ORIGINAL ARTICLE

WILEY **Cancer Science**

Forkhead box C1 boosts triple-negative breast cancer metastasis through activating the transcription of chemokine receptor-4

Hongchao Pan[1] (iD) | Zhilan Peng[2] | Jiediao Lin[1] | Xiaosha Ren[1] | Guojun Zhang[1] |
Yukun Cui[1]

[1]Guangdong Provincial Key Laboratory for Breast Cancer Diagnosis and Treatment, Cancer Hospital of Shantou University Medical College, Shantou, China

[2]College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, China

Correspondence
Yukun Cui, Guangdong Provincial Key Laboratory for Breast Cancer Diagnosis and Treatment, Cancer Hospital of Shantou University Medical College, Shantou, China.
Email: yukuncui@yahoo.com

The transcription factor forkhead box C1 (FOXC1) has recently been proposed as a crucial regulator of triple-negative breast cancer (TNBC) and associated with TNBC metastasis. However, the mechanism of FOXC1 in TNBC development and metastasis is elusive. In this study, overexpression of FOXC1 in MDA-MB-231 cells significantly enhanced, whereas knockdown of FOXC1 in BT549 cells significantly reduced, the capabilities of TNBC cell invasion and motility in vitro and metastasis to the lung in vivo, when compared to their respective control cells. Mechanistic studies revealed that FOXC1 increased the expression of CXC chemokine receptor-4 (CXCR4), probably through transcriptional activation. AMD3100, an inhibitor of CXCR4, could block cell migration. In a zebrafish tumor model, AMD3100 could suppress cell invasion and metastasis. In addition, overexpressing CXCR4 in FOXC1-knockdown BT549 cells increased the capabilities of TNBC cell invasion and motility. In contrast, inhibition of CXCR4 with either AMD3100 or siRNA in MDA-MB-231 cells overexpressing FOXC1 reduced the capabilities of invasion and motility. Taken together, our results reveal a potential mechanism for FOXC1-induced TNBC metastasis.

KEYWORDS
CXCR4, FOXC1, invasion, metastasis, triple-negative breast cancer

1 | INTRODUCTION

Recent microarray technologies have enabled the molecular classification of breast cancer into four subtypes: estrogen receptor-positive/luminal-like, human epidermal growth factor receptor 2-overexpressing (Her2/ErbB2-positive), normal-like, and basal-like.[1,2] Basal-like breast cancer accounts for 15%-20% of all breast cancers. It is the most aggressive breast cancer, accounting for 75% of TNBC and easily metastasizing to distant organs, such as lung, bone, and liver.[3,4] Although advanced treatments have dramatically improved the survival rate of breast cancer, TNBC, especially those with distal metastasis, has no effective treatments due to lack of therapeutic target(s).[5] Currently, toxic chemotherapy is still the only form of TNBC management. Hence, it is urgent to identify crucial biomarkers of TNBC and design effective therapies for this disease.

Abbreviations: BLBC, basal-like breast cancer; CXCR4, chemokine receptor-4; FOXC1, Forkhead box C1; NF-κB, nuclear factor-κB; PCMT1, protein-L-isoaspartate O-methyltransferase; RT-PCR, real-time PCR; TNBC, triple-negative breast cancer.

- -

Forkhead box C1 is a transcription factor belonging to the fork-head box family that plays multiple functions in the formation of vasculature and organic development during embryogenesis.[6,7] It is also involved in cell growth and invasion as well as regulation of metabolism, and longevity.[8,9] In vivo, FOXC1 knockout mice die at birth and have anterior eye segment malformations.[10,11] Accumulating evidence has shown that FOXC1 exerts an important function in cancer tumorigenesis and progression. Overexpression of FOXC1 increases cell invasiveness and promotes distal metastasis in many cancer types.[4,12-14] Further examinations show that FOXC1 is a potential biomarker for BLBC and predicts poor survival in breast cancer patients.[4,15,16] Forkhead box C1 promotes BLBC cell proliferation and invasion through regulating NF-κB signaling or the expression of MMP7, which is downstream of NF-κB.[17,18] Forkhead box C1 activates Smoothened-independent Hedgehog signaling and enhances BLBC cancer stem cell properties.[15] In addition, FOXC1 induces epithelial-mesenchymal transition through inhibition of E-cadherin expression in mammary epithelial cells.[19,20] The mechanisms of how FOXC1 exerts these effects are not well understood. Therefore, we chose two cell lines in this study to examine the potential mechanisms and to determine whether FOXC1 could be a promoter for TNBC.

CXC chemokine receptor-4, a G protein–coupled cell surface receptor with seven transmembrane-spanning domains, selectively binds the stromal cell-derived factor 1 to play a crucial function in various cancers, including esophageal cancer, ovarian cancer, gastric cancer, and breast cancer.[21,22] Additionally, CXCR4 is overexpressed in various tumor cells and mediates cell survival, proliferation, and migration.[23-25] Recent researches disclose that CXCR4 is a crucial regulator facilitating breast cancer cell metastasis.[22,26-29] Moreover, inhibition of CXCR4 in basal-like MDA-MB-231 breast cancer cells blocks breast cancer metastasis to the lung.[27,30]

Our group reported previously that CXCR4 expression is associated with TNBC distal metastasis.[31] Previous works suggested that FOXC1 could transcriptionally enhance CXCR4 expression in mouse embryonic endothelial cells.[32] This study aims to reveal whether FOXC1 could promote the metastatic capability of TNBC cells through regulating the expression of CXCR4. We discovered that overexpression of FOXC1 in MDA-MB-231 cells significantly enhanced, whereas knockdown of FOXC1 in BT549 cells significantly reduced, the capabilities of TNBC cell invasion and motility in vitro and metastasis to the lung in vivo, when compared to their respective control cells. We then determined that FOXC1 enhanced the expression of CXCR4, probably through transcriptional upregulation of CXCR4. Moreover, we found that CXCR4 might mediate FOXC1-induced capabilities of invasion and motility in BLBC cells. Collectively, these data suggest that FOXC1 promotes TNBC cells metastasis by upregulating expression of CXCR4.

2 | MATERIALS AND METHODS

2.1 | Cell culture and specimens

The human breast cancer cell lines MDA-MB-231 and BT549 were obtained from ATCC (Manassas, VA, USA) and cultured according to the instructions of the manufacturer. These cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FCS. Seven TNBC specimens frozen in liquid nitrogen were selected from Cancer Hospital of Shantou University Medical College (Shantou, China) and verified by immunohistochemistry. This study was carried out with the approval of the ethical committee of Cancer Hospital of Shantou University Medical College.

2.2 | Transient and stable transfection

For siRNA-mediated knockdown, cells were transiently transfected with siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. MDA-MB-231 cells stably overexpressing FOXC1 or BT549 cell with stable knockdown of FOXC1 and their respective control transfectants were generated using standard procedures.[4] Briefly, cells were stably transfected with the pLKO.1 shFOXC1 or the control shRNA construct using Lipofectamine 2000 and selected with 5 μg/mL puromycin. A full-length human FOXC1 cDNA was inserted into the pCMV6 plasmid and stably transduced into MDA-MB-231 cells using Lipofectamine 2000. Stable transfected MDA-MB-231 cells were selected with 800 μg/mL G418. Overexpression or knockdown efficiency was confirmed by quantitative RT-PCR and immunoblotting analysis. An siRNA targeting CXCR4 and a control scramble siRNA were purchased from GenePharma (Shanghai, China). The sequence for si-CXCR4 was 5′-UAAAAUCUUCCUGCCCACCdTdT-3′.[30] The non-specific scramble siRNA (5′-UUCUCCGAACGUGUCACGUdTdT-3′) was used as the negative control. The human FOXC1 shRNA sequence is CCGGCAAGAAGAAGGACGCGGTGAACT- CGAGTTCAC CGCGTCCTTCTTCTTGTTTTTG, corresponding to 523-543 of the FOXC1 cDNA.

