



FOXF2 deficiency accelerates the visceral metastasis of basal-like breast cancer by unrestrictedly increasing TGF- β and miR-182-5p

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Received: 24 September 2019 / Revised: 29 April 2020 / Accepted: 30 April 2020
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Abstract

The mesenchymal transcription factor forkhead box F2 (FOXF2) is a critical regulator of embryogenesis and tissue homeostasis. Our previous studies demonstrated that FOXF2 is ectopically expressed in basal-like breast cancer (BLBC) cells and that FOXF2 deficiency promotes the epithelial–mesenchymal transition and aggressiveness of BLBC cells. In this study, we found that FOXF2 controls transforming growth factor-beta (TGF- β)/SMAD signaling pathway activation through transrepression of TGF- β -coding genes in BLBC cells. FOXF2-deficient BLBC cells adopt a myofibroblast-/cancer-associated fibroblast (CAF)-like phenotype, and tend to metastasize to visceral organs by increasing autocrine TGF- β signaling and conferring aggressiveness to neighboring cells by increasing paracrine TGF- β signaling. In turn, TGF- β silences FOXF2 expression through upregulating miR-182-5p, a posttranscriptional regulator of *FOXF2* and inducer of metastasis. In addition to mediating a reciprocal repression loop between FOXF2 and TGF- β through direct transrepression by SMAD3, miR-182-5p forms a reciprocal repression loop with FOXF2 that directly transrepresses *MIR182* expression. Therefore, FOXF2 deficiency accelerates the visceral metastasis of BLBC through unrestricted increases in autocrine and paracrine TGF- β signaling, and miR-182-5p expression. Our findings provide novel mechanisms underlying the roles of TGF- β , miR-182-5p, and FOXF2 in accelerating BLBC dissemination and metastasis, and may facilitate the development of therapeutic strategies for aggressive BLBC.

Introduction

Breast cancer is a heterogeneous disease with different biological and histological characteristics that lead to distinct prognoses and therapeutic responses. Based on gene

expression profiling, breast cancers can be divided into normal-like, luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and basal-like intrinsic subtypes. Basal-like breast cancer (BLBC) is characterized by a gene expression profile similar to that of basal/myoepithelial cells of the breast [1, 2]. The majority of BLBCs are triple-negative breast cancers that lack expression of estrogen receptor, progesterone receptor, and HER2 [3]. BLBC is the most aggressive subtype, and tends to exhibit hematogenous dissemination and visceral metastasis [4, 5]. However, the underlying molecular mechanisms are not well understood, and the absence of effective targeted therapies remains a clinical challenge.

BLBC cells have intrinsic phenotypic plasticity for mesenchymal transition [6]. Epithelial–mesenchymal transition (EMT) is a major phenotypic change characterized by the loss of epithelial features and the gain of a mesenchymal phenotype, with enhanced migratory and invasive properties, thus endowing cancer cells with increased aggressiveness [7]. EMT is a process leading to distinct intermediate phenotypes that are determined by the cellular

Edited by J. P. Medema

Supplementary information The online version of this article (<https://doi.org/10.1038/s41418-020-0555-7>) contains supplementary material, which is available to authorized users.

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context and response to stimulation by cytokines [8]. Accumulated evidence has demonstrated that a myogenic program can be activated during EMT, leading to the acquisition of a myofibroblast- or cancer-associated fibroblast (CAF)-like phenotype characterized by increased expression of myofibroblast/CAF markers, the formation of stress fibers, and cellular contractility. This process is referred to as epithelial–myofibroblastic transition (EMyoT) [9–12]. Transforming growth factor- β (TGF- β) has been considered a key inducer of EMT, and long-term exposure to TGF- β elicits EMyoT [13]. However, there is insufficient evidence as to whether and how BLBC cells undergo EMyoT to gain the ability to invade and metastasize.

Forkhead box F2 (FOXF2) is a mesenchymal transcription factor (TF) specifically expressed in the mesenchyme adjacent to the epithelium, and that functionally maintains mesenchymal cell differentiation and inhibits the mesenchymal transformation of adjacent epithelial cells in multiple organs and tissues [14]. Our previous studies showed that FOXF2 is ectopically expressed in basal-like breast cells [15], while FOXF2-deficient BLBC exhibits mesenchymal/myofibroblast/CAF-like morphology and tends to metastasize to visceral organs. FOXF2 functions as a transrepressor of EMT-inducing TFs (EMT-TFs), e.g., TWIST1 [16], FOXC2 [17], and FOXQ1 [18] in basal-like breast cells. However, whether or how FOXF2 plays a pivotal role in controlling BLBC progression and metastasis has not been fully elucidated.

In the present study, we investigated the regulatory relationship among FOXF2, TGF- β /SMAD signaling pathway components, and TGF- β -regulated microRNAs (miRNAs) potentially targeting *FOXF2*. We also provide in vitro and in vivo experimental evidence for the role of the reciprocal repression loop between FOXF2 and TGF- β in initiating a vicious cycle, resulting in aggressive progression of BLBC. Our findings will hopefully facilitate the development of effective therapeutic strategies for aggressive BLBC.

Materials and methods

Cell culture and treatment

The human breast cancer cell lines MDA-MB-231, BT-549, and MCF7 were obtained from American Type Culture Collection (Manassas, VA, USA). MDA-MB-231-Luc (231-Luc), a subline of MDA-MB-231 cells that stably expresses firefly luciferase (Luc), was established by our laboratory via infection with recombinant lentiviruses carrying the Luc gene. All cell lines were authenticated by short tandem repeat profiling and tested for mycoplasma contamination. Cells were grown in RPMI 1640 or Dulbecco's modified Eagle medium supplemented with 10%

fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Gaithersburg, MD, USA). The conditioned medium (CM) of feeder cells was collected and diluted with an equal volume of normal medium for the induction of parental cells for 7 days. For TGF- β signaling activation, cells were cultured in medium supplemented with 10 ng/mL TGF- β 1, TGF- β 2, or TGF- β 3 (Peprotech, Rocky Hill, NJ, USA) for 3, 6, or 9 days. For TGF- β signaling inhibition, cells were grown in medium with 10 μ M SB431542 (Sigma-Aldrich, St Louis, MO, USA) for 2 days.

Lentiviral infection

MDA-MB-231 and 231-Luc cells were infected with recombinant lentiviruses carrying enhanced green fluorescent protein (EGFP)-tagged short hairpin RNA targeting human *FOXF2* (231-shFOXF2-Green and 231-Luc-shFOXF2) or negative control (231-shControl-Green and 231-Luc-shControl). The shFOXF2 sequences were GCGTCATGTGAACGGAAAG (shFOXF2#1) and GTCCTCAACTTCAATGGGATT (shFOXF2#2). BT-549 cells were infected with recombinant lentiviruses carrying EGFP-tagged human full-length *FOXF2* cDNA (NM_001452.2; 549-FOXF2-Green) or vector (549-Vector-Green). These cells were selected in 1.0 μ g/mL puromycin for 2 weeks to stably silence endogenous FOXF2 or express exogenous FOXF2. Red fluorescent-labeled MDA-MB-231 (231-Red) and BT-549 (549-Red) cells were established by infection with lentiviruses expressing mCherry fluorescent protein (Shanghai GeneChem Co., Ltd., China).

Plasmids, small interfering RNA, miRNA, and transfection

The human full-length *FOXF2* cDNA (GeneChem Co., Ltd.) was subcloned into the pcDNA3.1-FLAG vector (FOXF2-FLAG). The small interfering RNAs (siRNAs) targeting distinct sequences of human *FOXF2* mRNA (siFOXF2#1: GCGTCATGTGAACGGAAAG and siFOXF2#2: GTCCTCAACTTCAATGGGATT) and nuclear receptor corepressor 1 (*NCOR1*) mRNA (siNCOR1: GGA-CAAGTTTATCCAGCAT), nonspecific control siRNA (siControl), miR-182-5p mimic, mimic control, miR-182-5p inhibitor, and inhibitor control were synthesized by RiboBio Co., Ltd. (Guangzhou, China). Plasmids and oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Reverse transcription-quantitative PCR

Total RNA extraction, reverse transcription (RT), quantitative PCR (qPCR), and quantification of target gene expression were performed as described previously [19].

Bulge-loop miRNA-specific RT primers and the internal control U6 (RiboBio Co., Ltd.) were used for miRNA amplification. All primers and probes for mRNA amplification were synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), and the sequences are provided in Supplementary Table 1. All primers for miRNA amplification were synthesized by RiboBio Co., Ltd., and commercial reference numbers are listed in Supplementary Table 2. The Platinum Quantitative PCR SuperMix-UDG system (Invitrogen; TaqMan system) or SYBR Premix DimerEraser system (TaKaRa, Beijing, China; SYBR system) was used for qPCR. The expression level of the target gene was calculated by normalizing the cycle threshold (Ct) values of target gene to the Ct values of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; ΔC_T) and was determined as $2^{-\Delta C_T}$.

