

# Fucoidan inhibits epithelial-to-mesenchymal transition via regulation of the HIF-1 $\alpha$ pathway in mammary cancer cells under hypoxia

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**Abstract.** This study examined the effects of fucoidan on epithelial-to-mesenchymal transition (EMT) in a human triple-negative breast cancer (TNBC) cell line in a hypoxic microenvironment. Transwell and wound-healing assays were performed to analyze the invasion and migration of MDA-MB-231 human mammary cancer cells, respectively. The expression levels of EMT markers and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) were detected through western blotting. Under hypoxia, fucoidan treatment inhibited proliferation of breast cancer cells. Fucoidan also suppressed the invasion and migration of MDA-MB-231 cells. Western blotting revealed that fucoidan treatment significantly reduced the protein expression levels of HIF-1 $\alpha$  and HIF-1 target genes. Furthermore, the nuclear translocation and activity of HIF-1 $\alpha$  were reduced. Fucoidan treatment significantly downregulated the expression levels of mesenchymal markers (N-cadherin and vimentin), but upregulated the expression levels of the epithelial markers zonula occludens-1 and E-cadherin. In addition, over-expression of HIF1- $\alpha$  protected cells from fucoidan-mediated suppression of migration and invasion. These data suggested that fucoidan may inhibit EMT in human TNBC cells via downregulation of the HIF1- $\alpha$  signaling pathway.

## Introduction

A hypoxic microenvironment is a common characteristic of solid tumors. It has been reported that hypoxia can

induce epithelial-to-mesenchymal transition (EMT) via hypoxia-induced factor-1 $\alpha$  (HIF-1 $\alpha$ ) in various types of cancer, including gastric cancer, hepatoblastoma, pancreatic carcinoma, colon carcinoma and mammary cancer (1-3). EMT is an important process in cancer metastasis; during EMT, epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells. To complete the metastatic process, cancer cells must migrate out of the primary tumor. Studies in mammary cancer have shown that the EMT phenomenon increases the migration and invasion of cancer cells, and is closely associated with tumor occurrence, infiltration and distant implantation (4,5). Cancer cells must detach from the neighboring epithelial cells by reducing E-cadherin expression to start invading the surrounding extracellular matrix. It has been reported that HIF-1 $\alpha$  expression is rarely observed in normal tissues but is increased in various cancer tissues or cells, and is closely associated with the migration and invasion of tumor cells. Therefore, inhibition of HIF-1 $\alpha$  activation may reduce the development and progression of tumors (6-8).

HIF-1 $\alpha$  can directly or indirectly regulate EMT regulators, including TWIST, Snail, carbonic anhydrase IX (CAIX), glucose transporter protein-1 (GLUT-1), and other transcription factors (9-11). These transcription factors then transactivate EMT-related genes, including vimentin, E-cadherin and N-cadherin, to regulate progression of the EMT (12,13). HIF-1 $\alpha$  levels in mammary cancer tissues are associated with pathological stages, and high HIF-1 $\alpha$  levels may result in high multiplication rates and the formation of more aggressive tumors (14).

The metastasis of triple-negative breast cancer (TNBC) is associated with poor prognosis and high mortality due to ineffective treatment. Fucoidan is a complex sulfated polysaccharide extracted from brown seaweed. It has been reported to exert antitumor activity in various types of cancer, including acute prostate cancer cells, myeloid leukemia, lung cancer and hepatocellular carcinoma cells (15-18). Our previous study reported that fucoidan induces apoptosis of different breast cancer cell lines, including mouse breast cancer 4T1 cells, human breast cancer MCF-7 cells and MDA-MB-231

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cells (19-21). However, the effects of fucoidan on the metastasis of TNBC and the underlying mechanisms remain elusive. The present study aimed to examine the effects of fucoidan on EMT and the underlying molecular mechanisms in a TNBC cell line. Since hypoxia may promote EMT in mammary cancer cells via regulation of the expression of EMT regulators, the present study also aimed to clarify whether fucoidan can directly affect HIF-1 $\alpha$  in a TNBC cell line.

## Materials and methods

**Materials.** Fucoidan was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). It was dissolved in normal saline at a concentration of 20 mg/ml and then stored at -20°C. A human mammary cancer cell line (MDA-MB-231) was purchased from the Cell Bank of the Shanghai Institute of Cell Biology (Shanghai, China). RPMI-1640 medium was obtained from HyClone (GE Healthcare Life Sciences; Logan, UT, USA) and was supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibodies against CAIX (cat. no. A1463-100), HIF-1 $\alpha$  (cat. no. TA301442), vimentin (cat. no. 3634-100), N-cadherin (cat. no. 119-14215) and histone H3 (cat. no. A310-257A) were obtained from Abcam (Cambridge, MA, USA). Antibodies against zonula occludens-1 (ZO-1, cat. no. sc-33725), E-cadherin (cat. no. sc-71008), TWIST (cat. no. sc-81417), Snail (cat. no. sc-393172), GLUT-1 (cat. no. sc-377228), Na, K-ATPase (cat. no. sc-71638) and  $\beta$ -actin (cat. no. sc-70319) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary anti-rabbit (cat. no. sc-2491) and anti-mouse (cat. no. sc-516102) antibodies were obtained from Santa Cruz Biotechnology, Inc.

**Cell culture.** MDA-MB-231 cells were cultured in Roswell Park Memorial Institute 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and were allowed to attach onto the dish. All cells were cultured to ~90% confluence in 6-well plates prior to treatment. A hypoxic humidified incubator with a gas mixture of 94% N<sub>2</sub>, 5% CO<sub>2</sub> and 1% O<sub>2</sub> was used for hypoxia exposure. All cells were incubated under hypoxia during or after fucoidan treatment.