2.3 | Colony formation assay

Cells (400 cells/well) were seeded onto 6-well plates. After incubation at 37°C for 14 days, the colonies were fixed with methanol for 20 minutes, stained with 0.4% crystal violet, and photographed using phase-contrast light microscopy. An accumulated growth of more than 50 cells was identified as the formation of a colony. We used ImageJ software (NIH, Bethesda, MD, USA) to calculate the colony numbers.

2.4 | Wound healing assay

MDA-MB-231 cells were seeded at a density of 4×10^4 cells per well in 96-well plates. After treatment with 40 μmol/L AMD3100, the monolayer of cells was scratched with a 10-μL plastic pipette tip to create a uniform wound. The wound width was then examined after 24 hours of incubation under a phase-contrast microscope (IX51; Olympus, Tokyo, Japan). Photographs of at least 3 random fields were taken, and the cell migration ability was expressed by the closure of the gap distance. The distance was measured by ImageJ software.

2.5 | Migration and invasion assays

Migration was assessed using culture well inserts from Costar (Corning, NY, USA). Invasion was assayed using Matrigel-coated Transwell chambers. For migration and invasion experiments, 5×10^4 cells in serum-free DMEM were plated on the upper chamber of the insert. The lower chamber was filled with DMEM supplemented with 10% FCS. Twenty-four hours after being plated, cells that had migrated or invaded were fixed by methanol, stained with 0.1% crystal violet, and then photographed under ×20 magnification. Cells were counted in quadruplicate fields of view in triplicate membranes.

2.6 | Luciferase reporter assay

MDA-MB-231 cells stably overexpressing FOXC1 or BT549 cells with stable knockdown of FOXC1 and their respective control transfectants were plated at a density of 1×10^5-5×10^5 cells/mL into 24-well plates and transfected with 0.2 μg pGL3-CXCR4 promoter-luciferase plasmid and 0.02 μg SV40 *Renilla* luciferase plasmid (Promega, Madison, WI, USA) After 48 hours, cells were lysed with lysis buffer, and cell debris were removed by centrifugation at 14 500 *g* for 1 minute in an Eppendorf microcentrifuge. The luciferase activity was detected with the Dual Luciferase Assay (Promega) according to the manufacturer's instructions. The relative luciferase intensity was measured by a Modulus TD20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA). All luciferase assays were carried out in triplicate. The promoter of CXCR4 was PCR amplified from genomic DNA using the primers 5′-*GGGGTACC*TTCCAGCCACCAC CCTCCA-3′ (forward), 5′-*GAAGATCT*CGGCGTCACTTTGCTACCT G-3′ (reverse), digested with *Kpn*I (FD0524; Fermentas) and Bgl II (fd0083; Fermentas), and ligated to the corresponding sites in pGL3 Basic vector (Promega).

2.7 | Real-time PCR

Total RNA was extract using TRIzol (Invitrogen), and reverse transcription was carried out using an RT-PCR kit (RR047A and RR820A; Takara, Beijing, China) according to the manufacturer's instructions; GAPDH was used as a loading control. The reactions were undertaken with a 7300 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The data were displayed as $2^{-\triangle\triangle Ct}$ values. The experiments were repeated three times.

The primer sequences as follows: FOXC1 forward, 5′-CCCGTTTATGAAAGTCGC -3′; FOXC1 reverse, 5′-GTCCACGAC ATCCAACTACA-3′; CXCR4 forward, 5′-CCGTGGCAAACTGGTACT TT-3′; CXCR4 reverse, 5′-GACGCCAACATAGACCACCT-3′; GAPDH forward, 5′-CAATGACCCCTTCATTGACC-3′; and GAPDH reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′.

2.8 | Immunoblotting analysis

Equal amount of proteins (50 μg) were separated onto SDS-polyacrylamide gels and were electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary Abs; GAPDH was used as the loading control. Primary Abs used included anti-FOXC1 (1:500, AP8907B; Abgent, San Diego, CA, USA), anti-CXCR4 (1:1000, ab58176; Abcam, Cambridge, UK), anti-PCMT1 (1:1000, ab97446, Abcam), anti-GAPDH (1:2000, sc-47724; Santa Cruz Biotechnology, Dallas, TX, USA). After that, the membranes were incubated with corresponding HRP-conjugated secondary Abs. The blot signals were visualized using the ECL western blotting substrate (Promega).

2.9 | Chromatin immunoprecipitation-coupled RT-PCR

Chromatin immunoprecipitation was carried out using a kit (17-10086; Merck, Kenilworth, NJ, USA) according to the instructions. Briefly, 37% formaldehyde was added directly to the cultured BT549 cells to a final concentration of 1% and then incubated at room temperature for 10 minutes. Glycine (final concentration = 0.125 mol/L) was added to the reaction for 5 minutes at room temperature to stop cross-linking, then the cells were washed by cooled PBS and lysed directly in cell lysis buffer for 15 minutes. The samples were pelleted, resuspended in nuclear lysis buffer, and sonicated to obtain chromatin fractions with an average size of 200-1000 bp using a Biosafer sonicator (1200-98C; Hong Kong, China). The chromatins were incubated with magnetic A-G beads, 2 μg goat anti-human FOXC1 Ab (ab5079; Abcam, Cambridge, UK) or 2 μg normal goat IgG overnight at 4°C. The bound chromatin was washed extensively and eluted twice with elution buffer. After addition of 20 μL of 5 mol/L NaCl, the cross-linking was reversed by 4 hours of incubation at 65°C. The immunoprecipitated DNAs as well as whole cell extract DNAs (input) were preliminarily purified by treatment with RNase A and then proteinase K followed by further purification with a DNA Purification Kit (CW2301M; CWBio, Beijing, China). The purified DNA was used for quantitative PCR (RR820A; Takara) analyses using the specific primers of CXCR4 containing the potential FOXC1 binding site. The sequences were forward, 5′-CCTTGTCTGGTTCACGCTGGA-3′, and reverse, 5′-AA TCACTCATTCATTCTCACAACAC-3′. The amplification conditions were: initial denaturation, 95°C for 1 minute; denaturation, 95°C for 5 seconds; annealing, 60°C for 34 seconds, extension, 72°C for 30 seconds, altogether 40 cycles; 72°C extension for 2 minutes, by using the Applied Biosystems 7500 Real-Time PCR System.

2.10 | Zebrafish tumor metastasis model

All animal experiments were approved by the Animal Care Committee of Shantou University Medical College. Zebrafish embryos were fed at 28°C under standard experimental conditions. Zebrafish embryos were incubated in E3-medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl$_2$, and 0.33 mmol/L MgSO$_4$) and supplemented with 0.2 mmol/L phenylthiourea (Sigma-Aldrich, St. Louis, MO, USA) at 24 hours postfertilization. At 48 hours postfertilization, zebrafish

embryos were dechorionated with a pair of sharp-tip forceps and anesthetized with MS-222 (0.03 mg/mL; Sigma-Aldrich). Anesthetized embryos were subjected to microinjection. BT549 cells were treated with 40 µmol/L AMD3100 or PBS for 48 hours and then labeled with 2 µg/mL CellTracker CM-DiI dye (Invitrogen) in vitro. Tumor cells were washed and resuspended in DMEM (Hyclone, Thermo, Waltham, MA, USA), and approximately 150 tumor cells in 5 nL DMEM were injected into 1 embryo yolk by an Eppendorf microinjector (FemtoJet 5247, Hauppauge, CT, USA). The injected zebrafish embryos were washed once with fish water and transferred into E3 medium containing phenylthiourea. After incubation at 31°C for 72 hours, we monitored the tumor cell invasion and metastasis using a fluorescent microscope. The intensity of DiI-labeled tumor cells was measured using ImageJ software.