Immunoblot, immunohistochemistry, and immunofluorescence assays

Immunoblot of intracellular proteins, immunohistochemistry, and immunofluorescence were performed as previously described [20]. For immunoblot of secreted proteins, CM was collected from cultured cells, concentrated with 30% ammonium sulfate overnight at 4 °C, and centrifuged at 20,000×g for 1 h. For immunofluorescence of the F-actin cytoskeleton, cells were incubated with Alexa Fluor 594-conjugated phalloidin (Invitrogen), and the fluorescence intensity was quantified using ImageJ software (NIH, Bethesda, MD, USA). Nuclear DNA was stained using DAPI. All antibodies and their dilutions are listed in Supplementary Table 3.

ChIP-PCR, ChIP-qPCR, and Re-ChIP-PCR

The *TGFB2*, *TGFB3*, and *MIR182* promoter regions containing putative FOXF2-binding elements in cells transfected with FOXF2-FLAG plasmids were enriched by chromatin immunoprecipitation (ChIP) using an anti-Flag antibody. Anti-Flag-enriched *TGFB2* and *TGFB3* promoter regions were used to perform Re-ChIP with an anti-NCoR1 antibody as previously described [18]. The *MIR182* promoter region containing a SMAD3-binding element in cells was enriched using an anti-SMAD3 antibody. ChIP-PCR assays were performed as previously described [16]. The quantity of ChIP-enriched DNA fragments was calculated according to the percent input and fold enrichment methods.

Dual-luciferase reporter assays

TGF- β signaling pathway activity was determined using the Cignal SMAD Reporter Assay Kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. The

TGFB2, *TGFB3*, and *MIR182* promoter regions containing or lacking a FOXF2- or SMAD3-binding element were cloned into the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI, USA). The full-length *FOXF2* 3' untranslated region (UTR) containing the predicted miR-182-5p-binding element 5'-TTGCCAAA-3' or its mutated sequence 5'-AACGGTTT-3' was cloned into downstream of the luciferase-coding sequence in the pGL3-Promoter Vector (Promega). Promoter activation was measured using the Dual-Luciferase Reporter Assay System (Promega) and quantified as described previously [15].

Three-dimensional culture assay

Cells at a density of 1×10^4 cells/cm² were plated on Matrigel (BD Biosciences, San Jose, CA) and maintained in culture medium containing 5% Matrigel for 2 days. The number of invasive branching structures formed by cells was counted under a microscope in three predetermined fields at 100× magnification.

Collagen gel contraction assay

Cells embedded in three-dimensional (3D) collagen matrices were prepared by mixing rat tail type I collagen (BD Biosciences) and cells suspended in cell culture medium to achieve a final concentration of 2 mg/mL collagen and 1×10^7 cells/mL. A 500- μ L aliquot of this mixture was dispensed into each well of a 24-well plate and incubated at 37 °C for 30 min. After polymerization, the collagen gels were freed from the wells using a pipette tip, and complete culture medium was added to the wells. Then, the cells were incubated for 2 days. The contraction index was calculated using ImageJ software as the rate of decrease in the gel area.

Wound healing and transwell assays

For the wound healing assay, cells at 80% confluency were starved overnight, scratched with a pipette, and then cultured for 24 h. The distance migrated by the cells into the wound was measured. Transwell assays were performed with non-Matrigel-coated and Matrigel-coated transwell inserts (BD Biosciences) to assess cell migration and invasion, respectively, as described previously [20]. The number of migrating or invading cells was counted under a microscope in five predetermined fields at 400× magnification.

Xenograft tumor assays

A total of 5×10^6 231-Luc-shFOXF2 cells, or 2.5×10^6 231-Luc cells mixed with 2.5×10^6 231-shFOXF2 cells or their

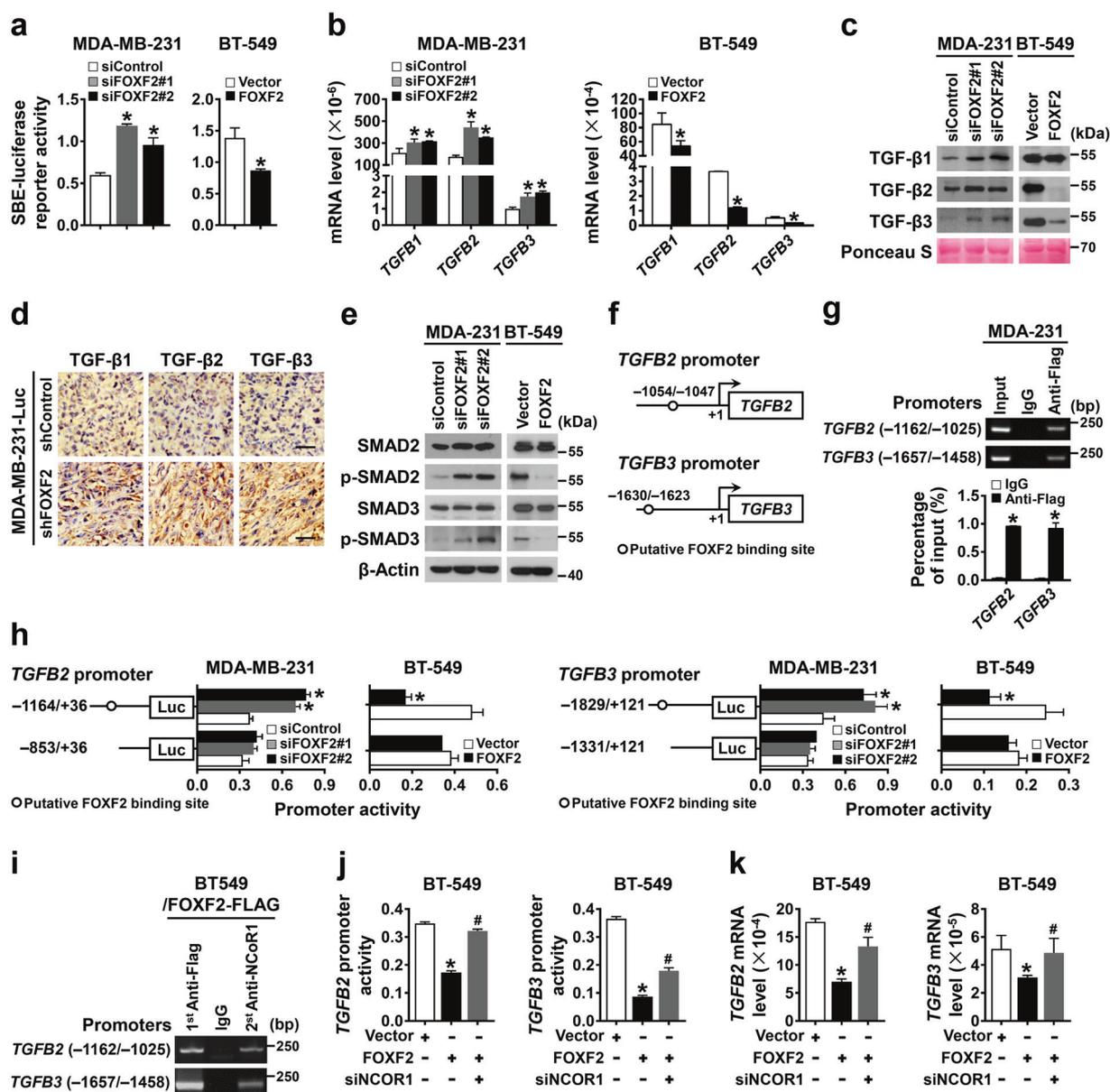


Fig. 1 FOXF2 suppresses TGF- β /SMAD signaling pathway activation through direct transrepression of *TGFB2* and *TGFB3* expression in BLBC cells. **a** SBE-luciferase reporter activity in the indicated cells was assessed by a dual-luciferase reporter assay. **b** *TGFB1*, *TGFB2*, and *TGFB3* mRNA levels in the indicated cells were measured by RT-qPCR. **c** The levels of secreted TGF- β 1, TGF- β 2, and TGF- β 3 protein in CM collected from the indicated cells were detected by immunoblot. **d** TGF- β 1, TGF- β 2, and TGF- β 3 protein levels in primary tumor tissue harvested from the xenograft mouse model were determined by immunohistochemistry. Scale bar: 200 μ m. **e** SMAD2, p-SMAD2, SMAD3, and p-SMAD3 protein levels were detected by immunoblot. **f** Schematics show the putative FOXF2-binding sites in the *TGFB2* and *TGFB3* promoter regions. **g** The binding of FOXF2 to the *TGFB2* and *TGFB3* promoters containing

putative FOXF2-binding elements was assessed by ChIP-PCR and ChIP-qPCR assays in MDA-MB-231 cells transfected with FOXF2-FLAG plasmid, using anti-Flag antibodies or IgG control. **h** The activity of the *TGFB2* and *TGFB3* promoters containing or lacking FOXF2-binding elements in the indicated cells was assessed by a dual-luciferase reporter assay. **i** The enrichment of the *TGFB2* and *TGFB3* promoter regions containing FOXF2-binding site in BT-549 cells was determined by Re-ChIP-PCR assay using antibodies against the indicated proteins. **j** The transcriptional activity of the *TGFB2* and *TGFB3* promoters in BT-549 cells was assessed by a dual-luciferase reporter assay. **k** *TGFB2* and *TGFB3* mRNA levels in BT-549 cells were detected by RT-qPCR. * $P < 0.05$ compared with control cells; # $P < 0.05$ compared with FOXF2-overexpressing cells.

control cells were injected into the lower abdominal mammary fat pads of 6-week-old female severe combined immunodeficiency (SCID) mice ($n = 5$ per group). For drug

treatment, the mice were intraperitoneally injected with 10 mg/kg SB431542 daily for 6 weeks starting on the seventh day after initial inoculation. The primary tumors and

metastases in visceral organs were imaged and counted based on bioluminescence, and the results were validated by hematoxylin and eosin (H&E), and immunohistochemistry staining of paraffin-embedded tissue sections as previously described [16]. Animals of similar age and weight were randomly selected and grouped. Histological evaluation was performed in a blinded fashion. All experimental procedures were approved by the Laboratory Animal Ethics Committee at Tianjin Medical University Cancer Institute and Hospital.

