Stem Cell Research & Therapy

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FBF1 maintains stem cell-like properties in breast cancer via PI3K/AKT/SOX2 axis

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Abstract

Background Considerable evidence suggests that tumor initiation, malignancy, metastasis and recurrence occur due to emergence of cancer stem cells (CSCs). Fas binding factor 1 (FBF1) is a multifunctional protein that plays essential roles in the regulation of development and cell fate decisions. However, the function in maintaining stem cell-like properties of breast cancer remains elusive.

Methods Tissue microarray was used to evaluate FBF1 expression. Cancer stemness assays were performed in FBF1 silencing and overexpressing cells *in vitro* and in a xenograft model *in vivo*. RNA sequencing, immunofluorescence and immunoprecipitation assays were performed to explore the underlying mechanism. Clinical expression and significance of FBF1 and stemness-associated factors were explored by analyzing datasets.

Results We report that FBF1 was highly expressed in breast cancer and significantly correlated with clinical progression. Silencing FBF1 in MDA-MB-231 cells restrained CSCs properties, including side population, sphere formation and migration, whereas ectopic FBF1 expression increased the side population proportion, enhanced the sphere formation ability, and promoted the expression of core stemness genes, such as SOX2, OCT4, KLF4 and NANOG, as well as facilitated metastasis of T47D breast cancer cells. Furthermore, mice bearing FBF1-overexpressed T47D xenografts had higher tumorigenic frequency and stronger metastasis potential. In addition, exploration of the underlying mechanism indicated that FBF1 binds PI3K which then activates PI3K-AKT phosphorylation cascades. Then the activated p-AKT interacts with stemness marker SOX2, elevates SOX2 and OCT4 activity, and finally forms PI3K/AKT/SOX2 axis, which mediates stem cell-like identities. Moreover, PI3K inhibitors abolished FBF1-mediated signaling pathway and diminished breast cancer stemness *in vitro* and *in vivo*. In 24 human breast cancer samples, we found a good positive correlation between the expression of FBF1 and p-AKT, as well as between FBF1 and SOX2 as determined by IHC. Clinical data showed that FBF1 expression was positively correlated with the expression of POU5F1 (OCT4), AKT1 and was negatively correlated with PTEN, which is a negative regulator of PI3K/AKT signaling.

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Conclusion Collectively, we identified a potential CSCs regulator and suggested a novel mechanism by which FBF1 governs cancer cell stemness. This study thus introduces an effective target for the diagnosis and treatment of breast cancer.

Highlights

- FBF1 is highly expressed in breast cancer and correlated with clinical progression.
- FBF1 maintains stem cell-like properties in breast cancer.
- FBF1 facilitates EMT and metastasis in breast cancer.
- FBF1 binds PI3K and then activates PI3K/AKT/SOX2 axis which mediates cancer stemness.
- FBF1 is a promising target for the diagnosis and treatment of breast cancer.

Keywords FBF1, Cancer stem cell, Breast cancer, Stemness, SOX2

Introduction

Breast cancer is one of the most common female malignancies and is the principal cause of cancer deaths in women around the world [1]. Although breast cancer mortality has declined in recent decades, there is still a lack of therapeutic regimens that effectively inhibit tumor recurring and remote metastasis, which are the leading causes of death from breast cancer and are the bottleneck in improving clinical cure rates [2]. To make matters even more complicated, the underlying mechanisms of these symptoms are still not well understood.

Recent research has indicated that cancer stem cells (CSCs) are a subpopulation of cells found within the tumors themselves. They are speculated to be the root cause of initiating tumorigenesis, causing tumor relapse and metastasis, as well as resistance to clinical therapeutics [3–5]. A wide variety of tumors have identified CSCs, such as breast cancer [6], pancreatic cancer [7], and acute myeloid leukemia [8]. Meanwhile, the CSCs phenotype is closely related to the process of epithelial-mesenchymal transition (EMT), which is a prominent step in metastasis [9]. Therefore, efforts to understand of which factor(s) drive the CSCs properties signaling pathway will offer the opportunity for cure of cancer patients with potential for metastasis or recurrence with more targeted therapeutic strategies.

Fas binding factor 1 (FBF1) is expressed in multiple tissues and mainly localized in cytoplasm [10]. It is a novel protein that spatiotemporally integrates multiple aspects of centrosome function that is essential for cell proliferation and differentiation [11, 12]. Current studies provide evidences that FBF1 could regulate centrosomes duplication and separation, thereby affecting cell cycle progression during mitosis [13]. In addition, it was reported that FBF1 is expressed in oocytes of the mouse, and silencing FBF1 showed inhibitory effects on the PLK1 expression and caused abnormal spindle assembly and chromosomal separation. Thus, which demonstrated that FBF1 is involved in spindle assembly and chromosome arrangement during meiosis in mouse oocyte [13]. Moreover, FBF1 has recently been reported as a key mediator of preadipocyte differentiation [14]. Furthermore, ten genes were downregulated by deslanoside, FBF1 was one of them and had an inverse correlation with recurrence-free survival (RFS) in prostate cancer patients [15]. However, so far, there are hardly any reports on the potential role of FBF1 in cancer development, and the specific function of FBF1 in breast cancer is even less clear.

The transcription factor SOX2 is indispensable for developing and maintaining cancer stemness [16]. Ectopic SOX2 increased CSCs population in breast, prostate and lung cancers, whereas knockdown SOX2 restrained CSCs properties and diminished tumorigenic frequency *in vivo* [17–20]. In addition, SOX2 is expressed at high levels in at least 25 types of cancer and could stimulate cancer cells to proliferate, migrate, invade and metastasize, ultimately facilitating neoplastic progression [21]. Furthermore, SOX2 accelerates EMT process through multiple mechanisms. SOX2 promotes *β-catenin* transcription by binding to its promoter, which then activates Wnt/β-catenin signaling and thus stimulate EMT in prostate and breast cancer [22]. In this way, it can be seen that SOX2 is not only a potent EMT inducer, but also a regulator of cancer stemness.

In this study, we investigate the function and mechanism of FBF1 in breast cancer progression *in vitro* and *in vivo*. We illustrate that FBF1 enhances stem cell-like properties in breast cancer cells as well as in mouse model. In terms of the mechanism, we demonstrate that FBF1 binds to PI3K and activates the PI3K/AKT signaling. Activated AKT can further interact with SOX2, which induces SOX2 expression. In addition, blockage of the PI3K/AKT/SOX2 signaling abolishes FBF1-mediated cancer cell stemness. Collectively, these findings provide evidence that FBF1 may be an effective target for breast cancer treatment.

Methods

Cell culture

MDA-MB-231 and T47D cells were purchased from ATCC. MDA-MB-231 cells were cultured in L15 medium at 37 °C. T47D cells were maintained in DMEM

supplemented with 1% NEAA at 37 °C in 5% CO_2. Each medium contains 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Stable cell line establishment

Human FBF1 overexpression (FBF1-oe) and shRNA targeting FBF1 (shFBF1) plasmids were constructed following previous protocols. To stably silencing FBF1 in cells, MDA-MB-231 cells were infected with lentivirus carrying the shFBF1 plasmid. The cells were then treated with puromycin to obtain FBF1 diminished and control cell lines. For the stable overexpress FBF1 in cells, T47D were infected with lentivirus carrying FBF1-oe plasmid, then blasticidin treated to obtain cell lines. The primer sequences are summarized in Supplementary Table 1.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was conducted following the previous protocol [23]. The used primers are listed in Supplementary Table 2.