2.11 | In vivo metastasis assay

This animal experiment for testing the effect of FOXC1 on BLBC cells metastasis to the lung was approved by the Animal Care Committee of Shantou University Medical College and was undertaken in accordance with national and international guidelines. Cells described in the figure legend were suspended in a density of 1×10^7 per mL PBS; 0.1 mL cell suspension was injected into the tail veins of 6-week-old SCID mice (Vital River Laboratory Animal Technology, Beijing, China). Eight weeks later, the mice were killed and the lungs were fixed in 10% formalin for tissue sectioning. The numbers of lung metastatic nodules were counted by H&E staining.[33]
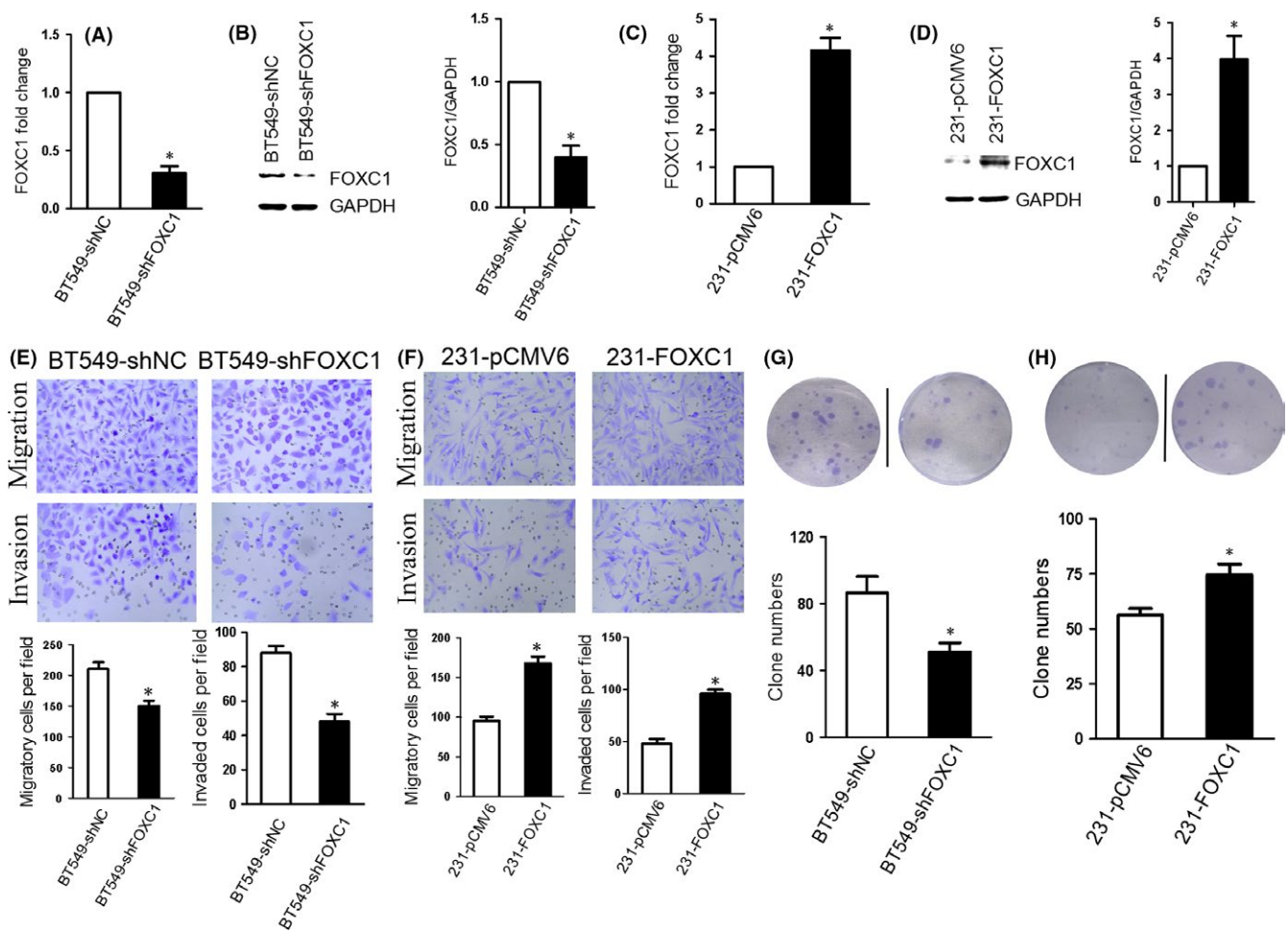

FIGURE 1 Forkhead box C1 (FOXC1) improves cell migration and invasion of basal-like breast cancer cells. A, Real-time PCR shows the efficiency of FOXC1 knockdown in BT549 cells. *P < .05, n = 3. B, Immunoblotting and quantitative analyses show the efficiency of FOXC1 knockdown in BT549 cells. GAPDH served as control. C, Real-time RT-PCR shows the efficiency of FOXC1 overexpression in MDA-MB-231 cells. D, Immunoblotting and quantitative analyses show the efficiency of FOXC1 overexpression in MDA-MB-231 cells. GAPDH served as control. E, Cell migration and invasion were evaluated after FOXC1 knockdown. Inhibition of FOXC1 expression significantly reduced cell migration and invasion compared with the control group. Three independent experiments were carried out. *P < .05. F, Overexpression of FOXC1 significantly increased cell migration and invasion compared with the control group. *P < .05, n = 3. G, Results of colony formation assays show that FOXC1 knockdown reduced the numbers of formed colonies. *P < .05, n = 3. H, Results of colony formation assays showed that FOXC1 overexpression increased the numbers of formed colonies. *P < .05, n = 3

2.12 | Statistical analyses

Data were reported as mean values ± SEM. Differences were assessed for statistical significance by a two-tailed *t* test. All statistical analyses were carried out using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). *P* values < .05 were considered to be statistically significant.

3 | RESULTS

3.1 | Forkhead box C1 promotes the capabilities of invasion, motility, and colony formation of TNBC cells

To examine whether FOXC1 could affect the metastatic ability of BLBC cells, we first carried out Boyden chamber or modified Boyden chamber assays to examine the in vitro migration and invasiveness of MDA-MB-231 cells stably overexpressing FOXC1 (231-FOXC1) or BT549 cells with FOXC1 knockdown (BT549-shFOXC1) and their respective control transfectants (231-CMV6 or BT549-shNC, respectively). Before starting the experiments, the mRNA and protein levels of FOXC1 in these cells were examined by RT-PCR or immunoblotting to confirm the overexpression or knockdown of FOXC1 (Figure 1A-D). Consistent with previous reports,[4,15] FOXC1 knockdown significantly decreased the numbers of migrated BT549 cells (150 ± 15.13 cells per field) compared to the scramble control (210 ± 19.31 cells per field, *P* < .05; Figure 1E) and also reduced the numbers of invaded BT549 cells (48 ± 7.94 cells per field) when compared to the scramble control (88 ± 7.21 cells per field, *P* < .05; Figure 1E). In contrast, overexpressing FOXC1 boosted the migration (168.33 ± 14.51 vs 95.33 ± 9.51; *P* < .05) and invasion (96 ± 6.56 vs 48.67 ± 7.94; *P* < .05) of MDA-MB231 cells in comparison with control transfectants (Figure 1F). In addition, we examined whether FOXC1

could influence the in vitro clonogenic capacities of BLBC cells and revealed that FOXC1 knockdown reduced the colony-forming efficiency (86.33 ± 17.16 vs 52 ± 9.64, *P* < .05; Figure 1G) of BT549 cells, whereas FOXC1 overexpression improved the colony-forming efficiency (56.35 ± 5.13 vs 74.67 ± 8.34, *P* < .05; Figure 1H) of MDA-MB-231 cells. These data suggest that FOXC1 could enhance the in vitro metastatic capabilities of TNBC cells.