**MTT assay.** MTT (Sigma-Aldrich; Merck KGaA) was used for the cell proliferation assay. Each well of a 96-well plate was seeded with 1x10<sup>4</sup> cells/ml. After overnight incubation at 37°C, a total of 6.25, 12.5 or 25  $\mu$ g/ml fucoidan was added to the breast cancer cells. The plates were incubated in a hypoxic chamber at 37°C. Following incubation for 24, 48 or 72 h, 20  $\mu$ l MTT reagent was added to all wells at a concentration of 5 mg/ml at 37°C for 4 h, and the crystals were dissolved in DMSO. The absorbance was detected at 490 nm on an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Wound-healing assay.** Breast cancer cells were grown until confluent in 6-well plates. Following treatment with fucoidan (0, 6.25, 12.5 or 25  $\mu$ g/ml) for 24 h in a hypoxic chamber, the

cell monolayers were scratched using a sterile 200  $\mu$ l plastic pipette tip. Following aspiration of the medium, the displaced cells were washed with PBS. Subsequently, the cells were continually incubated in a hypoxic chamber for 24 h. Five images were captured from randomly selected microscope fields at different time points using an OLYMPUS CX31 microscope (Tokyo, Japan). The area of the wound was measured using ImageJ 1.51p software (National Institutes of Health, Bethesda, MD, USA).

**Transwell invasion assay.** For the invasion assay, 24-well Transwell units were used. The lower units of a 6.5-mm Transwell filter with 0.4  $\mu$ m pore size were precoated with Matrigel (BD Biosciences, San Jose, CA, USA). Following treatment with fucoidan (0, 6.25, 12.5 or 25  $\mu$ g/ml) for 24 h in a hypoxic chamber, the cancer cells were suspended in serum-free medium, and 1x10<sup>4</sup> cells were seeded onto the upper wells. The lower units were filled with medium containing 10% FBS as a chemoattractant. After incubation in a hypoxic chamber for 12 h, the cancer cells on the upper units of the membrane were removed using a cotton swab. The invading cells on the bottom surface of the membrane were fixed with 70% ethanol for 10 min. After staining with 0.1% crystal violet for 10 min at room temperature, the cells were counted using an OLYMPUS CX31 microscope.

**HIF-1 activation assay.** MDA-MB-231 cells treated with fucoidan (0, 6.25, 12.5 or 25  $\mu$ g/ml) for 48 h in a hypoxic chamber were washed with PBS. HIF-1 $\alpha$  activation was evaluated using a HIF-1 activation assay kit (cat. no. 47096; Active Motif, Carlsbad, CA, USA), according to the manufacturer's protocols. Finally, the absorbance was detected at 450 nm with a SpectraMax 190 plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Overexpression of HIF-1 $\alpha$ .** Breast cancer cells were transfected with HIF-1 $\alpha$  overexpression vector (HIF-1 $\alpha$ -pcDNA3.0; Shanghai Genechem Co., Ltd., Shanghai, China) or empty plasmid (pcDNA3.0; Shanghai Genechem Co., Ltd.) using Lipofectamine<sup>®</sup> 2000 (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Briefly, the cells were prepared into a single cell suspension with 0.2-1 x10<sup>6</sup>/ml density by trypsin digestion and then inoculated into the 6-well plate. The transfection was conducted when the cells had reached 90-95% confluence. Each well contained 4  $\mu$ g plasmid and 10  $\mu$ l Lipofectamine<sup>®</sup> 2000. The follow-up experiments were continued 24 h after transfection. The sequence information of overexpression vector was shown in Fig. 1.

**Western blotting.** Nuclear extracts, cytosolic extracts and membrane extracts were prepared using the Nuclear and Cytosolic Protein Extraction Kit and Membrane and Cytosol Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). The membrane protein samples were used to detect the levels of ZO-1 and E-cadherin, and Na, K-ATPase was used as a reference for determination. The cytoplasmic protein samples were used to detect the expression levels of vimentin, TWIST, Snail, CAIX, N-cadherin and GLUT-1, and  $\beta$ -actin was used as a reference for determination. The

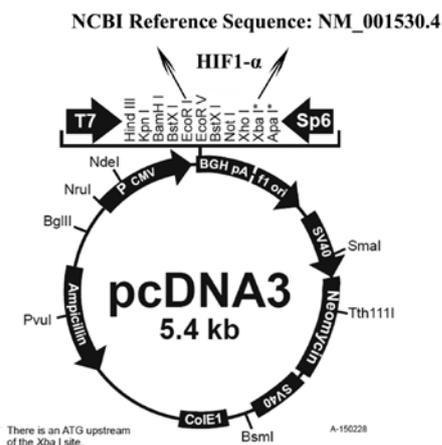


Figure 1. Sequence information of the overexpression vector HIF-1 $\alpha$ -pcDNA3.0. HIF-1 $\alpha$  (NCBI Reference Sequence, NM\_001530.4) was inserted between the *EcoRI* and *XbaI* restriction sites. HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

nuclear protein samples were used to detect HIF-1 $\alpha$  levels, and histone H3 was used as a reference.