Statistical analysis

All in vitro experiments were performed at least two independent times each in duplicate, and data are presented as the mean \pm standard deviation. Two-tailed Student's *t*-test or the rank-sum test was used to compare the differences between the experimental and control groups. Survival analysis of mice in the xenograft tumor assay was evaluated using Kaplan–Meier survival curves, and the log-rank test was used to assess statistical significance. Differences with $P < 0.05$ were considered statistically significant.

Results

FOXF2 functionally represses the TGF- β /SMAD signaling pathway and directly targets *TGFB2* and *TGFB3* in BLBC cells

To investigate whether FOXF2 plays a role in the regulation of the TGF- β /SMAD signaling pathway, we silenced FOXF2 in MDA-MB-231 cells (high FOXF2 expression) with two independent siFOXF2 sequences, and used a FOXF2 plasmid to increase FOXF2 expression in BT-549 cells (low FOXF2 expression) or to ectopically express FOXF2 in MCF7 cells (no FOXF2 expression; Supplementary Fig. 1a). SMAD-binding element (SBE) reporter assays revealed that FOXF2 negatively regulated TGF- β /SMAD signaling pathway activity in MDA-MB-231 and BT-549 cells (Fig. 1a), but had no significant effect on MCF7 cells (Supplementary Fig. 1b). These results indicate that FOXF2 negatively regulates TGF- β /SMAD signaling pathway activation in BLBC cells.

To explore how FOXF2 regulates TGF- β /SMAD signaling pathway activation in breast cancer cells, the mRNA levels of genes encoding the TGF- β /SMAD signaling pathway components TGF- β 1/2/3, TGF- β receptor 1/2 (TGF- β R1/2), and SMAD2/3/4 were detected in the above cells by RT-qPCR. The results showed that FOXF2 negatively regulated the transcription of genes encoding all three TGF- β isoforms, *TGFB1*, *TGFB2*, and *TGFB3* in

MDA-MB-231 and BT-549 cells (Fig. 1b), but did not affect the other genes in the BLBC cell lines and did not affect the expression of any of these genes in MCF7 cells (Supplementary Fig. 1c–e). Consistently, TGF- β 1, TGF- β 2, and TGF- β 3 protein levels were also negatively regulated by FOXF2 in the CM of MDA-MB-231 and BT-549 cells (Fig. 1c), and in MDA-MB-231-shFOXF2 tumors harvested from xenograft SCID mice (Fig. 1d). FOXF2 also negatively regulated the phosphorylation of SMAD2 and SMAD3 in MDA-MB-231 and BT-549 cells (Fig. 1e). These results indicate that FOXF2 negatively regulates the transcription and translation of TGF- β s to control TGF- β /SMAD signaling pathway activation in BLBC cells.

To clarify whether FOXF2 directly regulates the transcription of *TGFB1*, *TGFB2*, and *TGFB3*, the proximal promoters of these genes were analyzed for typical FOXF2-binding elements. We found potential FOXF2-binding elements in the *TGFB2* and *TGFB3* proximal promoters, but not in the *TGFB1* promoter (Fig. 1f). ChIP-PCR and ChIP-qPCR assays confirmed that FOXF2 was recruited to the *TGFB2* and *TGFB3* promoters containing FOXF2-binding sites in MDA-MB-231 cells transfected with FOXF2-FLAG (Fig. 1g). Luciferase reporter assays showed that FOXF2 negatively regulated *TGFB2* and *TGFB3* promoter activity only in the presence of the FOXF2-binding site in MDA-MB-231 and BT-549 BLBC cells (Fig. 1h). These results indicate that FOXF2 directly transrepresses *TGFB2* and *TGFB3*, but indirectly represses *TGFB1* transcription.

To further investigate whether the FOXF2 deficiency-induced increase in expression of TGF- β 1 was regulated by TGF- β 2 and/or TGF- β 3 stimulation, we treated MDA-MB-231 cells with TGF- β 2 and TGF- β 3. Indeed, *TGFB1* expression was upregulated by TGF- β 2 and TGF- β 3 stimulation (Supplementary Fig. 1f), suggesting that TGF- β 2 and TGF- β 3 initially released in FOXF2-deficient cells could induce TGF- β 1 expression. Taken together, these results indicate that FOXF2 controls TGF- β /SMAD signaling pathway activation in BLBC cells by directly repressing *TGFB2* and *TGFB3* transcription and expression, which further induce *TGFB1* expression.

We have recently reported that FOXF2 transrepresses target genes through interacting with NCoR1, which recruits histone deacetylase 3 to lead to histone deacetylation and the formation of repressive chromatin structures in basal-like breast cells. Thus, we tested whether NCoR1 mediates the transrepression of *TGFB2* and *TGFB3* by FOXF2. Re-ChIP-PCR assays showed that FOXF2-enriched *TGFB2* and *TGFB3* promoters by anti-Flag antibody could be further enriched with an antibody against NCoR1 in BT-549 cells transfected with FOXF2-FLAG plasmid (Fig. 1i). Consistent with these findings,

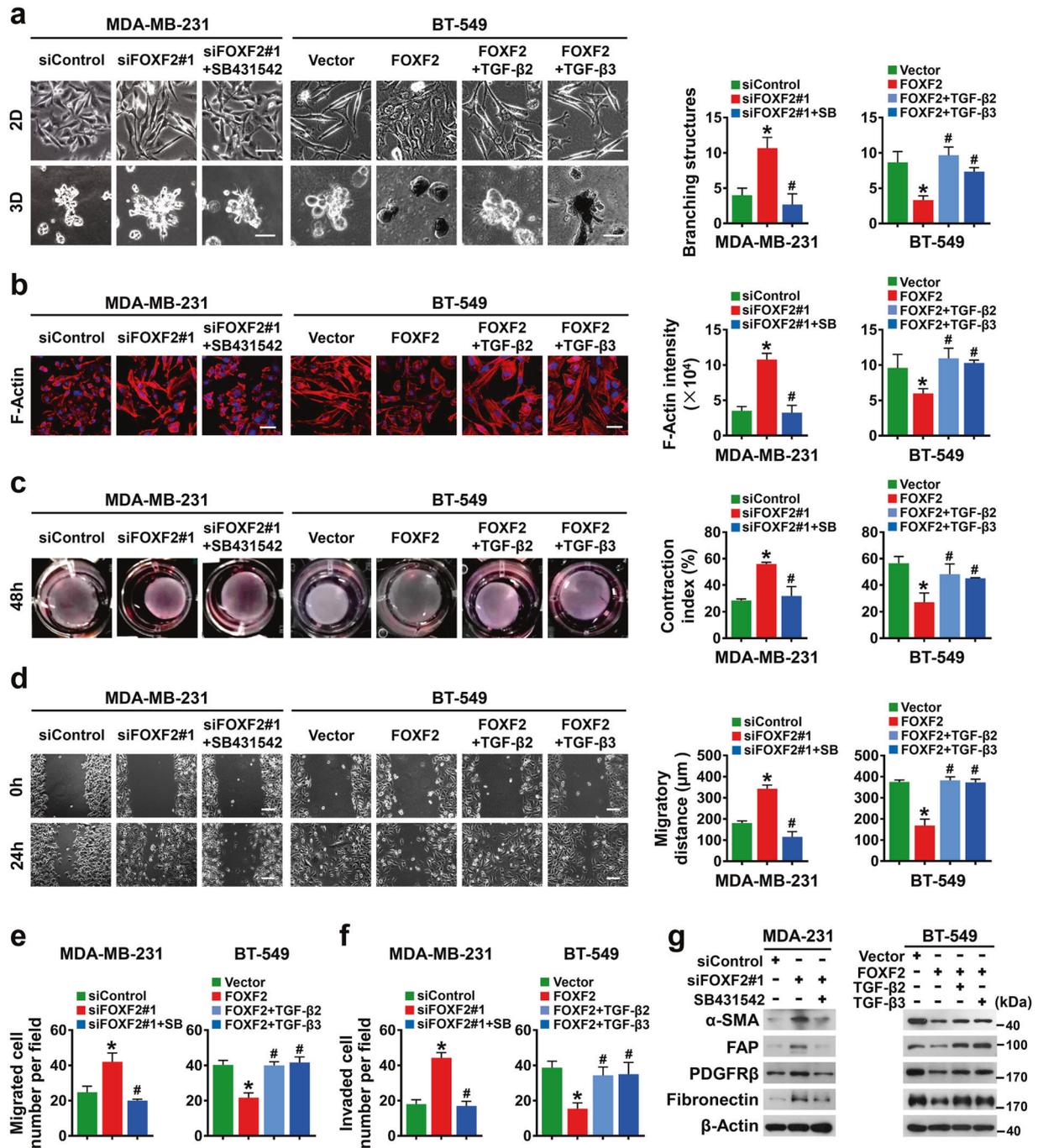


Fig. 2 FOXF2-deficient BLBC cells adopt CAF-like phenotype through autocrine TGF-β signaling. **a** The morphology and structure of the indicated cells in 2D culture and 3D Matrigel culture are shown in the images. The invasive branching structures formed by cells in 3D Matrigel culture were quantified. Scale bar: 100 μm. **b** F-actin cytoskeleton reorganization in the indicated cells was visualized by Alexa Fluor 594-conjugated phalloidin (red). DAPI was used to stain nuclei (blue). The fluorescence intensity of F-actin was quantified. Scale bar: 50 μm. **c** Gel contraction by the indicated cells embedded in collagen