3.2 | Forkhead box C1 facilitates TNBC cells metastasis to the lung

We next investigated whether FOXC1 could affect TNBC cells lung metastasis in vivo. BT549 cells with knockdown FOXC1 or MDA-MB-231 cells overexpressing FOXC1 and their respective controls were injected into the tail vein of SCID mice. Hematoxylin and eosin staining of lung sections revealed that knockdown of FOXC1 in BT549 cells completely inhibited breast cancer lung metastasis, whereas the mice injected with control cells showed 3.21 ± 1.38 metastatic foci under the microscope (Figure 2A, *P* < .05). Conversely, the lungs of the mice injected with MDA-MB 231 cells overexpressing FOXC1 developed 21.52 ± 3.38 metastatic foci, whereas the mice injected with control cells showed 4.31 ± 2.87 metastatic foci under the microscope (Figure 2B, *P* < .05). These results indicate that FOXC1 could promote breast cancer lung metastasis in vivo.

3.3 | Forkhead box C1 transactivates the expression of CXCR4

CXC chemokine receptor-4 is associated with distal metastasis in BLBC,[31] and is transcriptionally regulated by FOXC1 in vascular endothelial cells in mice.[32] Forkhead box C1 might also regulate the expression of CXCR4 in breast cancer cells. To examine this hypothesis,
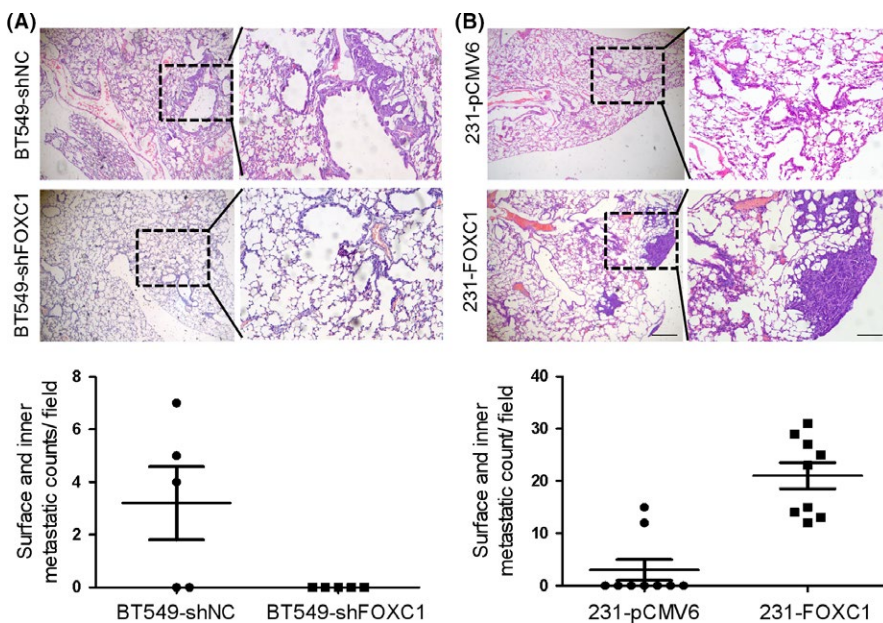

FIGURE 2 Forkhead box C1 (FOXC1) promotes cell metastasis to the lung. A, Inhibition of FOXC1 suppresses lung metastasis of triple-negative breast cancer (TNBC) cells in SCID mouse models. Representative images of H&E staining for lung micrometastasis (top) and quantification of lung metastatic nodules (bottom). Right panels show a higher magnification. *P < .05, n = 5. B, Overexpression of FOXC1 promotes lung metastasis of TNBC cells. Representative images of H&E staining for lung micrometastasis (top) and quantification of lung metastatic nodules (bottom). Right panels show a higher magnification.*P < .05, n = 9. Scale bar = 200 μm (low magnification) and 50 μm (high magnification)
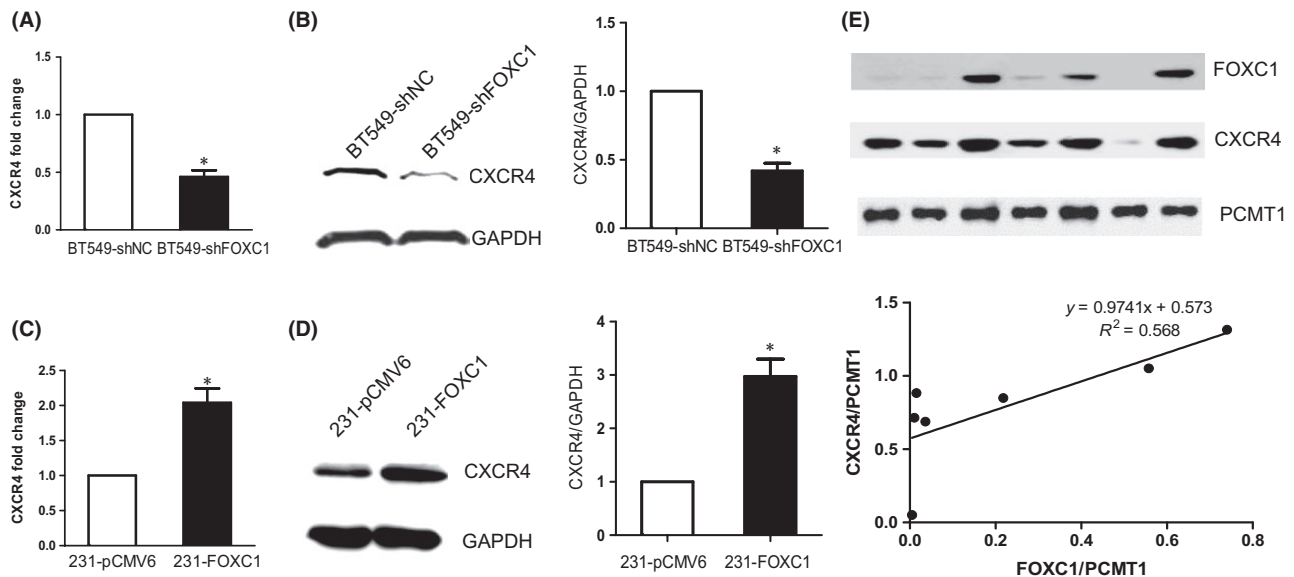
FIGURE 3 Forkhead box C1 (FOXC1) affects CXC chemokine receptor-4 (CXCR4) expression. A, Real-time RT-PCR shows the *CXCR4* gene expression in BT549 cells with downregulated FOXC1. *P < .05, n = 3. B, Western blot and quantitative analyses show CXCR4 expression in BT549 cells with downregulated FOXC1. GAPDH served as control. *P < .05, n = 3. C, Real-time RT-PCR shows the *CXCR4* gene expression in MDA-MB-231 cells transfected FOXC1 vector. GAPDH served as an internal control. *P < .05, n = 3. D, Western blot and quantitative analyses show the *CXCR4* expression in MDA-MB-231 cells transfected FOXC1 vector. GAPDH served as control. *P < .05, n = 3. E, Western blotting shows the FOXC1 and CXCR4 expression in clinical samples (top), and the correlation of the FOXC1 and CXCR4 expression was analyzed (bottom)
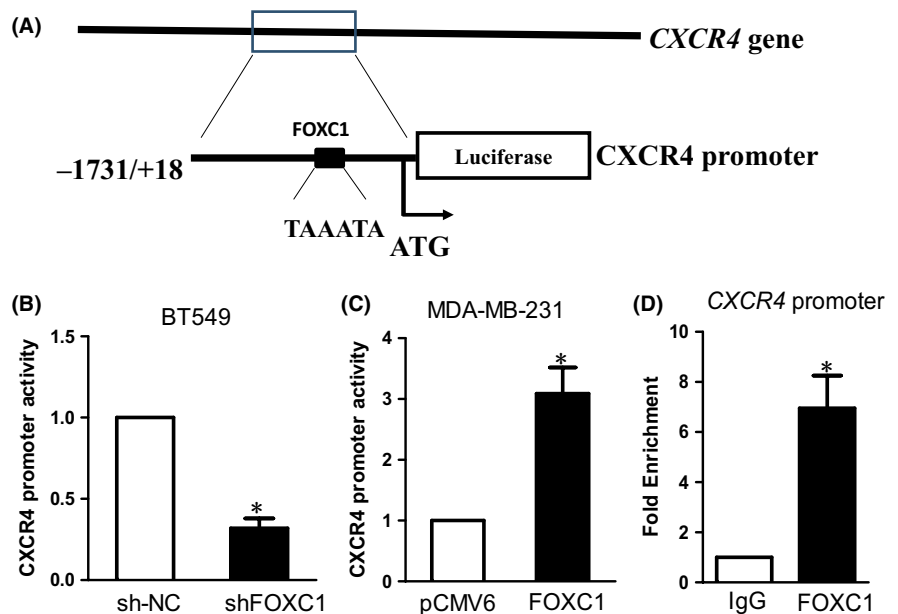