Western blot

Cell lysates were prepared from MDA-MB-231 and T47D cell lines. Proteins (10–40 μg) were loaded and subjected to SDS-PAGE, transferred onto PVDF membrane, and then blotted with antibodies. Primary antibodies included: anti-FBF1 (Proteintech, 11531-1-AP), anti-β-actin (Santa Cruz, 47778), anti-SOX2, OCT4, KLF4, NANOG, Vimentin, E-cadherin, ZO-1, Claudin-1, PI3K, p-PI3K, AKT, p-AKT (Cell Signal Technology, 3579, 2840, 4038, 4903, 5741, 3195, 8193, 13255, 4292, 4228, 4691, 4060).

Side population assay

Cells were collected and resuspended in PBS with 2% FBS at 1.0×10^6 cells/ml, and then cultured with 7 μg/ml (for MDA-MB-231), 8 μg/ml (for T47D) Hoechst 33342 at 37 °C for 60 min. Cells were analyzed by LSRFortessa flow cytometer.

Sphere formation assay

MDA-MB-231 and T47D cells were harvested, serum washed, and dissociated into single cell suspensions in sphere medium. Cells were then grown in 48-well plates with ultra-low attachment at a density of 500 cells per well.

Transwell and wound healing assay

Cell migration ability detected by Transwell and Wound healing assay were performed in accordance with previously described protocols [24].

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded specimens and human breast cancer tissue microarrays (Bioaitech Company, Xi'an, Shanxi, China). Protein expression in the tumor specimens was detected by means of antibodies against SOX2, OCT4, KLF4, NANOG, p-PI3K and p-AKT. For FBF1 tissue microarray staining, the percentage of $FBF1^+$ cells and staining intensity were used to evaluate the expression level. Specifically, ≤ 10%, 11-30%, 31-50%, and > 50% $FBF1^+$ cells were scored as 1, 2, 3, 4. Staining intensities were analyzed as negative, weak, moderate, and strong, scored 1, 2, 3, 4. The two scores were then multiplied to obtain IHC score.

Immunoprecipitation

Cell lysates were incubated with Protein A/G Agarose (Santa Cruz, 2003) and antibody at 4 °C for overnight. Then, bound proteins were boiled for SDS-PAGE, transferred onto PVDF membrane, and blotted with antibodies by Western blot.

RNA sequencing (RNA-Seq)

Total RNA was extracted from control and FBF1 overexpressed groups of T47D cells using TRIzol reagent (Invitrogen), and each group was prepared with three parallel replicates. The library construction, RNA sequencing and analysis work were performed at Beijing Genomics Institute. The accession number for the deposited RNA-seq data is PRJNA961214.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde and labeled with primary antibodies overnight at 4 °C, followed by incubation with species-appropriate secondary antibodies for 1 h. Nuclei were stained with DAPI, and images were acquired using a laser scanning confocal microscope.

Animal study

The work has been reported in line with the ARRIVE guidelines 2.0. All *in vivo* mouse experiments were approved by the Ethics Committee of Xinxiang Medical University. Six-eight weeks female nude mice randomized to each group and inoculated with cells to each mouse subcutaneously. The mice were anesthetized with isoflurane and were subsequently euthanized using CO_2 inhalation when necessary. Tumor volume was calculated by means of the formula: length × $width^2/2$. Tumor and lung tissues were harvested, followed by IHC or hematoxylin and eosin (H&E) staining was performed.

For limited dilution transplantation, 1×10^6, 5×10^5 or 1×10^5 of T47D-Ctrl and T47D-FBF1 cells were inoculated to each mouse, collected tumor tissues and analyzed tumorigenic capacity.

Patient datasets

FBF1 transcript levels in primary breast cancer and normal samples were analyzed at UALCAN (http://ualcan.path.uab.edu/index.html). The GEPIA was used to perform gene expression correlation analysis between FBF1 and stemness related genes in breast cancer samples (http://gepia.cancer-pku.cn/index.html).

Statistical analysis

Data were analyzed using GraphPad Prism 5 software. The values were showed as mean ± Standard Error of Mean (SEM). Student's *t*-test was used for the calculation of p-values. $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

FBF1 is highly expressed in human breast cancer

To investigate the clinical significance of FBF1 in breast cancer progression, we first performed immunohistochemistry to examine the expression of FBF1 in breast cancer tissue microarray containing 175 samples (145 breast cancer tissues and 30 para-carcinoma tissues). The results indicated that FBF1 immunostaining was stronger in tumor than in para-carcinoma (Fig. 1A, B). Meanwhile, we explored the associations between FBF1 immunostaining and clinicopathological characteristics. The findings suggested that FBF1 expression was positively associated with breast cancer TNM stage and histological grade (Fig. 1C, D). Next, we examined the mRNA levels of FBF1 in two cohorts from the TCGA database. These cohorts consisted of 1097 breast cancer specimens and 114 normal samples. Consistently, higher FBF1 levels were observed in breast cancer samples (Fig. 1E). Furthermore, elevated expression of FBF1 was also observed in the human breast cancer cell lines compared with the non-tumorigenic breast epithelial cell line MCF-10 A (Fig. 1F, G). Collectively, these findings indicate that FBF1 expression levels correlate with breast cancer malignancy.

FBF1 is essential for maintaining breast cancer cell stemness properties

To elucidate the role of FBF1 in CSCs performances, we first established stable MDA-MB-231 cell lines silencing FBF1, T47D and MCF7 cell lines overexpression of FBF1. We found that FBF1 knockdown decreased stemness markers level, including SOX2, OCT4, KLF4 and NANOG (Fig. 2A, B). We also investigated the changes in side population (SP) of shFBF1 and shCtrl cells. Our results showed that SP proportions was lower in shFBF1 compared to shCtrl cells (Fig. 2C, D). In addition, we also studied the differences in sphere formation between two groups. We discovered that silencing FBF1 showed inhibitory effects on the sphere formation ability (Fig. 2E, F).

It is reported that CSCs phenotype is closely related to the activation of EMT program [25–27]. For testing the regulatory effect of FBF1 on EMT, we detected the EMT-related protein expression and observed that FBF1 deficiency restrained EMT program, as the expression level of Vimentin was diminished in FBF1-deficient cells (Fig. 2B). Furthermore, wound healing assays were conducted to determine the function of FBF1 in migration, the results showed that silencing FBF1 suppressed cell migration properties (Fig. 2G, H). Transwell assay also proved that FBF1 knockdown diminished cell migration features (Fig. 2I, J).

In support of our conclusions, we investigated the effect of FBF1 overexpression in T47D and MCF7 cells. First in T47D cells, we found that FBF1 overexpression significantly increased SP cells proportion and strengthened sphere formation ability (Fig. 3A–D). Next, transwell and wound healing assays confirmed that ectopic FBF1 facilitated cell metastasis (Fig. 3E–H). Moreover, we used qPCR and western blot to analyze the expression of stemness markers, the results indicated that these markers were elevated in T47D-FBF1 cells (Fig. 3I, J). Furthermore, we observed that ectopic FBF1 contributes to mesenchymal traits through attenuation of E-cadherin, ZO-1 and Claudin-1 expression (Fig. 3J), indicating the EMT-promoting effects of FBF1. Consistently, the breast cancer cell stemness promotion effect of FBF1 was further supported in MCF7-FBF1 cells (Supplementary Fig. 1A–G). Taken together, these findings verify that FBF1 is essential for supporting CSCs performances.