matrices for 48 h. **d** The wound distances on confluent monolayers of the indicated cells at different time points. Scale bar: 100 μm. **e, f** The migration (**e**) and invasion (**f**) capabilities of the indicated cells were assessed by transwell assays. **g** The protein expression of the myofibroblast markers in the indicated cells was detected by immunoblot. SB431542 (10 μM) or TGF-β2/TGF-β3 (10 ng/mL) was added to the culture medium for the treatment of the indicated cells for 48 h. **P* < 0.05 compared with control cells; #*P* < 0.05 compared with FOXF2-depleted or FOXF2-overexpressing cells.

NCoR1 mediated the FOXF2-regulated repression of *TGFB2* and *TGFB3* transcription (Fig. 1j) and expression

(Fig. 1k). These results confirmed that FOXF2 transrepresses *TGFB2* and *TGFB3* in BLBC cells through

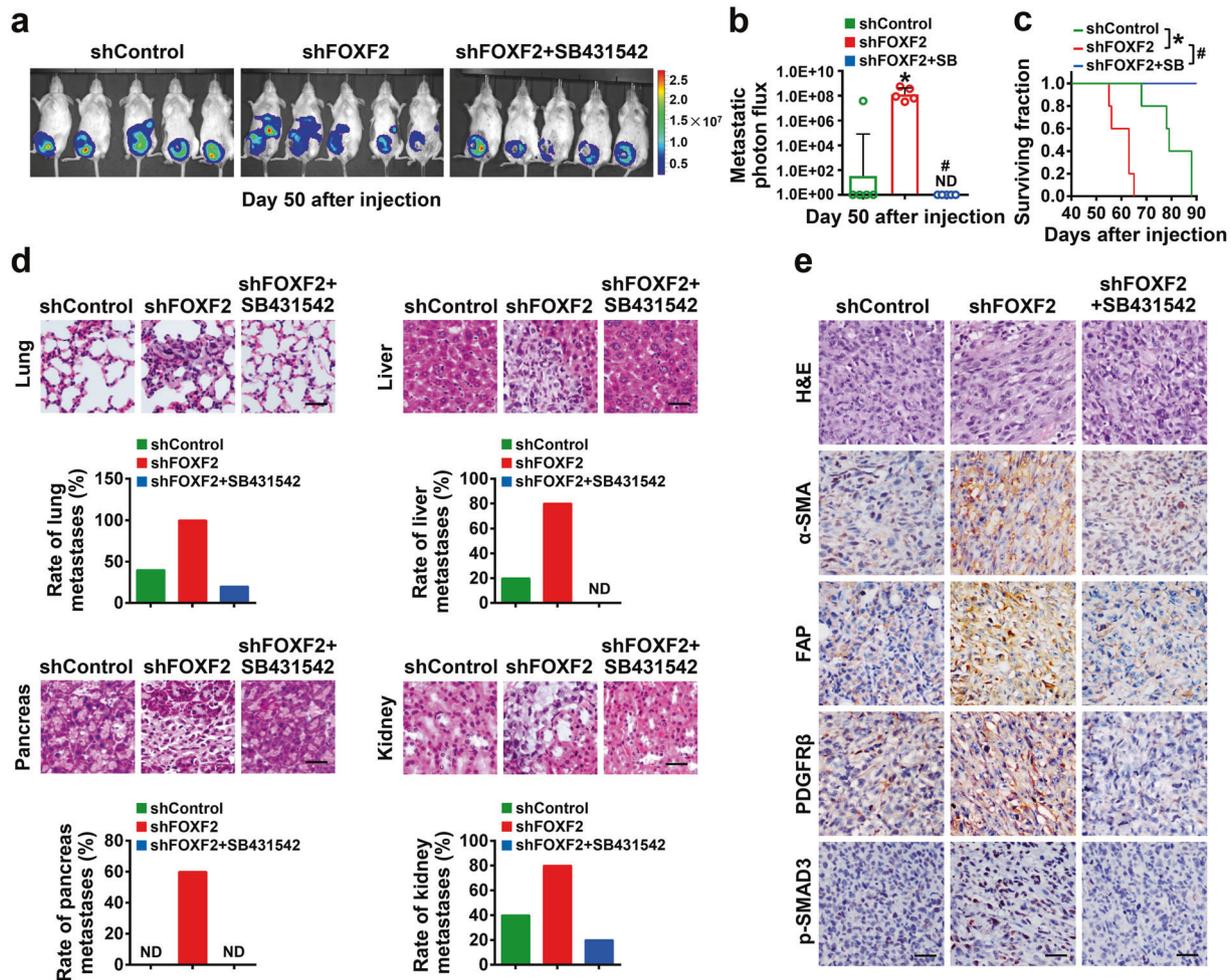


Fig. 3 The increase in BLBC cell metastatic ability induced by FOXF2 deficiency is abrogated by a TGF- β inhibitor in vivo. 231-Luc-shFOXF2 or 231-Luc-shControl cells were inoculated orthotopically into the abdominal mammary fat pads of 6-week-old female SCID mice ($n = 5$ each group), and a group of mice bearing 231-Luc-shFOXF2 tumors was treated with 10 mg/kg SB431542 daily by intraperitoneal injection for 6 weeks beginning at day 7 after the initial inoculation. **a**, **b** Representative bioluminescence images (**a**) and the quantification of photon flux (**b**) of metastases in xenograft mice at day

50 after cell inoculation. **c** Kaplan–Meier curves of the indicated xenograft mice ($n = 5$, each group). **d** Representative images and number of metastases formed by 231-Luc cells and identified by H&E staining in the lungs, livers, pancreases, and kidneys. Scale bar: 200 μ m. **e** H&E staining and immunohistochemistry for α -SMA, FAP, PDGFR β , and p-SMAD3 expression in primary tumors harvested from mice treated as indicated. Scale bar: 200 μ m. * $P < 0.05$ compared with mice bearing 231-Luc-shControl tumors; # $P < 0.05$ compared with mice bearing 231-Luc-shFOXF2 tumors.

recruiting NCoR1 to form a transcriptional corepressor complex.

FOXF2-deficient BLBC cells adopt a myfibroblast/CAF-like phenotype through autocrine TGF- β signaling

TGF- β signaling is a potent inducer of EMT [21, 22] and fibroblast–myofibroblast transition [23], and epithelial cells can also transdifferentiate to myofibroblast-like cells via EMyoT, a specialized EMT [24]. Therefore, we investigated whether FOXF2-deficient BLBC cells transdifferentiate into the myofibroblast/CAF-like phenotype through the induction of autocrine TGF- β signaling. Morphological

observations revealed that FOXF2-depleted MDA-MB-231 cells adopted a more mesenchymal/fibroblast-like morphology, with a spindle-like cell shape and scattered distribution in two-dimensional (2D) culture, and invasive branching in 3D Matrigel culture (Fig. 2a, Supplementary Fig. 2a). Accordingly, FOXF2 depletion in MDA-MB-231 cells led to increased stress fiber formation, as revealed by immunofluorescence staining of polymerized F-actin (Fig. 2b, Supplementary Fig. 2b), enhanced matrix contraction when the cells were embedded in type I collagen gel (Fig. 2c, Supplementary Fig. 2c), increased migration in wound healing assays (Fig. 2d), increased migration and invasion in transwell assays (Fig. 2e, f, Supplementary Fig. 2d, e), and upregulated expression of the