FIGURE 4 Forkhead box C1 (FOXC1) activates the transcription of CXC chemokine receptor-4 (CXCR4). A, Map of the CXCR4 promoter region with the positions of FOXC1 potential consensus binding sites. B, Inhibition of FOXC1 suppresses CXCR4 promoter activity in BT549 cells. *P < .05, n = 3. C, Overexpression of FOXC1 activates CXCR4 promoter activity in MDA-MB-231 cells. *P < .05 vs control, n = 3. D, ChIP real-time PCR assays showed that FOXC1 increases fold enrichment of CXCR4 promoter. *P < .05, n = 3

we first detected CXCR4 expression levels in BT549 cells with knockdown of FOXC1 or MDA-MB-231 cells overexpressing FOXC1 and their respective controls and found that the levels of CXCR4 mRNA and protein were reduced in BT549-shFOXC1 but increased in 231-FOXC1 cells when compared to their respective control transfectants (Figure 3A-D), suggesting that FOXC1 could upregulate the expression of CXCR4. Western blot analysis was used to determine FOXC1 and CXCR4 protein expression in 7 BLBC specimens with PCMT1 as

a loading control. The expression of FOXC1 was significantly varied in these specimens, and CXCR4 was expressed in all these specimens (Figure 3E). Following the normalizing of the protein expression levels of FOXC1 and CXCR4 to their respective PCMT1 expression levels in each specimen, we found that the FOXC1 protein level was positively correlated with CXCR4 protein level through linear regression analysis (Figure 3E), indicating that FOXC1 might transcriptionally upregulate the expression of CXCR4. We thus constructed a reporter with

a 1.7-kb promoter region of human CXCR4 (−1731 to +18) containing a putative FOXC1 binding site (Figure 4A). Reporter gene assays showed that the CXCR4 promoter activity normalized to that of the cotransfected *Renilla* luciferase was reduced by approximately 70% in BT549-shFOXC1 cells when compared with that in BT549-shNC cells (Figure 4B). However, the relative promoter activity was 3-fold higher in 231-FOXC1 than in 231-pCMV6 cells (Figure 4C). To confirm that *CXCR4* is a direct target gene of FOXC1, we carried out ChIP assays using a polyclonal Ab against FOXC1 in BT549 cells. As shown in Figure 4D, relative enrichment of CXCR4 promoter was markedly increased compared with the control normal goat IgG by quantitative PCR. These results are in line with those shown in Figure 3, further suggest that FOXC1 could upregulate the expression of CXCR4 at the transcription level, at least partially.

3.4 | Inhibition of CXCR4 suppressed cell migration, invasion, and metastasis

To identify the function of CXCR4, we chose AMD3100, a selective CXCR4 antagonist to treat MDA-MB-231 cells, and found that

expression of CXCR4 was inhibited by AMD3100 (Figure 5A). Wound healing assays showed that AMD3100 could suppress cell migration compared with the control group (Figure 5B). The zebrafish embryos were used to monitor human tumor cell invasion and metastasis in vivo. AMD3100 treatment decreased the ability of BT549 cells to metastasize to the trunk region of the zebrafish embryo (Figure 5C). Quantification analysis showed that the intensity of distal metastatic tumor cells treated with AMD3100 in the trunk was markedly lower than the intensity of the control cells (Figure 5D). These findings further verified that inhibition of CXCR4 reduced cancer cell metastasis.

3.5 | CXC chemokine receptor-4 mediates the FOXC1 enhancement of TNBC invasive capabilities in vitro

As CXCR4 has been reported to participate in the growth, migration, and invasion of cancer cells,[34-36] we further investigated whether FOXC1 expression would affect cell migration and invasion through CXCR4. First, western blot results showed that CXCR4 expression was downregulated by siRNA, and the inhibitory effect
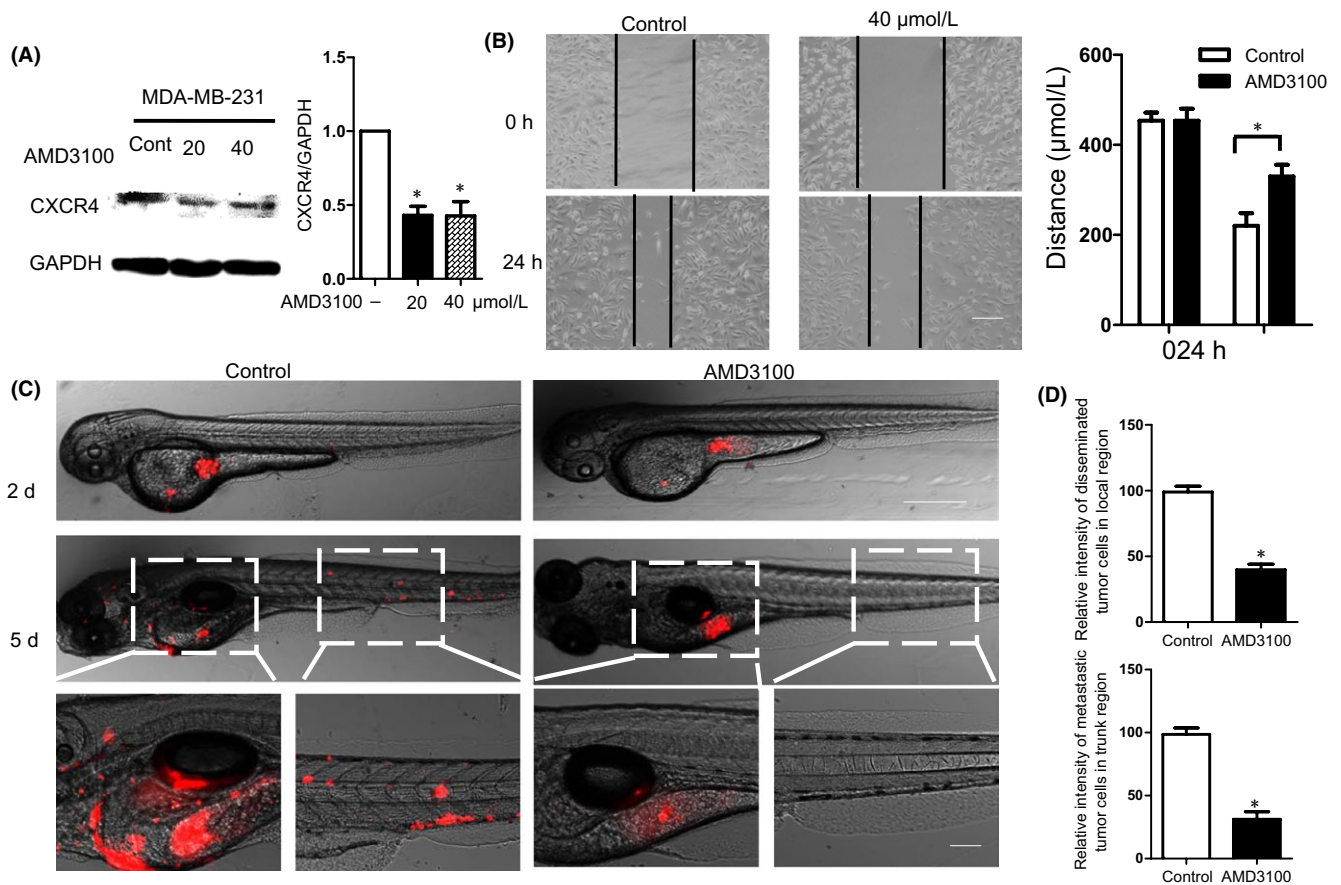