FBF1 mediates PI3K/AKT signaling in breast cancer

To gain better understanding of the potential mechanisms of FBF1-mediated breast cancer stemness properties, we performed RNA-seq to identify differentially expressed genes (DEGs) after FBF1 overexpression. The mainly upregulated DEGs associated with tumor progression were presented in Fig. 4A. To interpret the critical signaling pathway that are affected by FBF1, KEGG pathway classification showed that FBF1 was significantly associated with cellular processes including cell motility, cellular community, cell growth and death (Fig. 4B). And FBF1 is also associated with many human diseases, such as cancers, drug resistance and cardiovascular diseases (Fig. 4B). Additionally, KEGG pathway analysis indicated that the genes were concentrated in a number of signaling, including EGFR tyrosine kinase inhibitor resistance, Rap1 signaling pathway and PI3K-AKT pathway (Fig. 4C). Similarly, gene ontology (GO) enrichment analysis of biological processes showed that DEGs were concentrated in locomotion, cell proliferation, biological adhesion, growth and reproductive process (Fig. 4D). Molecular functions analysis indicated that gene terms associated with catalytic activity, molecular function

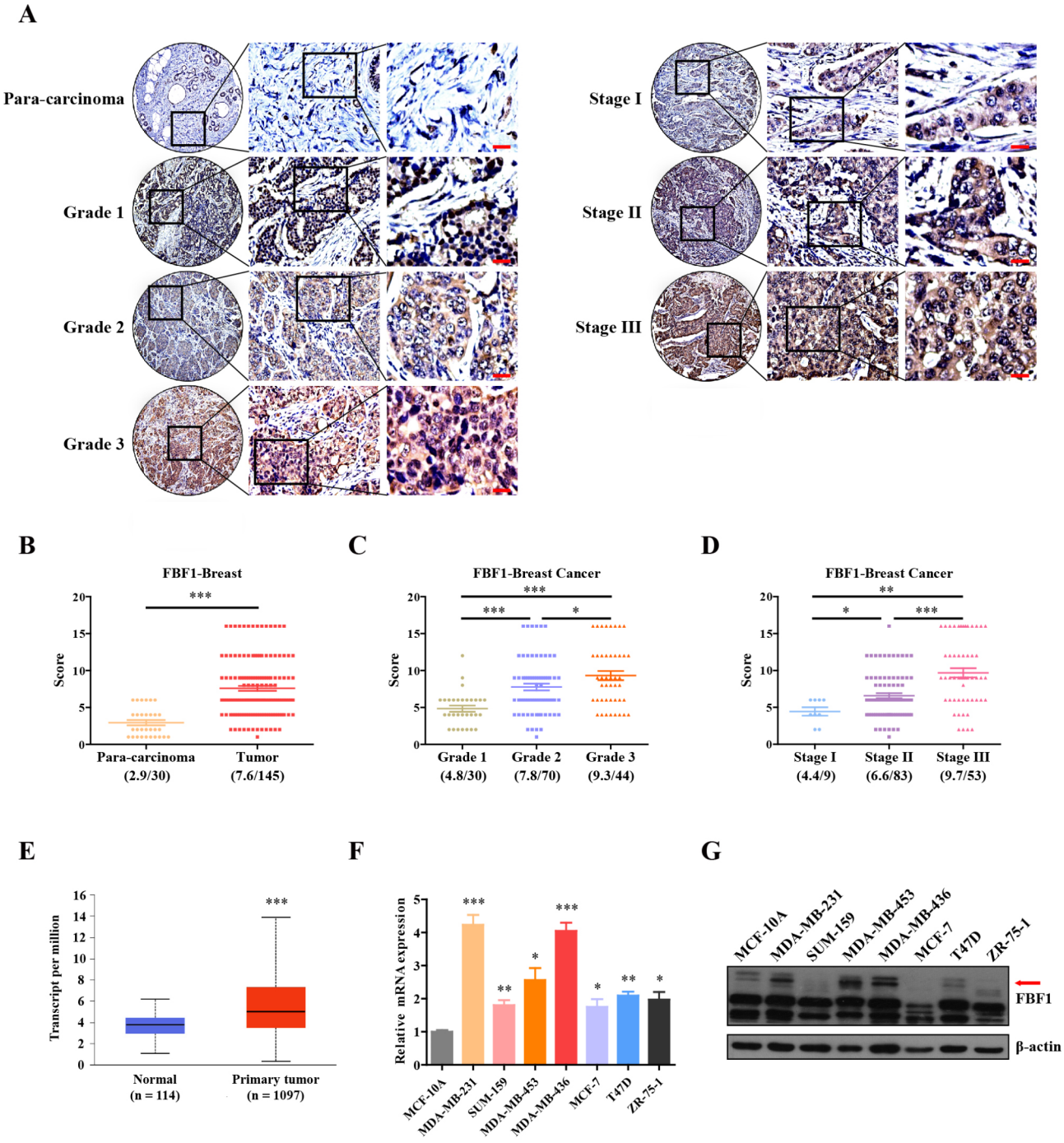


Fig. 1 FBF1 is highly expressed in human breast cancer and correlated with tumor malignancy. (**A**) Immunohistochemical analysis of FBF1 expression using tissue microarray. Scale bars: 20 μm. (**B**) Quantification results of FBF1 expression. (**C**, **D**) Quantification of FBF1 immunostaining with histological grades and TNM stages. (**E**) Transcript levels of FBF1 in 114 normal tissues and 1097 breast primary tumors from TCGA. (**F**, **G**) qRT-PCR and western blot showing FBF1 expression in non-tumorigenic breast epithelial cell line MCF-10 A compared with breast cancer cell lines

regulator and transporter activity have also been altered (Fig. 4E).

Subsequently, qRT-PCR was used to confirm the genes identified in RNA-seq. The expression of genes involved in tumor progression were enhanced in T47D-FBF1 cells, such as AKT3 (Fig. 4F). And it was consistent with RNA-seq data.

Based on these results, we speculated that FBF1 may regulate PI3K/AKT signaling pathway. To verified our hypothesis, we further investigated the role of FBF1 in activating PI3K/AKT pathway. We observed a striking