The total protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Each protein sample (30  $\mu$ g) was separated by SDS-PAGE (9% gel) and then transferred to nitrocellulose (NC) membranes, which were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). After blocking with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBST; 20 mM Tris-HCl, 120 mM NaCl, 0.1% Tween-20) at room temperature for 1 h, the NC membranes were incubated with primary antibodies at 4°C. The dilution ratio for anti-ZO-1, anti-vimentin and anti-E-cadherin was 1:1,000. The dilution ratio for anti-TWIST, anti-Snail, anti-CAIX, anti-N-cadherin and anti-GLUT-1 was 1:200. The dilution ratio for anti-HIF-1 $\alpha$ , anti-Na, K-ATPase, anti- $\beta$ -actin and anti-Histone H3 was 1:500. Following overnight incubation, the NC membranes were washed three times with TBST for 10 min and were then incubated with the corresponding secondary antibodies (dilution, 1:80) at room temperature for 1 h. After washing, protein bands were visualized by enhanced chemiluminescence with a reagent containing the horseradish peroxidase (HRP) substrate luminol, which was obtained from EMD Millipore (Billerica, MA, USA). Protein expression was quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Inc., Rockville, MD, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA in cells after transfection was extracted using an RNAprep pure tissue kit (Tiangen Biotech Co., Ltd., Beijing, China). cDNA was synthesized by RT using 200 ng total RNA. Each 20  $\mu$ l RT reaction was performed using a miScript II Reverse Transcriptase kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol in a GeneAmp PCR system 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) for 60 min at 37°C, followed by heat inactivation of the RT for 5 min at 95°C. The cDNA was then subjected to RT-qPCR amplification on a LightCycler 480 RT PCR system (Roche Diagnostics, Basel, Switzerland). The RT PCR Master mix

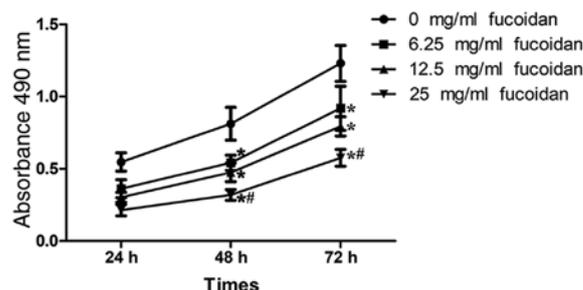


Figure 2. Effect of fucoidan treatment on the proliferation of MDA-MB-231 cells under hypoxia. The effects of fucoidan on the proliferation of breast cancer cells under hypoxia were detected via MTT assay. Fucoidan treatment inhibited the proliferation of MDA-MB-231 cells ( $P < 0.05$ ). Following treatment with 25  $\mu$ g/ml fucoidan for 72 h, cell proliferation declined by 53.2%. Data are presented as the means  $\pm$  standard deviation. \* $P < 0.05$  vs. control (0  $\mu$ g/ml fucoidan); # $P < 0.05$  vs. 6.25  $\mu$ g/ml fucoidan.

(Takara Biotechnology Co., Ltd., Dalian, China) and specific primer/probe sets were used to amplify HIF-1 $\alpha$  and GAPDH (TaqMan Gene Expression Assays; Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used for qPCR: HIF-1 $\alpha$ , forward, 5'-CCTGAGCCT AATAGTCCC-3', reverse, 5'-GCTGGCATTAGCAGT AGG-3'; and GAPDH, forward, 5'-ATTCCATGGCACCGT CAAGG C T-3' and reverse, 5'-TCAGGTCCACCACTGACA CGT T-3' (designed by Takara Biotechnology Co. Ltd.). The amplification consisted of 95°C for 30 sec and 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and 72°C for 2 min followed by a final extension at 72°C for 5 min. The expression levels of each gene were normalized to GAPDH expression levels using the  $2^{-\Delta\Delta C_q}$  method as previously described (22).

**Statistical analysis.** All results are expressed as the means  $\pm$  standard deviation. Each experiment was repeated three times. Statistical analysis was performed using SPSS software (version 17; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to determine the statistical significance and Tukey's post hoc test was used to analyze the differences between two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Fucoidan treatment inhibits the proliferation of MDA-MB-231 cells under hypoxia.** The effect of fucoidan on the proliferation of breast cancer cells under hypoxia was determined using an MTT assay. Fig. 2 indicated that fucoidan treatment reduced the proliferation of breast cancer cells ( $P < 0.05$ ). Following treatment with fucoidan at a concentration of 25  $\mu$ g/ml for 72 h, the cell proliferation had declined by 53.2%.

**Fucoidan inhibits the migration and invasion of breast cancer cells.** Wound-healing and Matrigel-coated Transwell invasion assays were used to detect whether fucoidan intervention exhibited any effect on EMT in MDA-MB-231 cells. As shown in Fig. 3A, fucoidan treatment reduced the migratory ability of breast cancer cells ( $P < 0.001$ ). Following treatment with fucoidan at a concentration of 12.5 or 25  $\mu$ g/ml for 24 h, cell invasion was also inhibited (Fig. 3B).