FIGURE 5 AMD3100 attenuated triple-negative breast cancer cell migration, invasion, and metastasis. A, CXC chemokine receptor-4 (CXCR4) expression was inhibited when treated with AMD3100 for 48 hours. *P < .05, n = 3. B, AMD3100 suppresses basal-like breast cancer cell migration in an in vitro wound healing assay. *P < .05, n = 3. Scale bar = 200 μm. C, Intravital fluorescence microscopy images of 5 days postfertilization zebrafish embryos taken 3 days postinjection (3 dpi). Scale bar = 500 μm. Bottom panels show a higher magnification of the boxes. Scale bar = 100 μm. D, Quantification of intensity of disseminated and metastatic cells in the primary tumor surroundings and in the trunk regions at 3 dpi. *P < .05, n = 22

was resisted by FOXC1 overexpression in MDA-MB-231 cells (Figure 6A). Transwell migration and invasion assay showed that FOXC1 overexpression could partially promote the invasion and motility of CXCR4-knockdown MDA-MB-231 cells (Figure 6B,C). To further identify whether FOXC1 mediated migration by regulating CXCR4, we chose AMD3100 to treat MDA-MB-231 cells overexpressing FOXC1 and found that FOXC1 attenuated the silencing of CXCR4 expression treated by AMD3100 in MDA-MB-231 cells (Figure 6D).

To explore the influence of FOXC1 on the aggressiveness of TNBC cells, we further investigated the effects of FOXC1 knockdown and CXCR4 overexpression on cultured BLBC cells. Our results indicated that transfection of CXCR4 resulted in its overexpression and restored the downregulation of CXCR4 induced by FOXC1 knockdown in BT549 cells as shown in western blot assays (Figure 7A). Moreover, overexpression of CXCR4 partially rescued the decrease of migration (Figure 7B) and invasion (Figure 7C) caused by shFOXC1 in BT549 cells. All these results indicate that upregulation of CXCR4 is involved in ectopic FOXC1 expression-promoted migration and invasion of TNBC cells.

4 | DISCUSSION

Breast cancer is a heterogeneous disease and comprises several molecular subtypes. TNBC, one of subtypes, is the most aggressive and has no targetd therapies currently.[1] The forkhead box transcription factor FOXC1, an evolutionarily conserved transcription factor, mediates multiple biological processes, including metabolism,

differentiation, development, and proliferation,[37] and participates in the progression of tumors.[38] Previous studies showed that FOXC1 is a special biomarker for BLBC and promotes cell proliferation and migration.[4,16] In this study, we also verify that FOXC1 enhances cell migration and invasion. We further show that FOXC1 improves the ability of cell colony formation in TNBC cells. Previous reports have shown that FOXC1 enhances the ability of cell migration and invasion in breast cancer.[4,18,39] In vivo studies reported that FOXC1-overexpressing cells have increased tumor growth and lung metastasis in nude mice, whereas FOXC1 knockdown completely suppressed tumorigenesis and lung metastasis.[13,15] These results show that FOXC1 plays a crucial role in TNBC. A recent study showed that FOXC1 exerts its promoting function through the NF-κB signaling pathway in BLBC.[18] Matrix metalloprotease-7, one of the downstream targets of NF-κB, is a novel regulatory mechanism that FOXC1 promotes in BLBC invasion and metastasis.[17] However, the regulatory mechanism of FOXC1 in TNBC remains to be elucidated.

In our studies, overexpression of FOXC1 could increase CXCR4 mRNA and protein expression in MDA-MB-231 cell. Conversely, inhibition of FOXC1 could reduce CXCR4 mRNA and protein expression. The level of FOXC1 expression was correlated with the expression of CXCR4 in clinical samples. In addition, the luciferase assay further showed that FOXC1 increases CXCR4 transcription activity. A recent study found that FOXC1 directly regulates CXCR4 expression by activating its promoter in mouse embryonic endothelial cells.[32] Indeed, our ChIP assay also identified that FOXC1 could bind the CXCR4 promoter and increase CXCR4 promoter fold enrichment in breast cancer. Furthermore, FOXC1-deficient endothelial cells show lower motility ability activated by CXCL12.[32] Interestingly, FOXO1, a member of
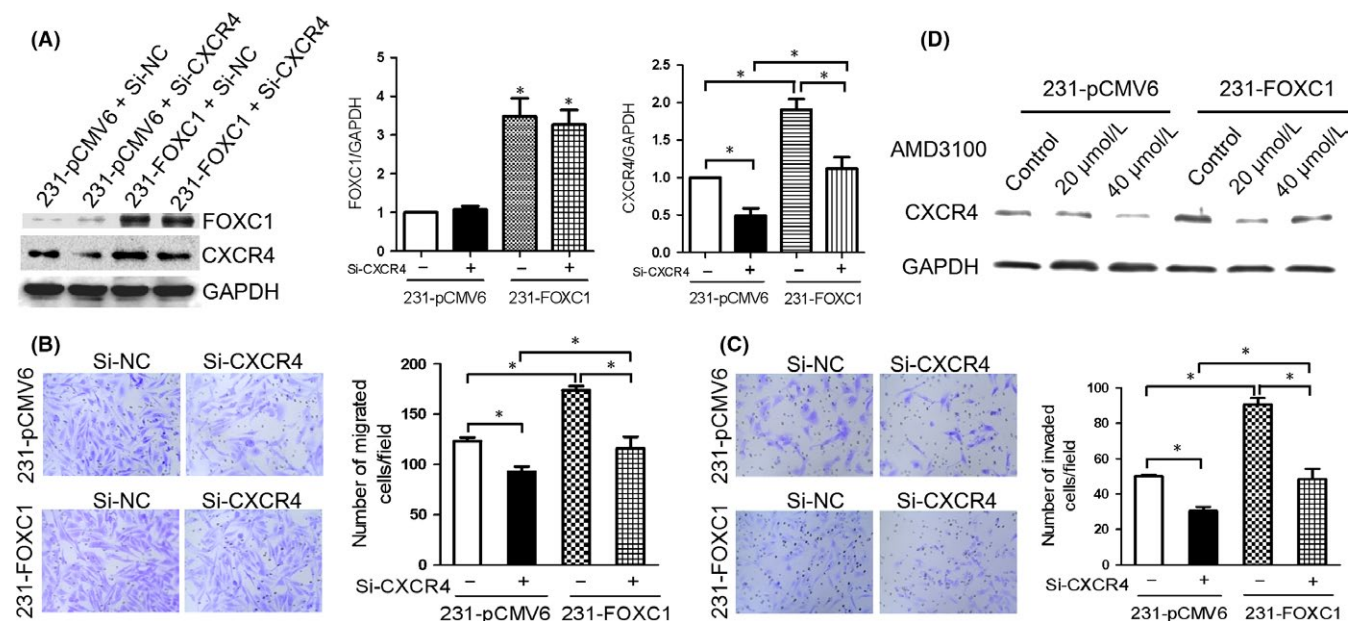