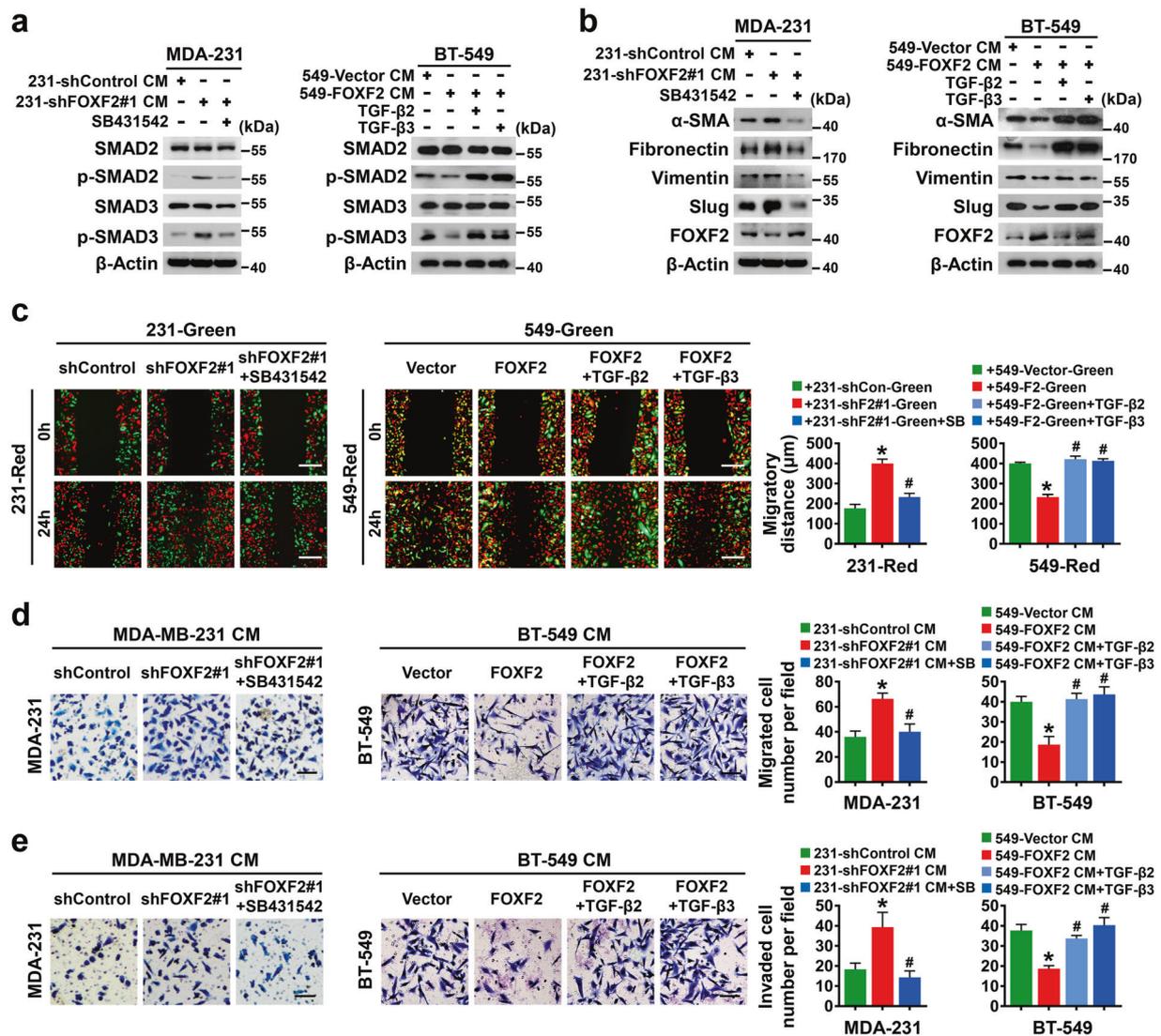


Fig. 4 FOXF2-deficient BLBC cells confer aggressiveness to neighboring cells by increasing paracrine TGF- β signaling. **a**, **b** The protein levels of SMAD2, p-SMAD2, SMAD3, and p-SMAD3 (**a**), as well as EMT markers (**b**), in MDA-MB-231 and BT-549 parental cells cultured with CM from the indicated cells were detected by immunoblot. **c** The migratory ability of MDA-MB-231 and BT-549 cells (red) cocultured with the indicated feeder cells (green) was assessed by wound healing assay. Scale bar: 200 μ m. **d**, **e** The migration (**d**) and invasion (**e**) abilities of MDA-MB-231 and BT-549 parental cells cultured with CM from the indicated cells were

assessed by non-Matrigel-coated and Matrigel-coated transwell assays, respectively. Scale bar: 100 μ m. SB431542 (10 μ M) and TGF- β 2/TGF- β 3 (10 ng/mL) were added to the CM of the indicated feeder cells to treat the parental cells for 48 h. * P < 0.05 compared with parental cells cultured with CM from control feeder cells or cocultured with control feeder cells; # P < 0.05 compared with parental cells cultured with CM from FOXF2-depleted or FOXF2-overexpressing feeder cells, or cocultured with FOXF2-depleted or FOXF2-overexpressing feeder cells.

myofibroblast/CAF markers alpha-smooth muscle actin (α -SMA), fibroblast activation protein (FAP), fibronectin, and platelet-derived growth factor receptor beta (PDGFR β ; Fig. 2g, Supplementary Fig. 2f). As expected, the myofibroblast/CAF-like morphology and phenotype of FOXF2-depleted MDA-MB-231 cells were reversed by the TGF- β signaling pathway inhibitor SB431542 (Fig. 2a–g, Supplementary Fig. 2a–f), and TGF- β 2 or TGF- β 3 knockdown (Supplementary Fig. 2d–f). Conversely, FOXF2 overexpression and treatment with TGF- β 1, TGF- β 2, or TGF- β 3

elicited the opposite effects in BT-549 cells (Fig. 2a–g, Supplementary Fig. 2a–f). These results indicate that FOXF2-deficient BLBC cells adopt a myofibroblast/CAF-like phenotype through autocrine TGF- β signaling.

Autocrine TGF- β signaling mediates the increased visceral metastasis of FOXF2-deficient BLBC cells

To investigate whether the TGF- β signaling pathway is crucial for the increased visceral metastasis of FOXF2-

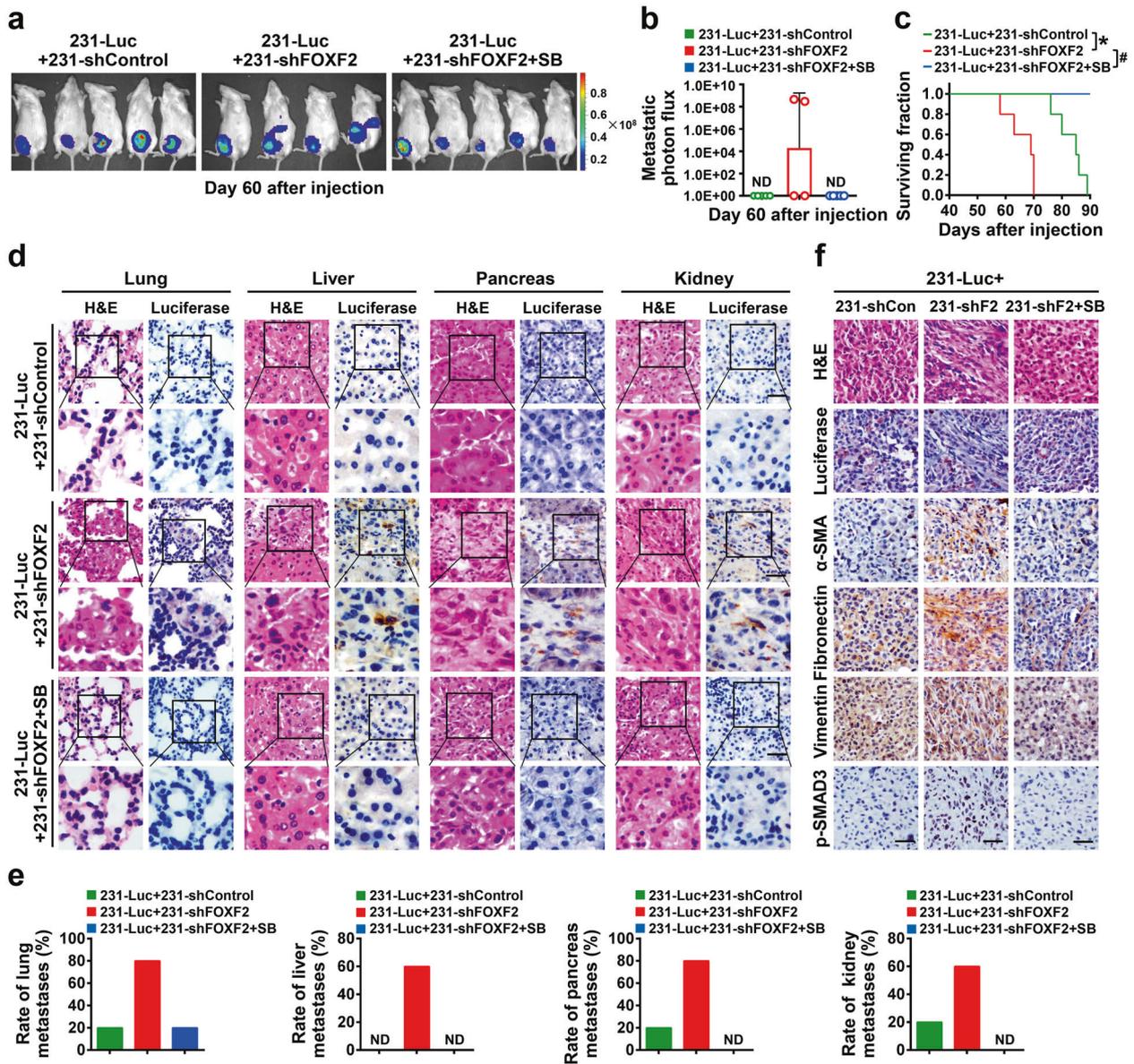


Fig. 5 FOXF2-deficient BLBC cells enhance the metastasis of neighboring cells by increasing paracrine TGF-β signaling. Mixes of equal numbers of 231-Luc cells with 231-shFOXF2 or 231-shControl cells were orthotopically inoculated into SCID mice, and the mice bearing 231-Luc plus 231-shFOXF2 tumors were treated with 10 mg/kg SB431542 daily by intraperitoneal injection for 6 weeks starting at day 7 after cell inoculation. **a**, **b** Bioluminescence images (**a**) and the quantification of photon flux (**b**) of metastases formed by 231-Luc cells in xenograft mice at day 60 after cell inoculation (a mouse bearing 231-Luc plus 231-shFOXF2 tumor died at day 58).