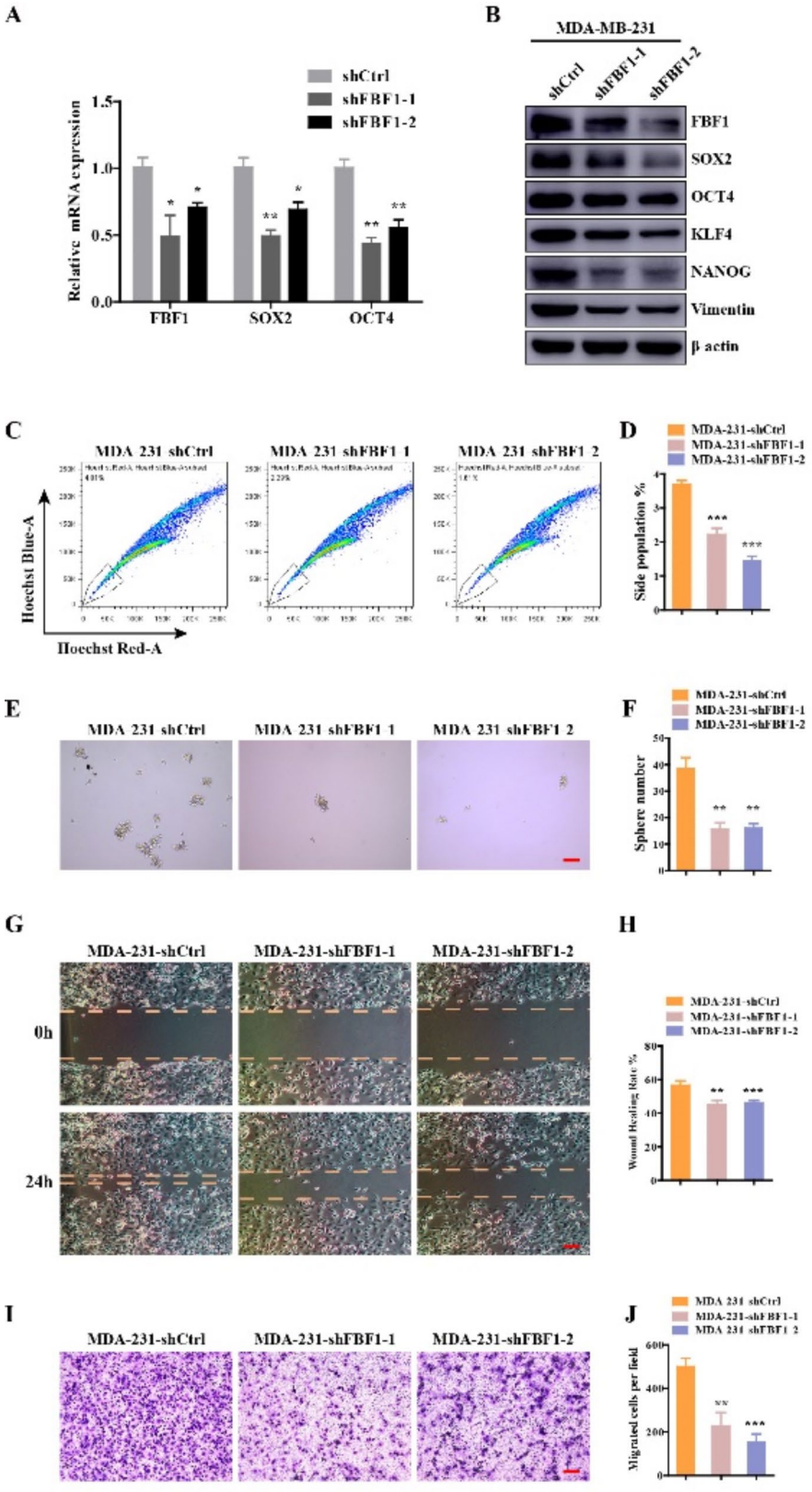


Fig. 2 Silencing FBF1 suppresses breast cancer cell stemness. **(A)** qRT-PCR analysis of *FBF1*, *SOX2* and *OCT4* mRNAs in MDA-MB-231 cells. **(B)** Western blot analysis of FBF1, SOX2, OCT4, KLF4, NANOG, and Vimentin in FBF1-silenced cells. **(C)** Flow cytometry analysis of side population in shFBF1 and shCtrl cells. **(D)** The statistical analysis of SP proportion. **(E)** Sphere formation ability of FBF1-deficient cells. Scale bars: 100 μm. **(F)** The statistical analysis of sphere number. **(G, H)** Migration properties of shFBF1 and shCtrl cells. Scale bars: 100 μm. **(I, J)** Migration traits of shFBF1 and shCtrl cells. Scale bars: 100 μm

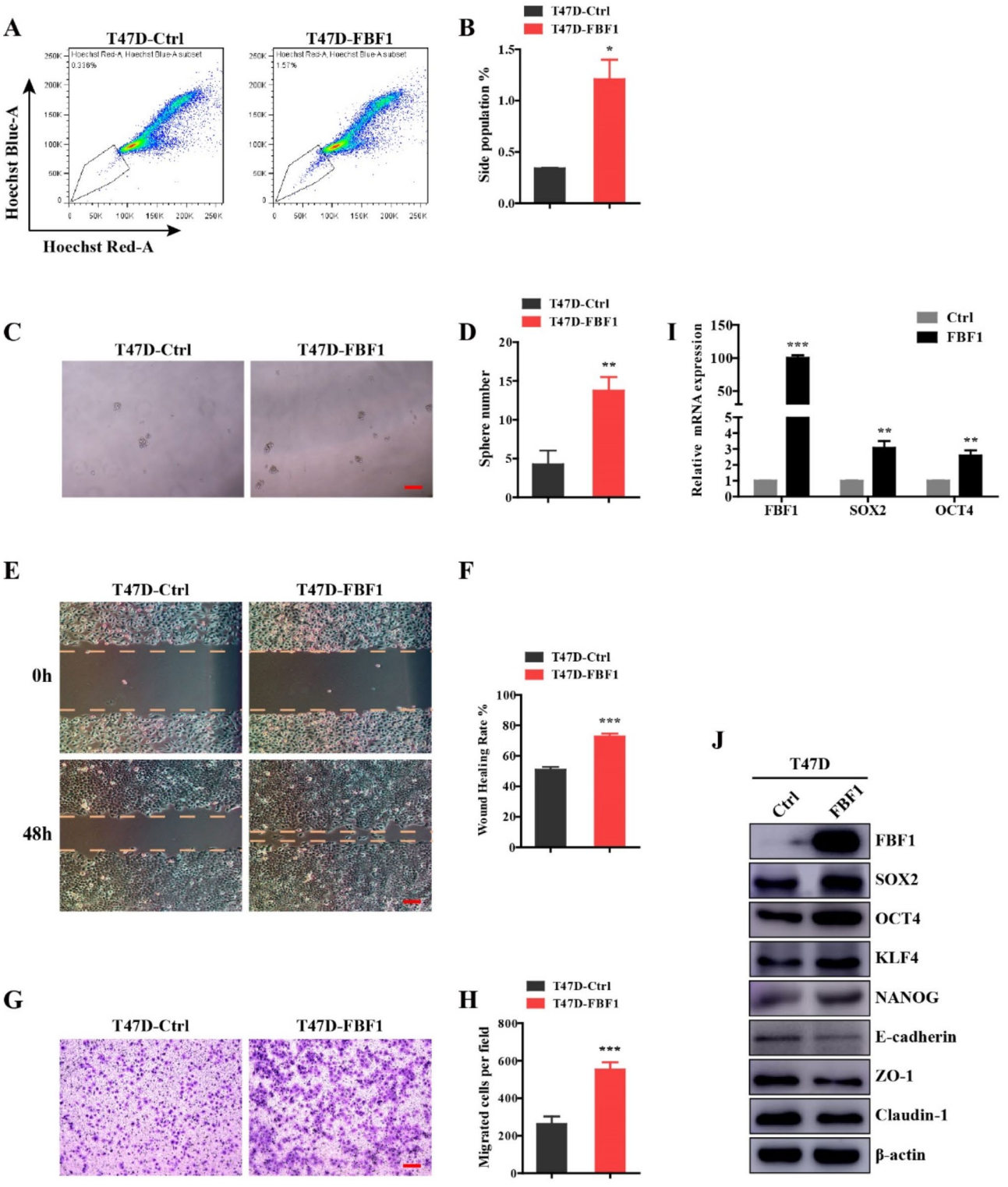


Fig. 3 Ectopic FBF1 promotes the CSCs-like characteristics of breast cancer cells *in vitro*. **(A)** Flow cytometry analysis of side population in T47D cells. **(B)** The statistical analysis of SP proportion. **(C)** Sphere formation ability of FBF1-overexpressed stable T47D cells. Scale bars: 100 μm. **(D)** The statistical analysis of sphere number. **(E**, **F)** Migration properties of T47D-FBF1 and control cells. Scale bars: 100 μm. **(G**, **H)** Migration traits of T47D-FBF1 and control cells. Scale bars: 100 μm. **(I)** qRT-PCR analysis of *FBF1*, *SOX2* and *OCT4* mRNAs in T47D cells. **(J)** Western blot analysis of FBF1, SOX2, OCT4, KLF4, NANOG, E-cadherin, ZO-1, and Claudin-1 in T47D cells