FIGURE 6 Inhibition of CXC chemokine receptor-4 (CXCR4) attenuated forkhead box C1 (FOXC1)-induced migration and invasion in MDA-MB-231 cells. A, Overexpression of FOXC1 restored CXCR4 expression in MDA-MB-231 cells transfected with CXCR4 siRNA. B, Migration of MDA-MB-231 cells with ectopic FOXC1 expression and CXCR4 knockdown was assessed by Transwell assays. *P < .05, n = 3. C, Invasion of MDA-MB-231 cells with ectopic FOXC1 expression and CXCR4 knockdown was assessed by Matrigel invasion assays. *P < .05, n = 3. D, FOXC1 overexpression attenuated the AMD3100-induced reduction of CXCR4. SiNC, nonspecific control siRNA
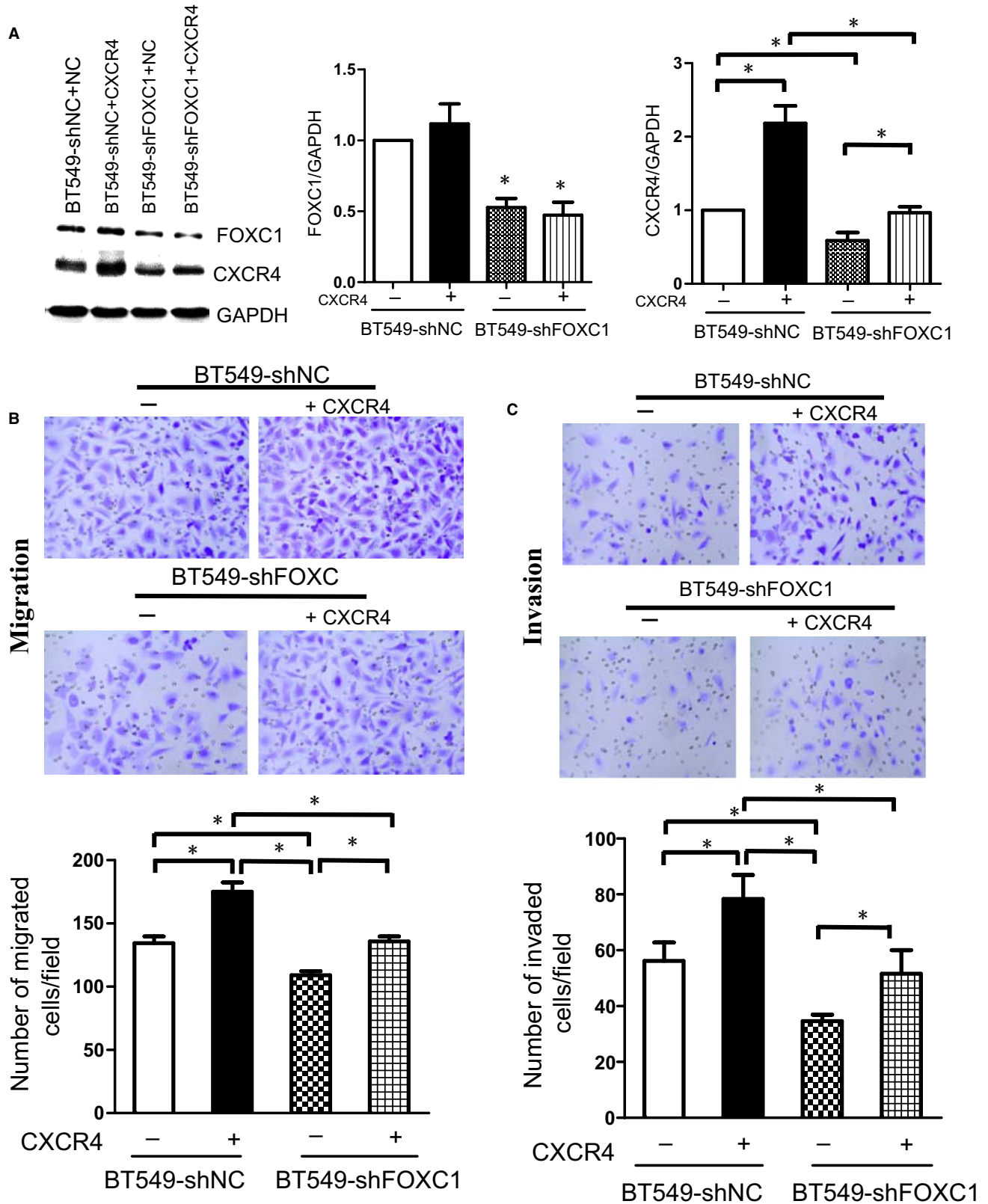
FIGURE 7 Overexpression of CXC chemokine receptor-4 (CXCR4) enhanced migration and invasion of forkhead box C1 (FOXC1) knockdown BT549 cells. A, Western blotting showed that transfection of CXCR4 attenuated the reduction of FOXC1-induced CXCR4 expression. B, Migration of FOXC1 knockdown BT549 cells with ectopic CXCR4 expression was assessed by Transwell assays and quantification of the number of migrated cells. *$P < .05$, n = 3. C, Invasion of FOXC1 knockdown BT549 cells with ectopic CXCR4 expression was assessed by Transwell assays and quantification of the number of invaded cells. *$P < .05$, n = 3. shNC, nonspecific control
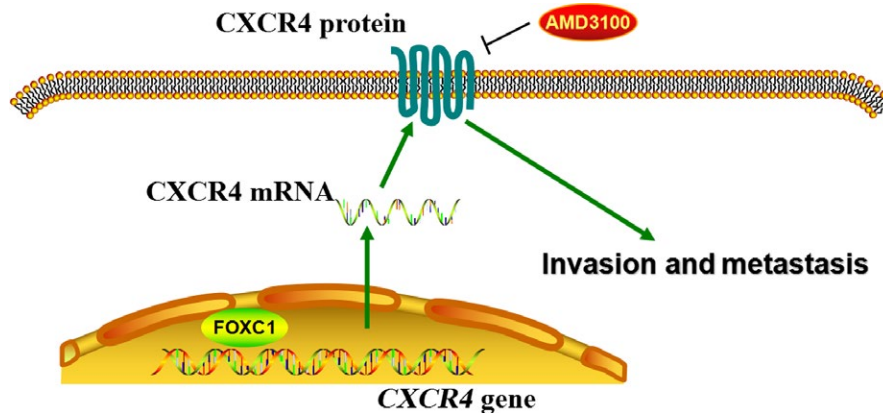
FIGURE 8 Schematic diagram delineating the promotion effects of forkhead box C1 (FOXC1) in triple-negative breast cancer cells. FOXC1 binds the promoter of the *CXCR4* gene, leading to activation of CXC chemokine receptor-4 (CXCR4) expression, which increases cell invasion and metastasis

the FOX transcription factor family, regulates CXCR4 expression and enhances postnatal vessel formation.[40] These results suggested that FOXC1 binds the CXCR4 promoter to regulate CXCR4 transcription, which indicates that CXCR4 was a downstream target of FOXC1.