c Kaplan–Meier curves of the indicated xenograft mice ($n = 5$, each group). **d**, **e** Representative images and number of metastases formed by 231-Luc cells, and identified by H&E staining and immunohistochemistry staining for luciferase in the lungs, livers, pancreases, and kidneys. Scale bar: 200 μm. **f** H&E staining and immunohistochemistry staining for luciferase, α-SMA, fibronectin, vimentin, and p-SMAD3 expression in primary tumors. Scale bar: 200 μm. * $P < 0.05$ compared with mice bearing 231-Luc plus 231-shControl tumors; # $P < 0.05$ compared with mice bearing 231-Luc plus 231-shFOXF2 tumors.

deficient BLBC cells, 231-Luc-shControl, and 231-Luc-shFOXF2 cells were used to establish a SCID mouse model by orthotopic transplantation, and the mice bearing 231-Luc-shFOXF2 tumors were treated daily with SB431542 by intraperitoneal injection for 6 weeks. Bioluminescence imaging (Fig. 3a) and metastatic photon flux analysis (Fig. 3b) at day 50 after injection revealed

that the number and extent of metastases were greater in mice injected with FOXF2-depleted cells than in control mice, and SB431542 treatment abolished FOXF2 depletion-enhanced metastases. The survival rates of the mice bearing 231-Luc-shFOXF2 tumors were significantly lower than those of the control mice, and survival improved by SB431542 treatment (Fig. 3c).

Histological examination by H&E staining identified that FOXF2 depletion significantly increased metastases in visceral organs, including the lungs, livers, pancreases, and kidneys, and SB431542 treatment abolished this effect (Fig. 3d). Immunohistochemical staining also confirmed that the protein expression levels of markers for myofibroblast/CAF and TGF- β signaling pathway activation α -SMA, FAP, PDGFR β , and p-SMAD3 were increased in shFOXF2 tumors compared with shControl tumors, and the increased expression of these proteins upon FOXF2 depletion was significantly repressed by SB431542 treatment (Fig. 3e). These results indicate that FOXF2 deficiency promotes BLBC visceral metastasis by increasing autocrine TGF- β signaling that induces cells to transdifferentiate into the myofibroblast/CAF-like phenotype.

FOXF2-deficient BLBC cells confer aggressiveness to neighboring cells by increasing paracrine TGF- β signaling

Because the secretion of TGF- β s was increased in FOXF2-deficient BLBC cells, we speculated that FOXF2-deficient cell populations could activate the TGF- β /SMAD signaling pathway and aggravate the malignancy of neighboring cancer cells. Indeed, when MDA-MB-231 and BT-549 cells were cultured with CM from 231-shFOXF2 and 549-FOXF2 cells, p-SMAD2 and p-SMAD3 levels were upregulated in MDA-MB-231 cells exposed to 231-shFOXF2 CM and downregulated in BT-549 cells exposed to 549-FOXF2 CM, and these alterations in p-SMAD2 and p-SMAD3 levels were abolished by SB431542 and TGF- β 1, TGF- β 2, or TGF- β 3, respectively (Fig. 4a, Supplementary Fig. 3a). The expression of the mesenchymal cell and myofibroblast markers fibronectin, vimentin and α -SMA, and the EMT-TF Slug changed in a consistent manner (Fig. 4b, Supplementary Fig. 3b). Interestingly, FOXF2 expression changed to be homogeneous with that in the feeder cells (Fig. 4b, Supplementary Fig. 3b). Cell migratory and invasive capabilities assessed by wound healing (Fig. 4c, Supplementary Fig. 3c) and transwell (Fig. 4d, e, Supplementary Fig. 3d, e) assays also changed consistently, when MDA-MB-231 and BT-549 cells were cocultured with MDA-MB-231-shFOXF2 and BT-549-FOXF2 feeder cells, respectively, or with CM derived from their feeder cells. These results indicate that FOXF2 in BLBC cells controls TGF- β /SMAD signaling pathway activation and phenotypic transition of neighboring cells toward mesenchymal cell/myofibroblast by negatively regulating paracrine TGF- β signaling that further negatively regulates FOXF2 expression. Thus, FOXF2 and TGF- β form a reciprocal negative feedback loop to maintain homeostasis and control aggressiveness in BLBC.

FOXF2-deficient BLBC cells enhance the metastasis of neighboring cells by increasing paracrine TGF- β signaling

To further test whether FOXF2-deficient BLBC cells affect the metastatic ability of neighboring cells, equal numbers of 231-Luc and 231-shFOXF2, or 231-shControl cells were mixed and then orthotopically inoculated into SCID mice. The mice bearing 231-Luc plus 231-shFOXF2 tumors were treated with SB431542 daily by intraperitoneal injection for 6 weeks from the seventh day after initial inoculation. Bioluminescence imaging (Fig. 5a) and metastatic photon flux analyses (Fig. 5b) at day 60 after injection revealed that 231-shFOXF2 cells induced 231-Luc cells to form more metastases than did 231-shControl cells, and SB431542 treatment abolished this effect. Consistently, the mice bearing 231-Luc plus 231-shFOXF2 tumors had worse survival than the mice bearing 231-Luc plus 231-shControl tumors, and SB431542 treatment extended the overall survival of the 231-Luc plus 231-shFOXF2 xenograft group (Fig. 5c). 231-shFOXF2 cells enhanced the metastasis of 231-Luc cells in the lungs, livers, pancreases, and kidneys, as identified by H&E staining and immunohistochemistry staining for luciferase, and SB431542 treatment reversed this effect (Fig. 5d, e). Immunohistochemical staining showed that the protein expression levels of α -SMA, fibronectin, vimentin, and p-SMAD3 were increased in tumors derived from 231-Luc cells mixed with 231-shFOXF2 cells compared with those derived from 231-Luc cells mixed with 231-shControl cells, and SB431542 treatment inhibited the increased expression of these markers in 231-Luc cells mixed with 231-shFOXF2 cells (Fig. 5f). These results confirm that FOXF2-deficient BLBC cells enhance the visceral metastasis of neighboring cells through increasing paracrine TGF- β signaling that induces cells to adopt a myofibroblast/CAF-like phenotype.

TGF- β represses FOXF2 expression through upregulating miR-182-5p

Because BLBC cells induced neighboring cells to homogeneously change FOXF2 expression by altering the TGF- β signaling pathway (Fig. 4b), we further investigated the underlying mechanism. First, we observed that sustained stimulation with TGF- β 1, TGF- β 2, and TGF- β 3 did not significantly change *FOXF2* mRNA expression, but reduced FOXF2 protein expression in MDA-MB-231 cells (Fig. 6a), indicating that TGF- β negatively regulates FOXF2 expression at the posttranscriptional level. Because miRNAs are capable of silencing gene expression at the posttranscriptional level, we selected miRNAs upregulated by TGF- β 1, TGF- β 2, or TGF- β 3 that potentially target