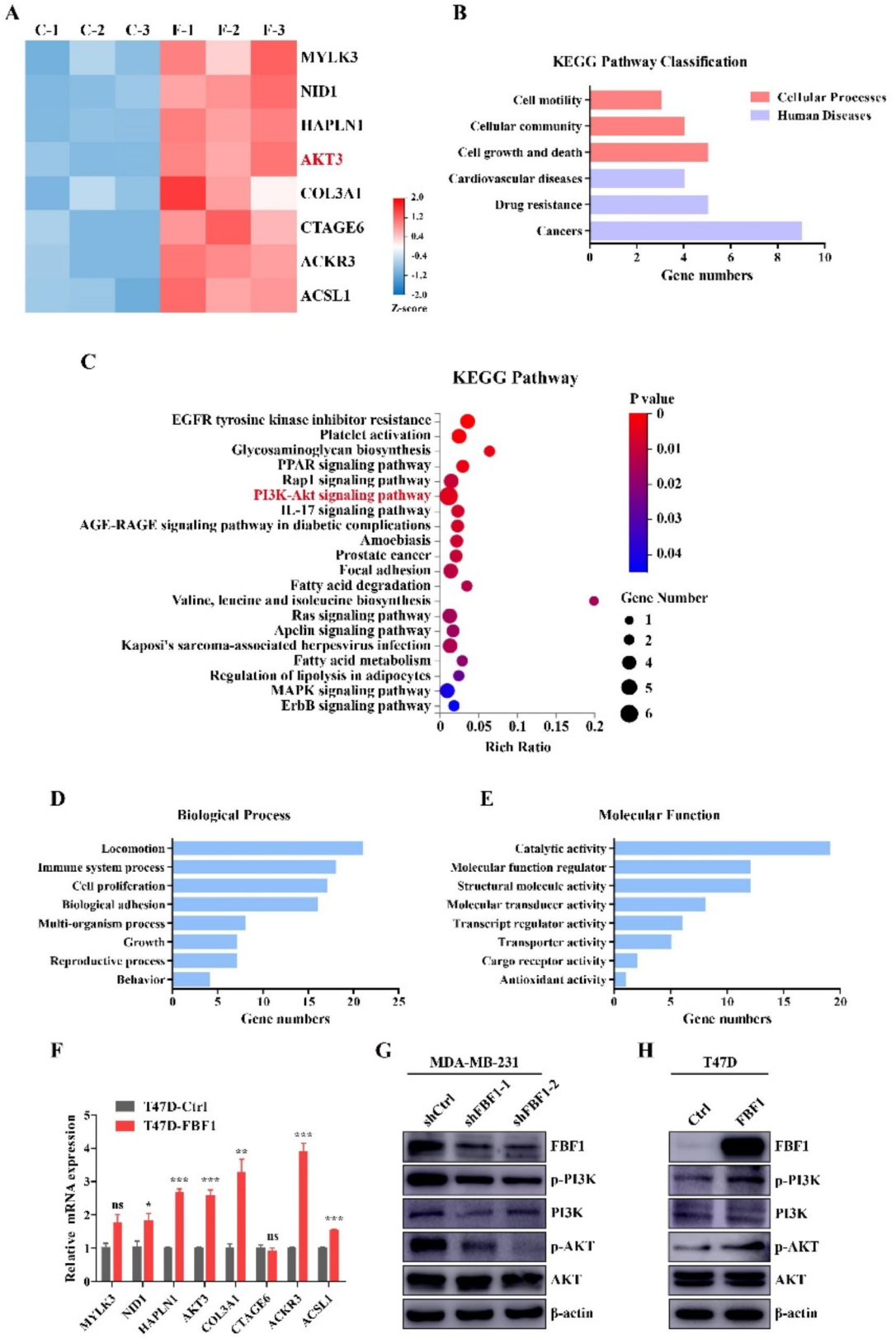


Fig. 4 FBF1 mediates PI3K/AKT signaling pathway in breast cancer. **(A)** Heatmap for the up-regulated genes between T47D-Ctrl and T47D-FBF1 cells. **(B)** KEGG pathway classification analysis in T47D cells. **(C)** Representative differential pathways were highlighted by KEGG pathway analysis in T47D cells. **(D, E)** Representative GO enrichment for analyzing biological process and molecular function. **(F)** qRT-PCR analyze the eight up-regulated genes. **(G, H)** Western blot analysis of PI3K/AKT signaling in MDA-MB-231 and T47D cells

decrease of phospho-PI3K and phospho-AKT expression in FBF1 silencing cells (Fig. 4G). By contrast, we found that PI3K/AKT signaling was activating in T47D-FBF1 cells (Fig. 4H) and MCF7-FBF1 cells (Supplementary Fig. 1H). Collectively, these findings provided the evidence that FBF1 could regulate PI3K/AKT signaling pathway.

Inhibitors targeted FBF1 mediated signaling constricts breast cancer cell stemness *in vitro*

To gain insight into the importance of FBF1 mediated pathway in boosting stem cell-like characteristics, we investigated PI3K inhibitor (LY294002) effects in T47D-FBF1 and MCF7-FBF1 cells. We confirmed that PI3K inhibitor turned SP cells proportion, sphere formation ability and migration facilitating effects of FBF1 in T47D cells (Fig. 5A–H) and MCF7 cells (Supplementary Fig. 2A–F). Consistent with this, treatment of cells with the inhibitor rescued Claudin-1 and ZO-1 expression, weakened the levels of pluripotency factors, such as SOX2, OCT4, KLF4, NANOG, and attenuated FBF1-induced PI3K/AKT pathway activation in T47D cells (Fig. 5I, J) and MCF7 cells (Supplementary Fig. 2G, H). Collectively, these findings verified that inhibitors targeting FBF1 mediated pathway restrained breast cancer stem cell identities *in vitro*.