Chemokine receptor-4, a G protein-coupled receptor that displayed 7 transmembrane helical domains, plays important functions in breast cancer. Studies showed that CXCR4 was expressed highly in both primary and metastatic breast cancer, but had a low level or even no expression in normal breast tissue.[28] Our results showed that inhibition of CXCR4 could block cell migration in vitro. In a zebrafish tumor model, we found that the cells treated with AMD3100 showed fewer metastatic tumor cells in the trunk region of the zebrafish embryos. This is consistent with reports that inhibition of CXCR4 in MDA-MB-231 cells reduced breast cancer metastases to the lung and lymph nodes in a mouse model.[27,30] High expression of CXCR4 in basal-like tumor patients resulted in higher recurrence incidence and related death compared with patients with low expression of CXCR4.[41,42] Hence, higher CXCR4 expression might predict a worse survival rate in patients with BLBC. Previous studies and our present data showed that FOXC1 promotes breast cancer cell migration and invasion. However, the mechanism by which FOXC1 enhanced CXCR4 expression remains unknown in TNBC. Our study shows that inhibition of CXCR4 expression significantly suppresses FOXC1-enhanced invasion and metastasis. Similarly, restoration of CXCR4 expression rescues the migration and invasion of TNBC cells reduced by FOXC1 knockdown. These results indicate that FOXC1 might exert its tumor promoting function, at least in part, through transcriptional regulation of CXCR4 in TNBC. Together, FOXC1 shows tumor-promoting activity that increases the growth, invasion, and metastasis of TNBC cells in vitro. Downregulation of CXCR4 expression inhibits FOXC1-induced migration and invasion. These data indicate that CXCR4 is partially involved in FOXC1-induced migration and invasion processes. Figure 8 shows a schematic model of the function of the FOXC1-CXCR4 axis in TNBC cells. This study also extends our knowledge about the regulation of CXCR4 at the transcriptional level by FOXC1 and suggests that FOXC1 could be of potential value as a therapeutic target for TNBC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Hongchao Pan http://orcid.org/0000-0001-7462-2230

REFERENCES

1. Engebraaten O, Vollan HK, Borresen-Dale AL. Triple-negative breast cancer and the need for new therapeutic targets. *Am J Pathol*. 2013;183:1064-1074.
2. Perou CM, Sørlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.
3. Kreike B, van Kouwenhove M, Horlings H, et al. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. *Breast Cancer Res*. 2007;9:R65.
4. Ray PS, Wang J, Qu Y, et al. FOXC1 is a potential prognostic biomarker with functional significance in basal-like breast cancer. *Cancer Res*. 2010;70:3870-3876.
5. Rouzier R, Perou CM, Symmans WF, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res*. 2005;11:5678-5685.
6. Kume T, Jiang H, Topczewska JM, et al. The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev*. 2001;15:2470-2482.
7. Maclean K, Smith J, St Heaps L, et al. Axenfeld-Rieger malformation and distinctive facial features: clues to a recognizable 6p25 microdeletion syndrome. *Am J Med Genet A*. 2005;132A:381-385.

8. Benayoun BA, Caburet S, Veitia RA. Forkhead transcription factors: key players in health and disease. *Trends Genet*. 2011;27:224-232.

9. Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. *Dev Biol*. 2002;250:1-23.

10. Kidson SH, Kume T, Deng K, et al. The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. *Dev Biol*. 1999;211:306-322.

11. Smith RS, Zabaleta A, Kume T, et al. Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. *Hum Mol Genet*. 2000;9:1021-1032.

12. Taube JH, Herschkowitz JI, Komurov K, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci USA*. 2010;107:15449-15454.

13. Xia L, Huang W, Tian D, et al. Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Hepatology*. 2013;57:610-624.

14. Wei LX, Zhou RS, Xu HF, et al. High expression of FOXC1 is associated with poor clinical outcome in non-small cell lung cancer patients. *Tumour Biol*. 2013;34:941-946.

15. Han B, Qu Y, Jin Y, et al. FOXC1 activates smoothened-independent hedgehog signaling in basal-like breast cancer. *Cell Rep*. 2015;13:1046-1058.

16. Jensen TW, Ray T, Wang J, et al. Diagnosis of basal-like breast cancer using a FOXC1-based assay. *J Natl Cancer Inst*. 2015;107:djv148.

17. Sizemore GM, Sizemore ST, Pal B, et al. FOXC1 is enriched in the mammary luminal progenitor population, but is not necessary for mouse mammary ductal morphogenesis. *Biol Reprod*. 2013;89:10.

18. Wang J, Ray PS, Sim MS, et al. FOXC1 regulates the functions of human basal-like breast cancer cells by activating NF-κB signaling. *Oncogene*. 2012;31:4798-4802.

19. Ou-Yang L, Xiao SJ, Liu P, et al. Forkhead box C1 induces epithelial-mesenchymal transition and is a potential therapeutic target in nasopharyngeal carcinoma. *Mol Med Rep*. 2015;12:8003-8009.

20. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339:580-584.

21. Lippitz BE. Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol*. 2013;14:e218-e228.

22. Smith MC, Luker KE, Garbow JR, et al. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res*. 2004;64:8604-8612.

23. Marchesi F, Monti P, Leone BE, et al. Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res*. 2004;64:8420-8427.

24. Schmid B, Rudas M, Resniczek G, et al. CXCR4 is expressed in ductal carcinoma in situ of the breast and in atypical ductal hyperplasia. *Breast Cancer Res Treat*. 2004;84:247-250.

25. Zeelenberg I, Ruuls-Van Stalle L, Roos E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res*. 2003;63:3833-3839.

26. Kato M, Kitayama J, Kazama S, et al. Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. *Breast Cancer Res*. 2003;5:R144-R150.

27. Liang Z, Wu T, Lou H, et al. Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res*. 2004;64:4302-4308.

28. Müller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001;410:50-56.

29. Zlotnik A, Burkhardt AM, Homey B. Homeostatic chemokine receptors and organ-specific metastasis. *Nat Rev Immunol*. 2011;11:597-606.

30. Liang Z, Yoon Y, Votaw J, et al. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res*. 2005;65:967-971.

31. Chen HW, Du CW, Wei XL, et al. Cytoplasmic CXCR4 high-expression exhibits distinct poor clinicopathological characteristics and predicts poor prognosis in triple-negative breast cancer. *Curr Mol Med*. 2013;13:410-416.

32. Hayashi H, Kume T. Forkhead transcription factors regulate expression of the chemokine receptor CXCR4 in endothelial cells and CXCL12-induced cell migration. *Biochem Biophys Res Commun*. 2008;367:584-589.

33. Henkels KM, Boivin GP, Dudley ES, et al. Phospholipase D (PLD) drives cell invasion, tumor growth and metastasis in a human breast cancer xerograph model. *Oncogene*. 2013;32:5551-5562.

34. Fernandis AZ, Prasad A, Band H, et al. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene*. 2004;23:157-167.

35. Sobolik T, Su YJ, Wells S, et al. CXCR4 drives the metastatic phenotype in breast cancer through induction of CXCR2 and activation of MEK and PI3K pathways. *Mol Biol Cell*. 2014;25:566-582.

36. Raman D, Baugher PJ, Thu YM, et al. Role of chemokines in tumor growth. *Cancer Lett*. 2007;256:137-165.

37. Nishimura DY, Swiderski RE, Alward WL, et al. The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25. *Nat Genet*. 1998;19:140-147.

38. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer*. 2007;7:847-859.

39. Chung TK, Lau TS, Cheung TH, et al. Dysregulation of microRNA-204 mediates migration and invasion of endometrial cancer by regulating FOXC1. *Int J Cancer*. 2012;130:1036-1045.

40. Potente M, Urbich C, Sasaki K, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Investig*. 2005;115:2382-2392.

41. Bieche I, Chavey C, Andrieu C, et al. CXC chemokines located in the 4q21 region are up-regulated in breast cancer. *Endocr Relat Cancer*. 2007;14:1039-1052.

42. Chu QD, Panu L, Holm NT, et al. High chemokine receptor CXCR4 level in triple negative breast cancer specimens predicts poor clinical outcome. *J Surg Res*. 2010;159:689-695.