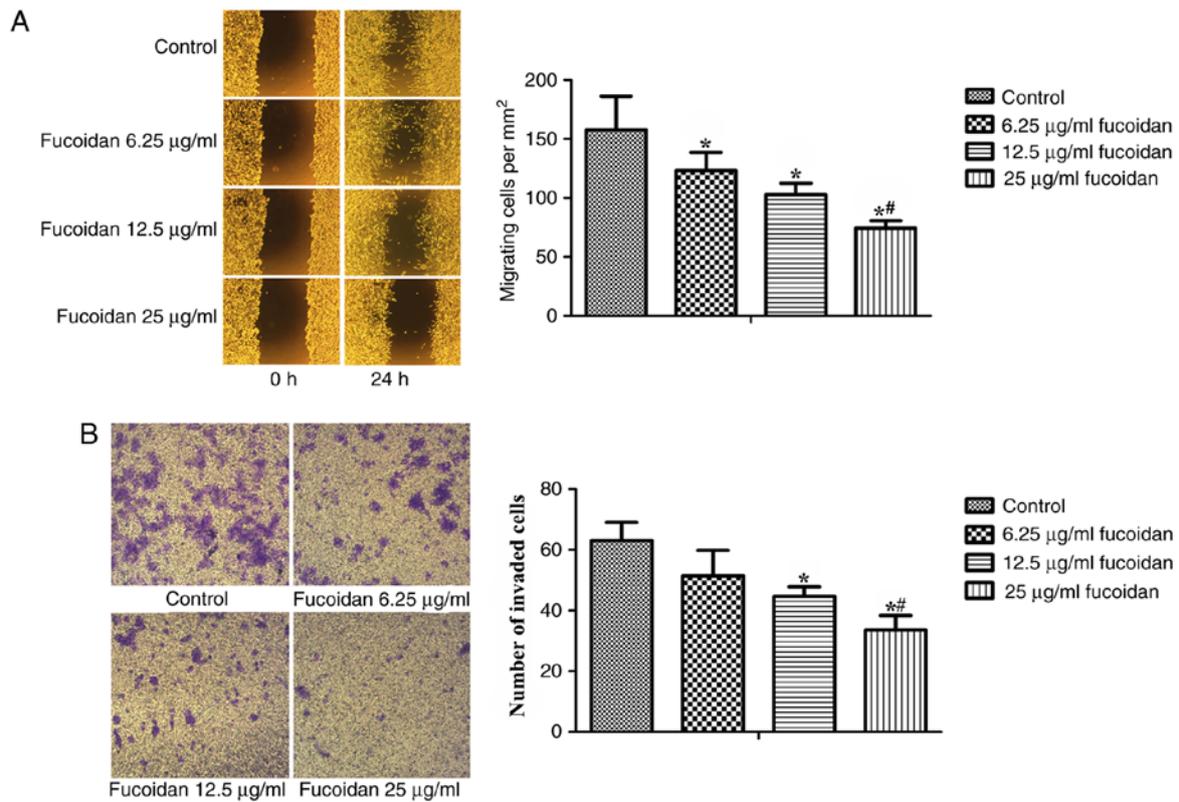


Figure 3. Fucoidan inhibits the migration and invasion of breast cancer cells. (A) A scratch assay was used to determine the migratory ability of breast cancer cells. Under a microscope, the relative wound closure was detected and images were captured (magnification, x100). Cell motility was quantified based on the wound width. Fucoidan treatment inhibited the migratory ability of breast cancer cells ( $P < 0.001$ ). (B) Transwell assays were used to determine the invasive ability of MDA-MB-231 cells. Representative fields showing invasive cells on the membrane are presented (magnification, x200). Following treatment with 12.5 or 25  $\mu\text{g/ml}$  fucoidan, cell migration was inhibited. All experiments were repeated three times. Data are presented as the means  $\pm$  standard deviation. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. 6.25  $\mu\text{g/ml}$  fucoidan.

*Fucoidan affects the expression levels of EMT markers.* The results revealed that fucoidan treatment downregulated the expression levels of the mesenchymal markers N-cadherin and vimentin (Fig. 4). In the membranes of MDA-MB-231 cells, the epithelial markers ZO-1 and E-cadherin were rarely expressed, but following treatment with fucoidan, the expression levels of ZO-1 and E-cadherin were significantly increased.

*Fucoidan inhibits protein accumulation in the nucleus and activation of HIF-1 $\alpha$ .* The effects of fucoidan on the expression and nuclear translocation of HIF-1 $\alpha$  were assessed by western blotting. The nuclear protein levels of HIF-1 $\alpha$  were decreased following treatment with fucoidan under hypoxic conditions (Fig. 5A). The activation of HIF-1 $\alpha$  was also assessed. As shown in Fig. 5B, fucoidan treatment decreased the activation of HIF-1 $\alpha$ .

*Fucoidan downregulates the expression of HIF-1 $\alpha$  target genes.* HIFs can regulate the expression of numerous genes involved in tumor growth, metastasis and metabolic reprogramming (23,24). HIF-1 $\alpha$  can directly or indirectly regulate the EMT regulators TWIST, Snail, CAIX and GLUT-1 (9-11). The expression levels of TWIST, Snail, CAIX and GLUT-1 were detected by western blotting. As shown in Fig. 6, following treatment with fucoidan, the expression levels of TWIST-1, Snail, CAIX and GLUT-1 were inhibited.

*Overexpression of HIF-1 $\alpha$  results in the reversal of fucoidan-mediated suppression of cell migration and invasion.* MDA-MB-231 cells were transfected with HIF-1 $\alpha$  overexpression plasmid (HIF-1 $\alpha$ -pcDNA3.0) to determine whether the anti-EMT effects of fucoidan depended on HIF-1 $\alpha$ ; empty plasmid (pcDNA3.0) was used as a control. The expression of HIF-1 $\alpha$  and its target genes (TWIST and Snail) in MDA-MB-231 cells was increased by HIF-1 $\alpha$ -pcDNA3.0 transfection (Fig. 7A). The mRNA expression levels of HIF-1 $\alpha$  in breast cancer cells were measured by RT-qPCR. Post-transfection with HIF-1 $\alpha$ -pcDNA3.0, the mRNA expression levels of HIF-1 $\alpha$  were increased (Fig. 7B). Post-transfection with HIF-1 $\alpha$ -pcDNA3.0, breast cancer cells were incubated with 25  $\mu\text{g/ml}$  fucoidan, and cell invasion and migration were detected. Wound-healing and Matrigel-coated Transwell invasion assays revealed that overexpression of HIF-1 $\alpha$  resulted in the reversal of fucoidan-mediated suppression of cell migration and invasion (Fig. 7C and D).

