppressing FOXJ2 transcription [Kehn et al., 2007]. However, it has been known that inhibition of BRCA1 function via mutations or downregulation is associated with poor prognosis and metastasis. Thus, our conclusion is contradictory to their inference. Hence, we will research the relationship among these molecules in our further study.

In summary, our studies support a novel role for FOXJ2 transcription factor in cell migration. From these data, we can expect that up-regulation of FOXJ2 might potentially be an effective therapeutic approach for the inhibition of cell migration and invasion of breast cancer. Therefore, FOXJ2 may serve as a novel molecular target for the detection and treatment of breast cancer and other human cancers.

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