FBF1 enhances breast cancer stemness characteristics *in vivo*

To further interrogate the effect of FBF1 on breast CSCs identities *in vivo*, we carried out xenograft experiments with T47D cells. To this end, stable T47D-Ctrl and T47D-FBF1 cells were injected into the mammary fat pad of female mice. Firstly, we confirmed that ectopic FBF1 promoted tumor volume and tumor weight, which was reversed by PI3K inhibitor (Fig. 6A–C). Then, we observed that more ectopically expressed FBF1 cells metastasized to the lung and initiated secondary tumor, which was also restored by downregulating PI3K expression (Fig. 6D–F). Additionally, IHC staining showed that upregulated FBF1 boosted stemness markers level, including SOX2, OCT4, NANOG, and reduced E-cadherin expression. Moreover, tumor tissues showed elevated levels of p-PI3K and p-AKT (Fig. 6G, H). However, the inhibitors reversed stemness marker expression and modulated PI3K/AKT pathway activation (Fig. 6G, H). Finally, limited dilution xenograft assay revealed that an increased incidence of tumor initiation in the T47D-FBF1 inoculation group compared to the T47D-Ctrl group. This increased incidence was reversed by decreasing PI3K levels (Fig. 6I). The above results collectively confirmed that FBF1 maintained breast cancer stemness characteristics via PI3K/AKT signaling *in vivo*.

FBF1 facilitates breast cancer stemness properties via interacting with PI3K kinase followed by activating PI3K-AKT-SOX2 axis

Since we found that FBF1 facilitates breast cancer stemness properties through PI3K-AKT pathway. We next asked that how FBF1 affects the PI3K signaling? And how the pathway mediates CSCs identities? In this regard, previous reports indicate that activation of PI3K mainly involves binding of the substrate near the inner side of plasma membrane [28]. Consequently, we determined to investigate the interaction between FBF1 and PI3K in cancer cells.

We have discovered that silencing FBF1 suppressed PI3K phosphorylation in MDA-MB-231 cells and ectopic FBF1 promoted phospho-PI3K expression in T47D cells (Fig. 4G, H). We also showed that FBF1 co-localizes with PI3K in T47D cells by means of double fluorescent staining (Fig. 7A). And we further demonstrated the interaction between FBF1 and PI3K, as well as p-PI3K in T47D cells performing co-immunoprecipitation (Fig. 7B–D). Together, these findings indicate that FBF1 interacts with PI3K and hastens PI3K phosphorylation, which then activating PI3K-AKT signaling.

The above leads one to ask the following question: Whether activated AKT can directly mediate the expression of pluripotency transcription factors SOX2 and thus influence cancer stemness? To answer this question, we conducted immunoprecipitation by means of anti-p-AKT antibody and immunoblotting using anti-SOX2 antibody in T47D cells. We identified that p-AKT could interact with SOX2, and enhance SOX2 activity (Fig. 7E).

In order to further confirm the correlation between FBF1 and p-AKT/SOX2, we studied the co-expression levels of FBF1, p-AKT and SOX2 using immunohistochemistry in 24 human breast cancer samples. Our results revealed that FBF1 has a good co-expression with p-AKT and SOX2. A positive correlation between the expression of FBF1 and p-AKT, as well as between FBF1 and SOX2 (Fig. 7F–K). Consistently, this was further supported by the immunofluorescence results, which revealed an elevation in the intensity of the p-AKT and SOX2 in FBF1 overexpression cells. These enhanced expressions were reversed by treatment of cells with the inhibitor (Fig. 7L, M).

Moreover, to determine whether FBF1 expression correlates with PI3K-AKT pathway, and stemness-associated factors expression, we analyzed the data in TCGA and GTEx database. The result revealed that FBF1 expression was positively correlated with the expression of AKT1, POU5F1 (OCT4) and was negatively correlated with PTEN (Supplementary Fig. 3A–C), which is a negative regulator of the PI3K/AKT pathway [29]. These results are consistent with our experimental data, and further demonstrate that FBF1 is closely associated with CSCs