## Discussion

The present study demonstrated that fucoidan treatment inhibited the proliferation of breast cancer cells, and suppressed the migration and invasion of mammary cancer cells in a hypoxic microenvironment. The nuclear translocation and activity of HIF-1 $\alpha$  were also reduced by fucoidan. In addition, fucoidan significantly downregulated the expression

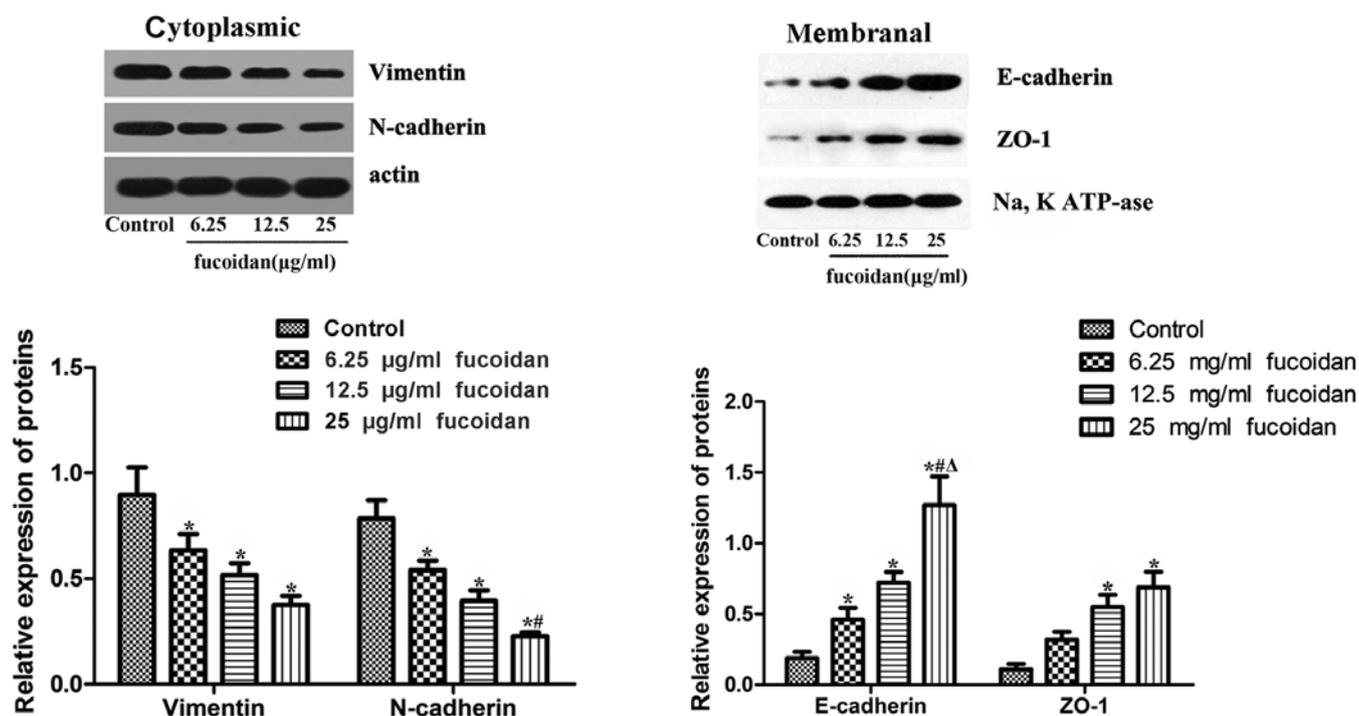


Figure 4. Effects of fucoidan on the expression levels of EMT markers. Following treatment with fucoidan, membrane and cytosolic extracts were prepared to measure the expression levels of EMT markers by western blot analysis. Fucoidan treatment significantly upregulated the expression levels of the epithelial markers ZO-1 and E-cadherin, but downregulated the expression levels of mesenchymal markers (N-cadherin and vimentin). All experiments were repeated three times. Data are presented as the means  $\pm$  standard deviation. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. 6.25  $\mu\text{g/ml}$  fucoidan;  $\Delta P < 0.05$  vs. 12.5  $\mu\text{g/ml}$  fucoidan. ZO-1, zonula occludens-1.

levels of N-cadherin and vimentin, but upregulated ZO-1 and E-cadherin expression.

During the process of EMT, cancer cells lose the characteristics of epithelial cells, such as polarity and cell adhesion. Subsequently, cancer cells acquire a mesenchymal cell-like morphology and a migratory ability. Once cancer cells cease to express epithelial markers, including E-cadherin (an adherens junction protein), they initiate the expression of mesenchymal markers, including N-cadherin and vimentin (25). ZO-1 is another epithelial marker and is a submembrane scaffolding protein. When ZO-1 relocates into the cell from tight junctions, it may promote the invasion activity (26).

HIFs are critical transcription factors that can regulate adaptive cellular responses to low  $\text{O}_2$  concentrations in Metazoa. HIFs have been reported to be upregulated in various cancer cells under hypoxia, which is commonly found in tumor microenvironments (23,24).

HIF-1 has two subunits, the HIF-1 $\alpha$  and HIF-1 $\beta$  subunits; these two subunits form a heterodimeric protein. The partial pressure of oxygen can regulate the expression of the HIF-1 $\alpha$  subunit; however, HIF-1 $\beta$  is constitutively expressed (27,28). At normal partial pressure of oxygen, HIF-1 $\alpha$  can be hydroxylated by  $\text{O}_2$  and  $\alpha$ -ketoglutarate-dependent prolyl hydroxylases. Hydroxylated HIF-1 $\alpha$  can be recognized by the von Hippel-Lindau tumor suppressor protein, which targets HIF-1 $\alpha$  to be degraded via ubiquitination. Therefore, HIF-1 $\alpha$  levels are kept low in the cytoplasm. However, under hypoxic conditions, HIF-1 $\alpha$  cannot be hydroxylated, resulting in accumulation of HIF-1 $\alpha$  in the cytoplasm (29,30). Once the presence of HIF-1 $\alpha$  is stable in

the cytoplasm, HIF-1 $\alpha$  can be transported into the nucleus. Subsequently, the two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ , can form a heterodimer. The HIF-1 $\alpha$ /1 $\beta$  heterodimer can bind to hypoxia-response elements (HREs). HREs exist in hypoxia target genes, and these genes are involved in tumor growth, metastasis, metabolic reprogramming, chemoresistance and radioresistance (23,24,31)