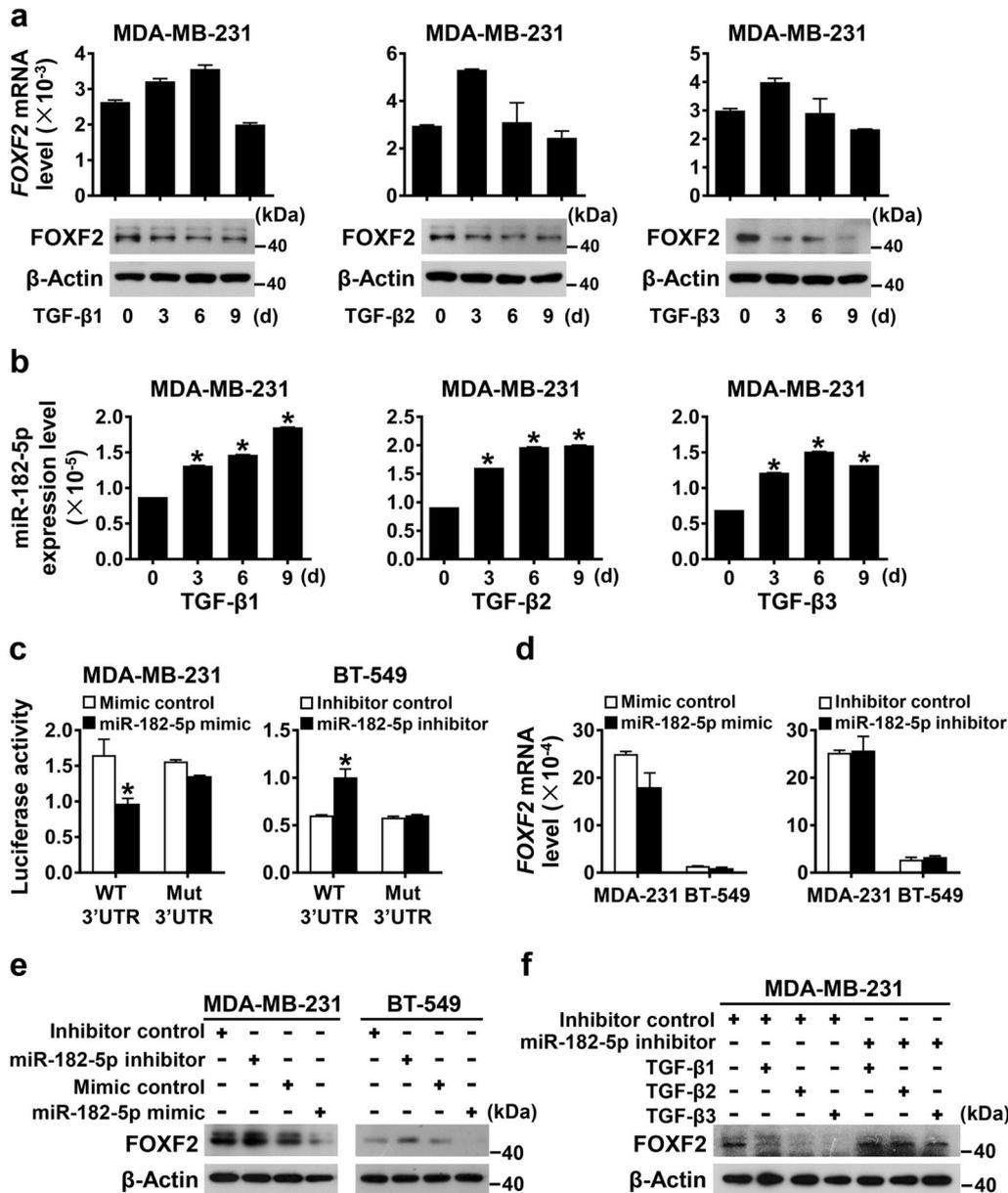


Fig. 6 MiR-182-5p is induced by TGF- β stimulation and represses FOXF2 translation. **a**, **b** FOXF2 mRNA and protein levels (**a**) and miR-182-5p expression levels (**b**) in MDA-MB-231 cells treated as indicated were detected by RT-qPCR and immunoblot. **c** The activity of the wild-type (WT) and mutated (Mut) FOXF2 3'-UTR luciferase reporters in MDA-MB-231 cells transfected with miR-182-5p mimic

or inhibitor was assessed by a dual-luciferase reporter assay. **d-f** FOXF2 mRNA (**d**) and protein (**e**, **f**) levels in the indicated cells were detected by RT-qPCR and immunoblot. TGF- β 1, TGF- β 2, or TGF- β 3 was added to the culture medium at a final concentration of 10 ng/mL. * $P < 0.05$ compared with the control.

FOXF2 (Supplementary Fig. 4a), and found that miR-182-5p and miR-200b-3p expression was upregulated by TGF- β stimulation in MDA-MB-231 cells (Fig. 6b, Supplementary Fig. 4b). Considering that miR-182-5p promotes EMT and metastasis in multiple types of cancer, including BLBC [25–27], we further validated the role of miR-182-5p in mediating FOXF2 silencing through TGF- β . Luciferase reporter assays showed that FOXF2 3'-UTR reporter activities were significantly reduced by miR-182-5p mimic

transfection and induced by miR-182-5p inhibitor treatment in MDA-MB-231 and BT-549 cells, respectively, and these effects were abrogated by a 7-bp mutation at the miR-182-5p-binding site in the FOXF2 3'-UTR reporter (Supplementary Fig. 4c, Fig. 6c). Consistently, the protein levels of FOXF2, but not the mRNA levels, were decreased by miR-182-5p mimic transfection and increased by miR-182-5p inhibitor treatment (Fig. 6d, e). Furthermore, TGF- β -induced silencing of FOXF2 expression was restored by

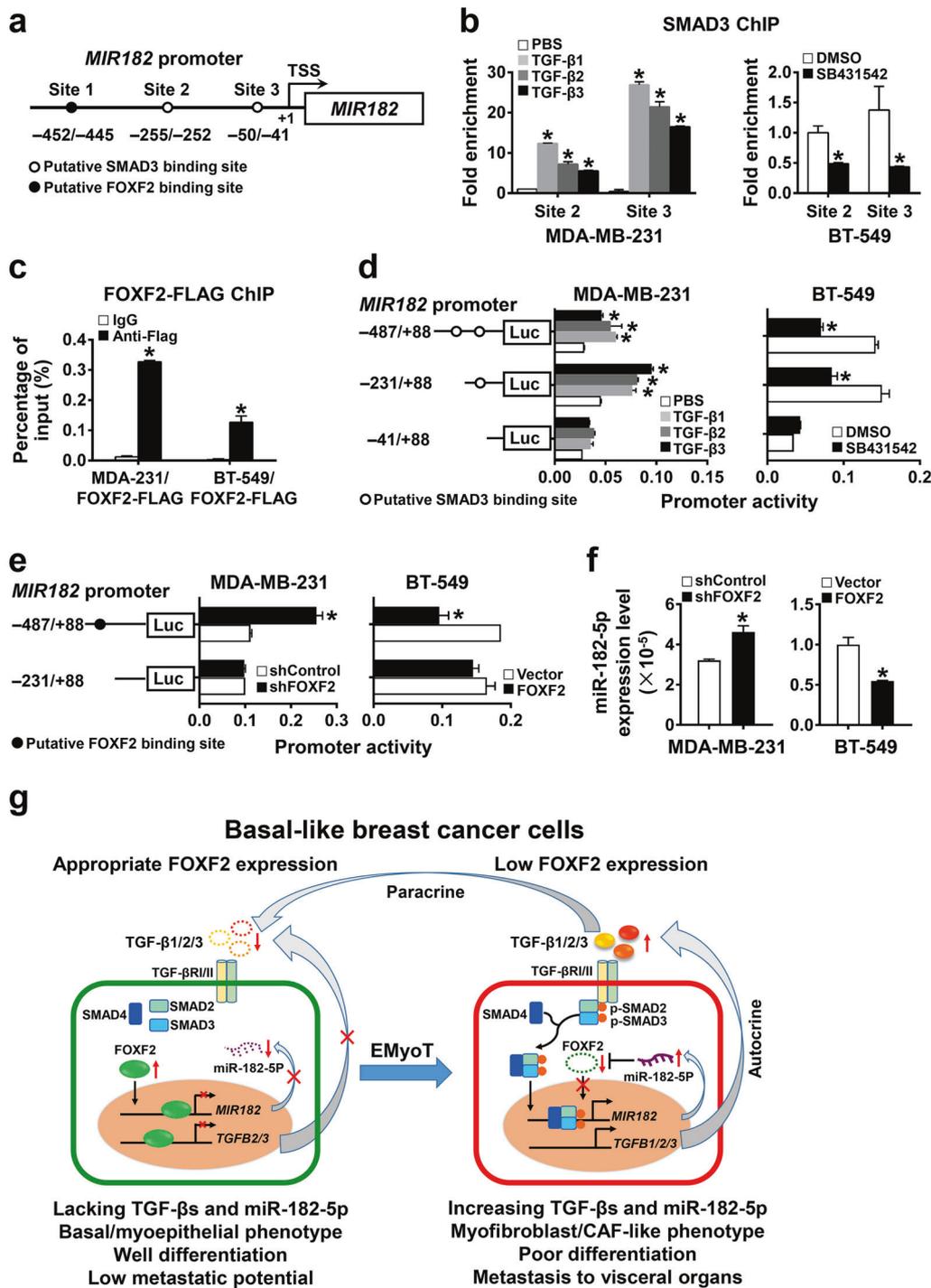


Fig. 7 *MIR182* is a common target of FOXF2 and SMAD3. **a** Schematic diagram showing the putative-binding sites of FOXF2 and SMAD3 in the *MIR182* proximal promoter region. **b**, **c** The binding of SMAD3 (**b**) and FOXF2 (**c**) to the *MIR182* promoter containing or lacking putative-binding elements in the indicated cells was assessed by ChIP-qPCR assays, using anti-SMAD3 (**b**) and anti-Flag (**c**) antibodies. **d**, **e** The activity of the *MIR182* promoter

containing or lacking SMAD3 (**d**) and FOXF2- (**e**) binding elements in the indicated cells was assessed by a dual-luciferase reporter assay. **f** MiR-182-5p expression in the indicated cells was measured by RT-qPCR. * $P < 0.05$ compared with the control. **g** The proposed model of reciprocal repression loops formed by FOXF2 and miR-182-5p or the TGF-β/SMAD/miR-182-5p axis in controlling BLBC aggressiveness.

miR-182-5p inhibitor treatment (Fig. 6f). These data indicate that FOXF2 and TGF-β undergo reciprocal repression

in BLBC cells, and that miR-182-5p mediates the silencing of FOXF2 expression in response to TGF-β.