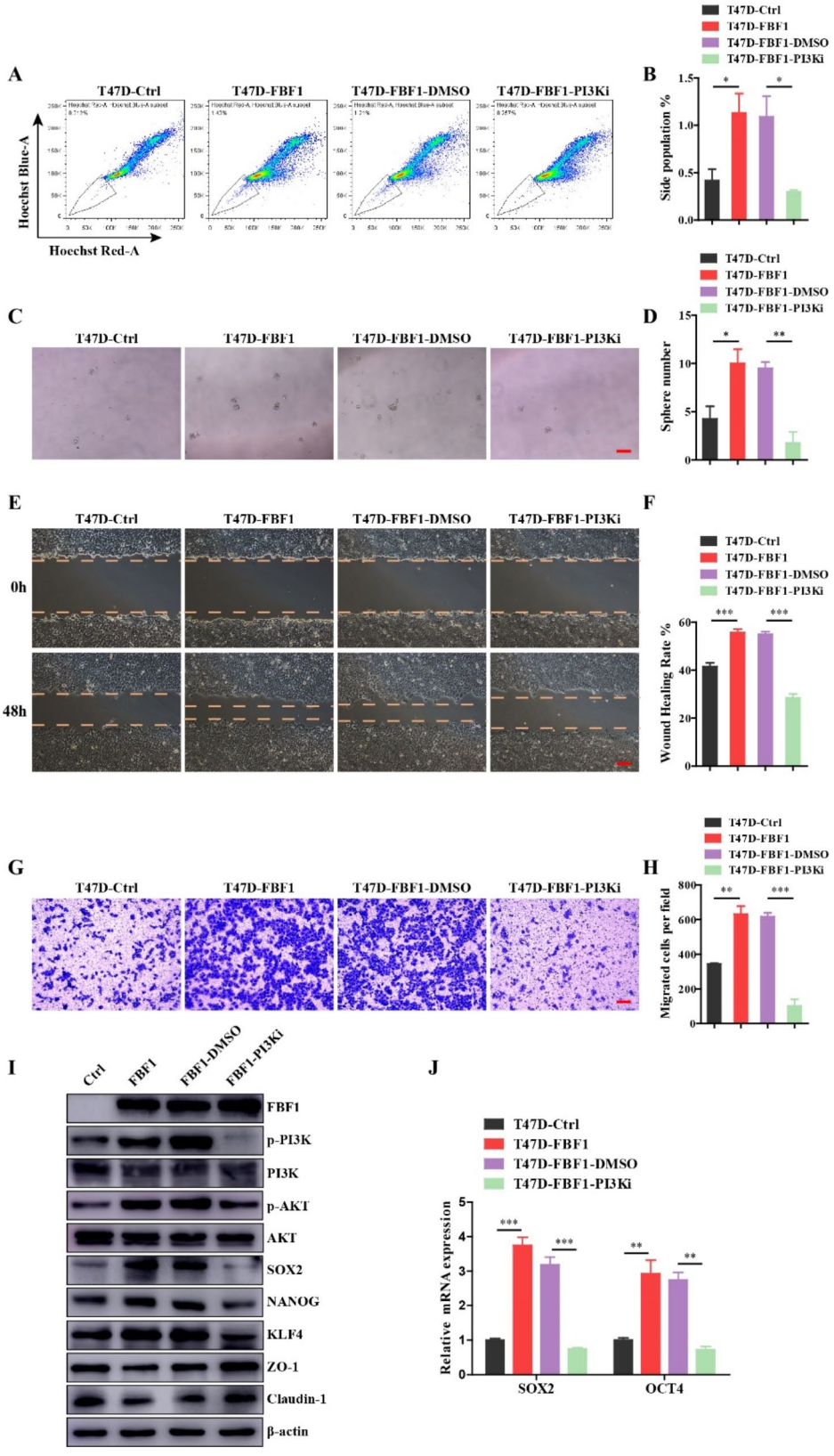
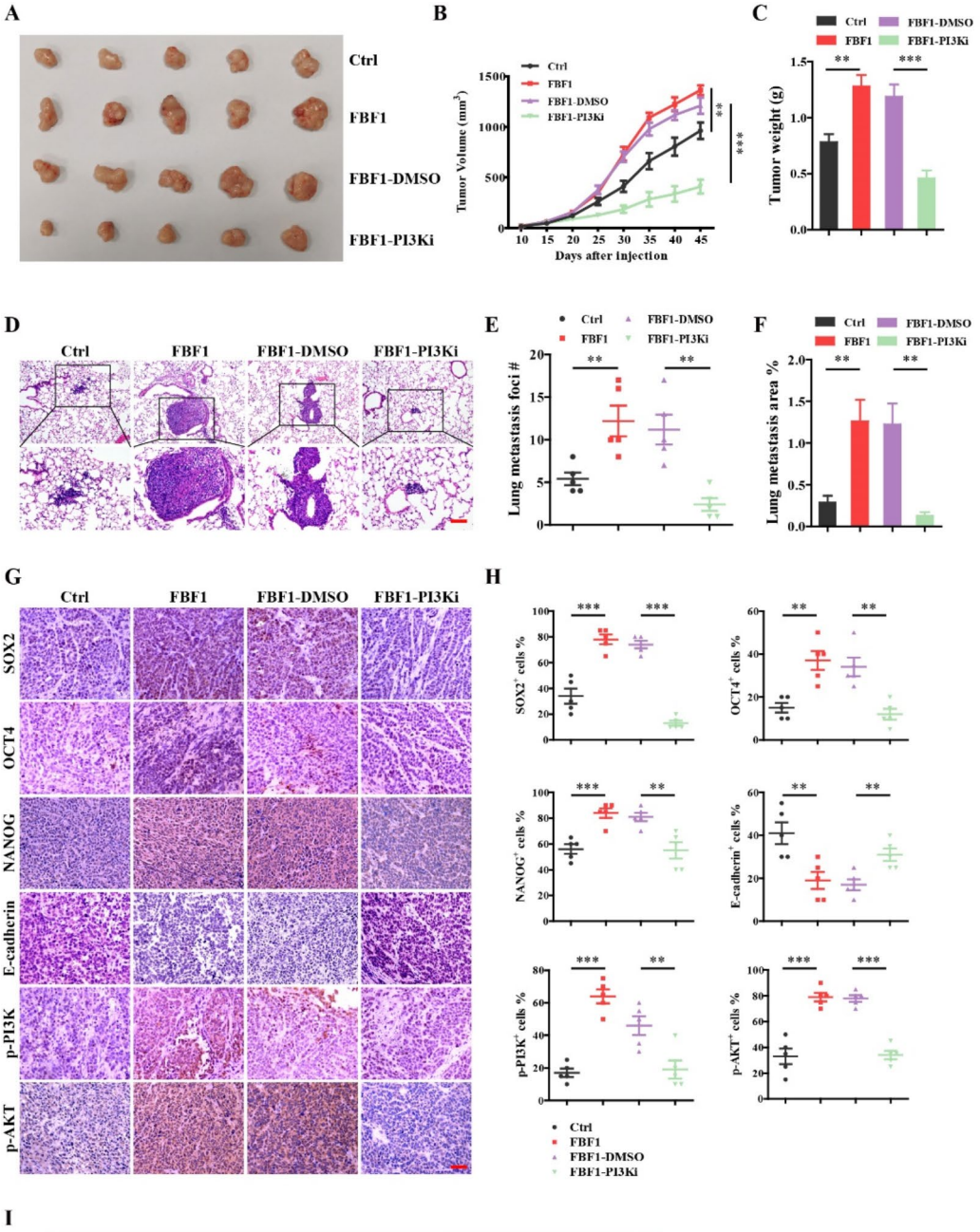


Fig. 5 Inhibitors targeted FBF1 mediated signaling pathway can rescue FBF1-induced stem cell-like properties *in vitro*. **(A, B)** Flow cytometry analysis of side population cells. **(C, D)** Sphere formation ability is identified. Scale bars: 100 μm. **(E-H)** Cell migration abilities are detected. Scale bars: 100 μm. **(I)** Western blot showed the expression of FBF1, p-PI3K, p-AKT, ZO-1, Claudin-1 and stemness markers. **(J)** qRT-PCR analysis of *SOX2* and *OCT4* expression



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Cell population	1×10^6	5×10^5	1×10^5	Frequency of tumorigenic cells (95% CI)	*p* value
Ctrl	4/6	3/6	0/6	1/918019 (1/1958942-1/430212)	* 0.0118
FBF1	6/6	5/6	2/6	1/248664 (1/509113-1/121454)	
FBF1-DMSO	6/6	5/6	2/6	1/248664 (1/509113-1/121454)	*** 0.000304
FBF1-PI3Ki	3/6	1/6	0/6	1/1927575 (1/5110131-1/727094)	

Fig. 6 FBF1 enhances the CSCs-like features of breast cancer *in vivo*. **(A-C)** Ectopic FBF1 promotes tumor growth, which is rescued by PI3K inhibitor. **(D-F)** H&E staining is used for analysis of lung metastasis and the metastases were quantified ($n=5$). Scale bars: 100 μm. **(G, H)** IHC staining of p-PI3K, p-AKT, E-cadherin, and stemness markers in tumor tissue sections and quantification ($n=5$). Scale bars: 50 μm. **(I)** The tumor initiation ability of T47D cells were analyzed with limited dilution assay ($n=6$). CI: confidence interval