It has been reported that HIF-1 $\alpha$  is hyperactivated in TNBC (32). High HIF-1 $\alpha$  levels in cancer tissues are associated with high mortality in several tumor types, including mammary cancer. Furthermore, elevated HIF-1 $\alpha$  levels in the mammary tumor tissues of patients have been associated with high rates of metastasis and mortality (27). The present results showed that fucoidan treatment in highly metastatic TNBC cell lines may inhibit the nuclear accumulation and activation of HIF-1 $\alpha$ .

HIF-1 $\alpha$  is an important transcription factor that can induce the expression of ~40 genes in hypoxic stromal and cancer cells, including glycolytic enzymes, glucose transporters, erythropoietin, and vascular endothelial growth factors. In addition, HIF-1 $\alpha$  can increase the expression of some genes, and the protein products of these genes can enhance the delivery of oxygen or promote metabolic adaptation to hypoxia, as well as increase metastasis and tumor invasion (33,34).

HIF-1 $\alpha$  can directly or indirectly regulate EMT regulators, and these transcription factors can transactivate EMT-related genes, including N-cadherin, vimentin and E-cadherin (12,13). It has been reported that hypoxia and continuously high levels of HIF-1 $\alpha$  can result in high levels of certain regulators, including TWIST, zinc finger E-box-binding homeobox (ZEB)2, Snail,

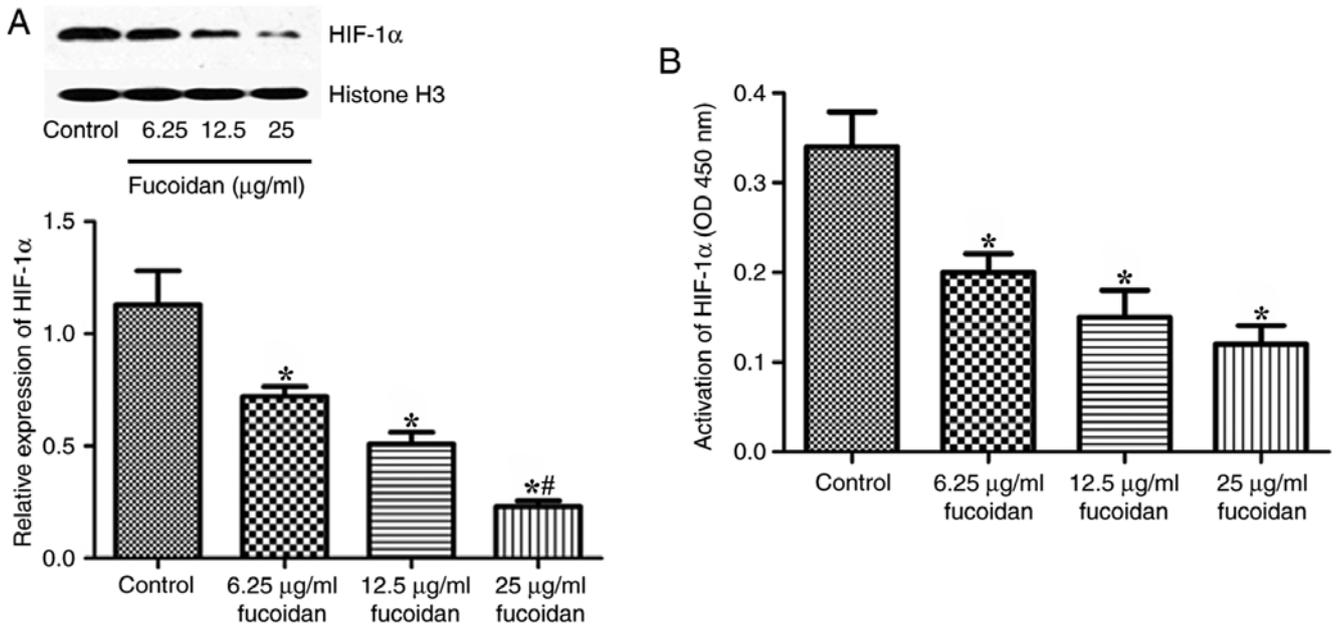


Figure 5. Fucoïdan inhibits HIF-1 $\alpha$  protein activation and nuclear accumulation. (A) Effects of fucoïdan on the expression and nuclear translocation of HIF-1 $\alpha$  under hypoxic conditions were evaluated by western blotting. Fucoïdan treatment inhibited the nuclear translocation of HIF-1 $\alpha$ . (B) Activation of HIF-1 $\alpha$  was detected. Fucoïdan treatment decreased the activation of HIF-1 $\alpha$ . All experiments were repeated three times. Histone H3 was used as a reference for nuclear protein determination. Data are presented as the means  $\pm$  standard deviation. \*P<0.05 vs. control; #P<0.05 vs. 6.25  $\mu\text{g/ml}$  fucoïdan. HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