FOXF2 and SMAD3 transcriptionally regulate *MIR182* expression

Because irreversible progression is a typical characteristic of aggressive BLBC, we further investigated whether the FOXF2/TGF- β /miR-182-5p regulatory axis forms a loop through the transcriptional functions of FOXF2 in BLBC. In addition, although TGF- β is known to positively regulate miR-182-5p expression, the underlying mechanism has not been established. Thus, we searched the *MIR182* promoter sequence for typical FOXF2-binding elements and SBEs, and found one FOXF2-binding element and two SMAD3-binding elements (Fig. 7a). ChIP-qPCR assays confirmed that both SMAD3 and FOXF2 bound the *MIR182* promoter regions, containing the predicted binding elements in MDA-MB-231 and BT-549 cells (Fig. 7b, c). The recruitment of SMAD3 to the *MIR182* promoter was significantly increased by TGF- β 1, TGF- β 2, or TGF- β 3 stimulation, and decreased by SB431542 treatment in MDA-MB-231 and BT-549 cells, respectively (Fig. 7b). Accordingly, the activity of the *MIR182* promoter containing the SMAD3-binding element was enhanced by TGF- β treatment and inhibited by SB431542 treatment (Fig. 7d). FOXF2 also negatively regulated the activity of the *MIR182* promoter containing the FOXF2-binding element (Fig. 7e) and miR-182-5p expression (Fig. 7f). These results demonstrate that *MIR182* is a target of FOXF2 and SMAD3. Therefore, in addition to mediating the reciprocal repression loop between FOXF2 and TGF- β in BLBC, miR-182-5p also forms a direct reciprocal repression loop with FOXF2. FOXF2 deficiency accelerates the visceral metastasis of BLBC by unrestrictedly increasing autocrine and paracrine TGF- β signaling, and miR-182-5p expression (Fig. 7g).

Discussion

EMT is a crucial cellular process for conferring cell plasticity and enabling the invasion-metastasis cascade. Cancer cells that have undergone EMT lose their epithelial cell-cell junctions and apical-basal cell polarity, and transition to a low proliferative state with a spindle-like cell shape, leading to increased cell migration, invasion, and survival [28]. EMT involves multiple transition stages that gradually progress from epithelial to completely mesenchymal. Cells in different stages of EMT display differences in differentiation status and metastatic potential [8]. The TGF- β /SMAD signaling pathway is recognized as a potent inducer of EMT. EMyoT occurs during TGF- β /SMAD pathway-induced EMT upon activation of a myogenic program and leads to the acquisition of a myofibroblast/CAF-like phenotype [10–13]. Our group previously reported that FOXF2 deficiency in BLBC cells promotes EMT and metastasis to

visceral organs through transactivation of the EMT-TFs *TWIST1* [16], *FOXC2* [17], and *FOXQ1* [18]. In this study, we further demonstrated that EMyoT occurs in the context of EMT in FOXF2-deficient BLBC cells, and confers myofibroblast/CAF-like properties and visceral metastasis capacity to the cells by increasing autocrine and paracrine TGF- β signaling.

Myocardin functions as a transcriptional coactivator of serum response factor (SRF), and modulates the expression of cardiac and smooth muscle-specific SRF target genes through interaction with myocardin-related TF (MRTF-A), an essential mediator of myogenic reprogramming during EMyoT [11, 12]. Myocardin ectopically expressed in non-muscle cells can induce smooth muscle differentiation. FOXF2 has been found to attenuate myocardin in smooth muscle cells through direct binding to the N-terminal region of myocardin [29]. The above evidence supports our finding that FOXF2 plays a key role in controlling the EMyoT of BLBC cells.

The TGF- β family belongs to the TGF- β superfamily and has three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3. TGF- β s are pleiotropic cytokines that regulate physiological embryogenesis and tissue homeostasis, as well as pathological inflammation and cancer. The TGF- β /SMAD signaling pathway plays a tumor suppressor role in early-stage tumors and an oncogenic role in advanced cancers [30]. However, the mechanisms underlying the dual role of the TGF- β /SMAD pathway have not been fully explored. Our previous studies have shown the dual role of FOXF2 in BLBC: the promotion of proliferation and the suppression of progression, which are opposite to the functions of the TGF- β /SMAD pathway [16, 31]. Hence, we investigated the transcriptional regulation of TGF- β /SMAD signaling pathway components by FOXF2, and found that FOXF2 directly bound to the proximal promoters of *TGFB2* and *TGFB3*, and repressed TGF- β 2 and TGF- β 3 protein expression and secretion in a BLBC cell subtype-specific manner. FOXF2 also repressed TGF- β 1 expression and secretion through the induction of TGF- β 2 and TGF- β 3. The study by Meyer-Schaller N et al. [32] shows that FOXF2 is activated by TGF- β signaling in a normal murine mammary gland cell line. As FOXF2 is a mesenchymal TF, it is logical that it mediates EMT in normal mammary epithelial cells, yet FOXF2 and TGF- β form a reciprocal repression loop to initiate a vicious cycle, resulting in aggressive progression of BLBC. TGF- β generated by either tumor cells, tumor-associated macrophages [33, 34], dendritic cells [35], or CAFs [20, 36] contributes to the evasion of the immune response and promotes metastasis in aggressive cancers. Thus, SB431542 treatment effectively suppressed metastasis in FOXF2-depleted BLBC cell-bearing mice in our study. The effects of FOXF2-depleted BLBC cells on stromal cells and stromal cell-

secreted TGF- β in primary tumors should be further deeply investigated.

MiR-182-5p plays an oncogenic role in multiple types of cancer [25, 26]. It is a downstream effector of TGF- β signaling and potentiates TGF- β -induced EMT, aggressiveness, and metastasis by targeting cylindromatosis (*CYLD*), cell adhesion molecule 1 (*CADMI*) and *SMAD7* [27, 37, 38]. FOXF2 is a known target of miR-182-5p [39, 40] and mediates miR-182-5p-regulated cancer progression [41]. However, how TGF- β regulates miR-182-5p expression and whether there is any other regulatory mechanism for miR-182-5p expression remain to be investigated. In this study, we observed that FOXF2 was silenced by TGF- β 1, TGF- β 2, or TGF- β 3 stimulation in BLBC cells and that this effect was mediated by miR-182-5p. We further demonstrated that *MIR182* is a direct transcriptional target of both FOXF2 and SMAD3. *MIR182* expression was transrepressed by FOXF2 and transactivated by SMAD3 via binding to its promoter in BLBC cells. Thus, we confirmed the regulatory cascade of the TGF- β /miR-182-5p/FOXF2 axis in BLBC cells, and proposed that reciprocal repression loops formed by FOXF2 and miR-182-5p or the TGF- β /SMAD/miR-182-5p axis control BLBC aggressiveness.

It is worth noting that TGF- β also significantly upregulated miR-200b-3p, a known upstream regulator of *FOXF2* [42]. Although miR-200 family members (miR-200s) play a role in regulating E-cadherin expression and EMT, prometastatic lung colonization that goes beyond their regulation of E-cadherin and epithelial phenotype has been revealed by Kang's group [43] and Lieberman's group [44]. Lieberman's group has also reported that metastatic breast cancer cells deliver miR-200s to poorly metastatic cells to promote lung colonization [45]. Because FOXF2 is a target of miR-200b-3p that is upregulated by TGF- β , miR-200b-3p may promote visceral metastasis of BLBC partially through direct targeting FOXF2. This inference should be validated by further experiments.

In summary, we found that FOXF2 is a repressor of both TGF- β and miR-182-5p, and is silenced by the TGF- β /SMAD/miR-182-5p axis. The reciprocal repression loops formed by FOXF2 and miR-182-5p or the TGF- β /SMAD/miR-182-5p axis control the aggressive behavior of BLBC. An imbalance in either of the loops will initiate a vicious cycle, resulting in tumor progression and visceral metastasis. This study not only contributes to our understanding of the molecular mechanism by which FOXF2 deficiency promotes BLBC aggressiveness and visceral metastasis, but also elucidates the regulatory relationships among FOXF2, TGF- β /SMAD, and miR-182-5p. In addition to the suppressive role of FOXF2 in visceral organ metastasis of BLBC, we also found a promoting role of FOXF2 in bone-specific metastasis of breast cancer [46]. Subtype-specific

and metastatic organ-specific patterns of FOXF2 expression, and its role in breast cancer reflect the pleiotropic regulatory function of FOXF2 in cancer development and metastasis. Deregulation of FOXF2 expression may lead to complex phenotypic transitions of cancer cells followed by refractory cancer metastasis. Thus, maintaining appropriate FOXF2 expression level may be an effective strategy for controlling breast cancer progression and metastasis leading by FOXF2 deregulation.

Acknowledgements This work was supported by the National Natural Science Foundation of China (nos. 81272357, 81472680, 81672894, and 81872403).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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