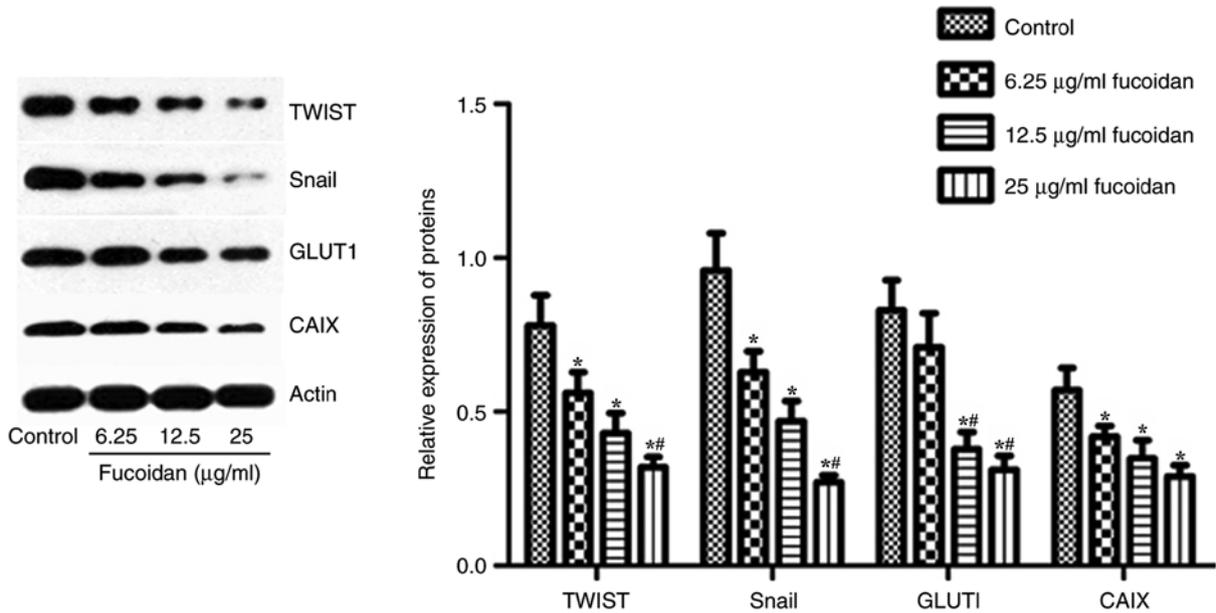


Figure 6. Fucoïdan downregulates the protein expression levels of hypoxia-inducible factor-1 $\alpha$  target genes. Western blotting suggested that fucoïdan treatment inhibited the expression levels of TWIST, Snail, CAIX and GLUT-1. Data are presented as the means  $\pm$  standard deviation. \*P<0.05 vs. control; #P<0.05 vs. 6.25  $\mu\text{g/ml}$  fucoïdan. CAIX, carbonic anhydrase IX; GLUT-1, glucose transporter protein type-1.

transcription factor 3, ZEB1, CAIX and GLUT-1, to moderate EMT and metastasis (9-11,35,36).

TWIST is an important factor that induces EMT and metastasis in cancer. HIF-1 $\alpha$  can directly increase TWIST levels via the response element of HIF1 located in the proximal promoter of the TWIST gene (9,37). TWIST can bind to the E-box in the promoter of E-cadherin and downregulate the expression of E-cadherin (38). Downregulation

of TWIST is important for invasion in TNBC (39). Snail is an important target gene of HIF, and overexpression of Snail is involved in EMT. Furthermore, high levels of Snail are associated with highly aggressive types of cancer in humans and mice (40). Snail overexpression is associated with a high rate of metastasis in mammary tumors, whereas Snail silencing decreases the invasiveness and cell motility of mammary cancer (41).

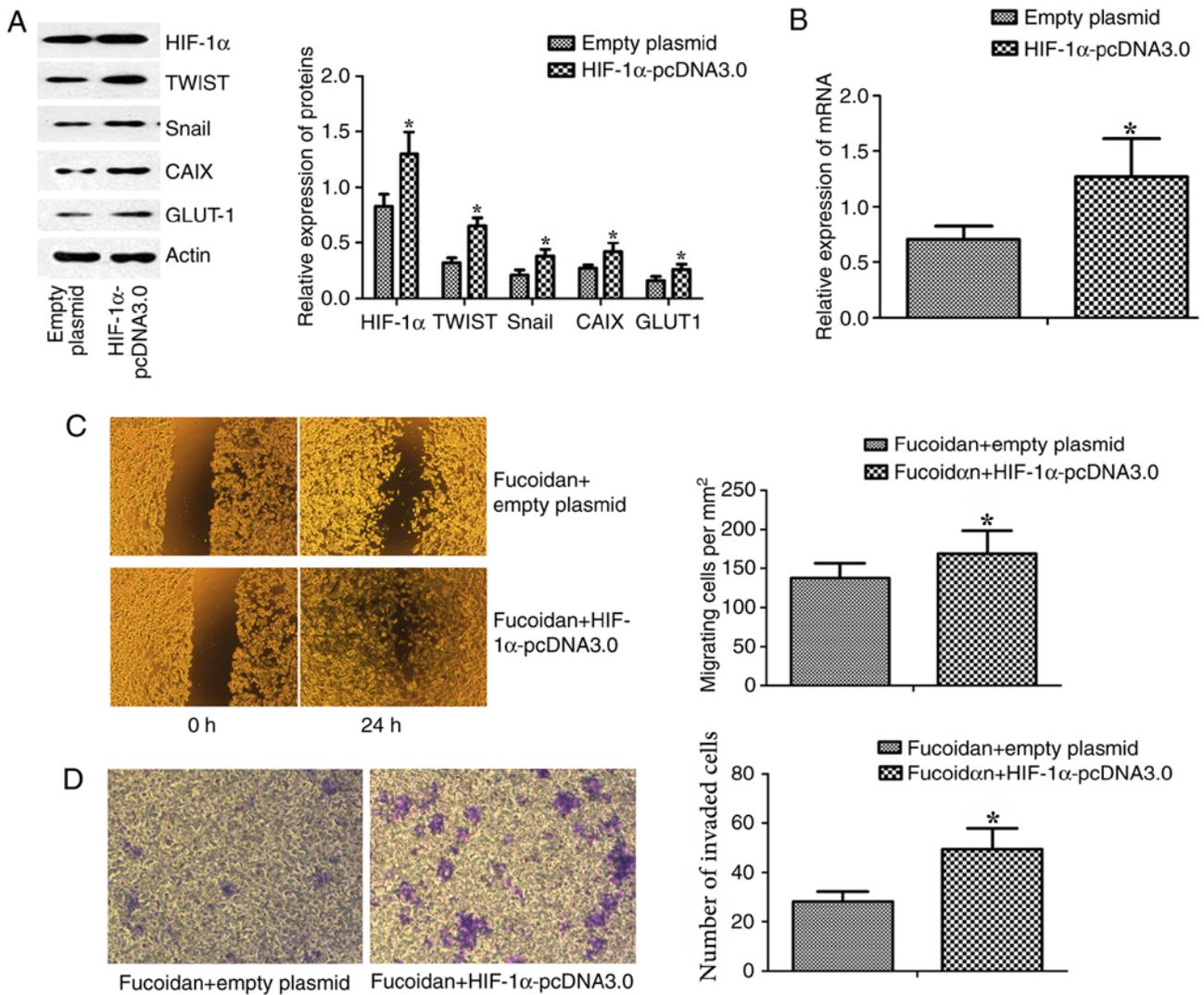


Figure 7. Overexpression of HIF-1α attenuates fucoidan-mediated suppression of cell invasion and migration. (A) Breast cancer cells were transfected with HIF-1α overexpression plasmid (HIF-1α-pcDNA3.0). Cell lysates from different groups were prepared and probed for HIF-1α and its target genes, including TWIST and Snail, through western blotting. (B) HIF-1α mRNA expression in breast cancer cells was measured by reverse transcription-quantitative polymerase chain reaction. GAPDH was used as a reference. Post-transfection with HIF-1α-pcDNA3.0, the mRNA expression levels of HIF-1α were increased. Data are presented as the means ± standard deviation. \*P<0.05 vs. empty plasmid. (C) Scratch assay (magnification, x100). (D) Transwell assay (magnification, x200). Breast cancer cells were transfected with HIF-1α-pcDNA3.0 and further treated with 25 μg/ml fucoidan. Migration and invasion of cancer cells were detected after 24 h. Data are presented as the means ± standard deviation. \*P<0.05 vs. fucoidan + empty plasmid. CAIX, carbonic anhydrase IX; GLUT-1, glucose transporter protein type-1; HIF-1α, hypoxia-inducible factor-1α.

HIF-1 can result in metabolic adaptation (or ‘reprogramming’) to hypoxic and energy-deprived conditions, and can enable tumor cells to survive. HIF-1 upregulates glucose influx through GLUT-1 and increases proton efflux via membranous CAIX. CAIX is involved in the hydration of carbon dioxide to form carbonic acid, which suggests a putative role of this enzyme in the acidification of the tumor environment. A high level of CAIX in patients with mammary cancer has been associated with a short disease-free survival time (42). Hypoxia in tumor tissues can markedly induce the expression of CAIX and has been proposed to be involved in acidification of the tumor microenvironment. In addition, hypoxia can increase cell adhesion and invasiveness. It has been reported that high levels of CAIX are linked to poor prognosis via regulation of the EMT (43). CAIX has the capacity to modulate cell adhesion mediated by E-cadherin through interactions with β-catenin (44). GLUT-1 levels are

positively associated with the expression of vimentin and N-cadherin in tumor tissues but are negatively associated with the levels of E-cadherin. Compared to the survival rates of patients with low levels of GLUT-1, vimentin and N-cadherin, those of patients with high levels of GLUT-1, vimentin and N-cadherin are much lower (45). In the present study, fucoidan treatment decreased the expression of HIF-1α target genes, including TWIST, Snail, CAIX and GLUT-1, in cells in a hypoxic environment.

The mechanism by which fucoidan downregulates the HIF-1α signaling pathway will be the next question explored by this research team. *In vivo* experiments are required to further confirm the effects of downregulation of this pathway on inhibition of migration and invasion. In addition, the present study suggested that HIF-1α may be a possible novel target for the treatment of TNBC. In animal experiments, we aim to further determine the antitumor effect of fucoidan on

TNBC, and focus on the preventative and inhibitory effects of fucoidan and its mechanism on tumor metastasis. Once the inhibitory effects and target of action of fucoidan on tumor metastasis have been confirmed and the appropriate dosage of fucoidan has been clarified, further clinical trials may be conducted. This would help to develop fucoidan into a natural antitumor drug.

In conclusion, to the best of our knowledge, the present study identified a novel mechanism by which fucoidan may inhibit EMT and metastasis through the HIF-1 $\alpha$  signaling pathway in mammary cancer. Fucoidan inhibited the activation and nuclear accumulation of HIF-1 $\alpha$ . EMT regulators downstream of HIF-1 $\alpha$ , including TWIST, Snail, CAIX and GLUT-1, were also downregulated. Subsequently, the expression levels of N-cadherin and vimentin, which are typical markers of EMT, were decreased, whereas the levels of ZO-1 and E-cadherin were increased, leading to the inhibition of EMT and migration in TNBC cells under hypoxic conditions. Therefore, the present study provides novel insight into the mechanisms of fucoidan in mammary cancer treatment.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

MX and HL analyzed and interpreted the data. WL, JZ and TS performed the cell culture, proliferation assay, overexpression of HIF-1 $\alpha$  and examination of protein factors. DX and YL performed migration and invasion assays. MX was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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