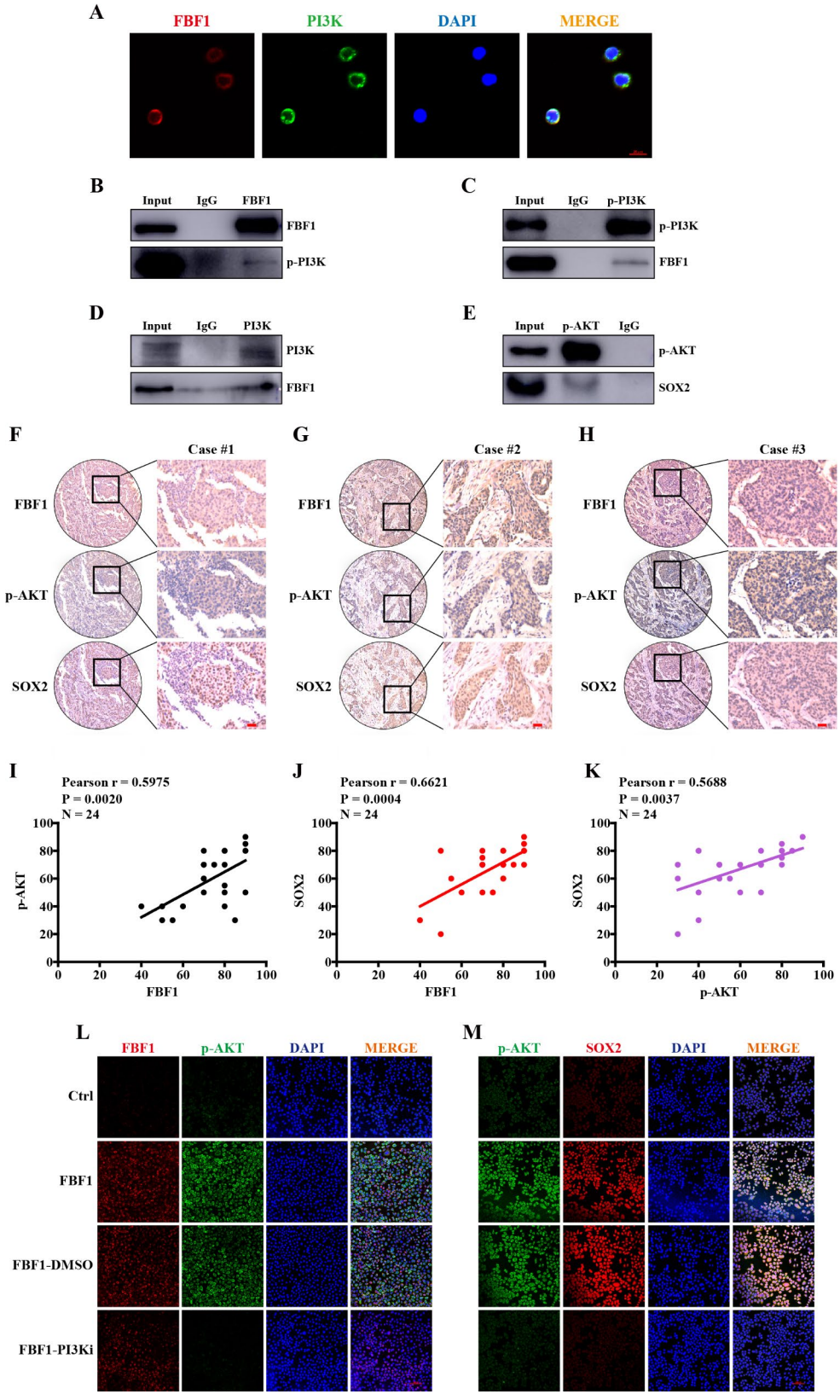

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Fig. 7 FBF1 facilitates breast cancer stemness properties by interacting with PI3K kinase followed by activating PI3K-AKT-SOX2 axis. **(A)** Co-localization of FBF1 and p-PI3K were examined in T47D cells by immunofluorescence staining. Scale bars: 20 μm. **(B-D)** IP-immunoblot was performed to confirm FBF1 and p-PI3K co-interaction. **(E)** IP-immunoblot was conducted to confirm p-AKT and SOX2 co-interaction. **(F-K)** Representative IHC images of FBF1, p-AKT, SOX2 and correlation graphs in human breast cancer tissue array. Scale bar: 50 μm. **(L, M)** Representative immunofluorescence staining images of FBF1, p-AKT and SOX2. Scale bar: 100 μm

characteristics. Taken together, these results indicated that FBF1 maintains stem cell-like properties in breast cancer via PI3K-AKT-SOX2 axis.

Proposed model of FBF1 in CSCs-like identities maintenance

Based on the totality of our findings, we propose the following model: FBF1 binds PI3K which then activates PI3K-AKT phosphorylation cascades. Then the activated p-AKT interacts with stemness marker SOX2, elevates SOX2 and OCT4 activity, and finally forms PI3K/AKT/SOX2 axis, which mediates breast cancer CSCs-like identities maintenance. Inhibitors targeted FBF1 mediated pathway suppressed breast cancer stemness (Fig. 8).

Discussion

Considerable evidence suggests that CSCs play pivotal roles in tumor initiation, malignancy, recurrence and metastasis [30–32]. Therefore, finding new crucial genes or signaling pathways that regulate the stem cell-like properties will form the foundation of developing novel therapeutic strategies to target CSCs and improve cancer treatment efficacy.

In the present study, we investigated the function of FBF1 in CSCs and identified the underlying mechanism. We observed that FBF1 was highly expressed in breast cancer and its expression correlated significantly with TNM stage and histological grade, suggesting that FBF1 levels are associated with breast cancer malignancy. Meanwhile, we found that FBF1 enhanced stem cell-like characteristics, including higher SP proportion, improved sphere formation ability, boosted cell migration features *in vitro*. FBF1 also promoted tumor-initiating capacity, tumor growth and lung metastasis *in vivo*. Furthermore, we explored the underlying mechanism and found that FBF1 maintains CSCs performance in breast cancer through PI3K/AKT/SOX2 axis.

CSCs are also considered as the driving force of tumorigenesis and the seeds of metastases [33]. These cells can not only evade the recognition of the immune system, but also have the ability to resist the conventional chemotherapy and radiotherapy, and finally formed tumor metastasis. Therefore, it can be seen that CSCs are closely correlate with tumor metastasis. It is reported that CSCs can promote tumor metastasis through epithelial-to-mesenchymal transition (EMT) [34, 35]. EMT can not only directly enhance the invasion and migration of various types of cancer, but also enable tumor cells to acquire CSCs characteristics, and then promoting tumor metastasis [36]. CD133 as a marker of hepatocellular carcinoma stem cells. Owing to the expression of E-cadherin was absent, while N-cadherin was upregulated in $CD133^+$ cells, $CD133^+$ cells are more aggressive than $CD133^-$ cells in hepatocellular carcinoma cells [37]. Moreover, recent evidence has revealed that certain types of cancer cell, such as lung, breast and ovarian cancers, can acquire the ability to initiate tumors after inducing EMT program. TWIST1 could boost the expression of BMI1, which is crucial for enhancing EMT and tumor initiation [38]. In our study, we demonstrated that FBF1 boosted breast cancer stemness and metastasis via EMT process.

The pluripotency-inducing transcription factor SOX2 is critical for reprogramming and homeostasis of the stem cells, and is also closely correlated with neoplastic progression [39]. Meanwhile, SOX2 plays an important role in mediating CSCs self-renewal and stemness [40]. The highly expressed of SOX2 correlates with colorectal cancer stem cell features [41]. SOX2 was elevated in osteosarcoma stem cells isolated through sphere formation assay, whereas silencing SOX2 suppressed cancer stemness [42]. In our study, we verified that FBF1 could upregulate SOX2 expression and multiple stemness markers level, such as OCT4, KLF4, NANOG, and thus facilitated CSCs properties.

To clarify the mechanism by which FBF1 facilitates CSCs characteristics, the role of PI3K signaling was specifically investigated by means of RNA-seq analysis. Our studies clarify that FBF1 promotes the activation of PI3K/AKT to mediate CSCs performances. As is well known that PI3K/AKT pathway can enhance cancer stem cell-like features as follows: (1) increase leukemia stem cells (LSCs) subpopulation via enhancing ABCG2 expression [43]; (2) enhance the stemness through AKT/β-catenin pathways [44]; as well as (3) maintain CSCs identity via PI3K/AKT/OCT4 signaling [45]. Furthermore, activating AKT signal transduction pathway in embryonal carcinoma cells can also prevent OCT4 degradation through induction of OCT4 phosphorylation, and further enhance NANOG expression [46].

In addition, recent evidence indicates that multiple inhibitors targeting PI3K/AKT axis have an attenuating effect on tumor progression [47]. In Figs. 5 and 6, we found that when PI3K/AKT pathway is blocked by its inhibitor LY294002, the facilitation of breast cancer stem cell properties by FBF1 induced will be attenuated. Taken

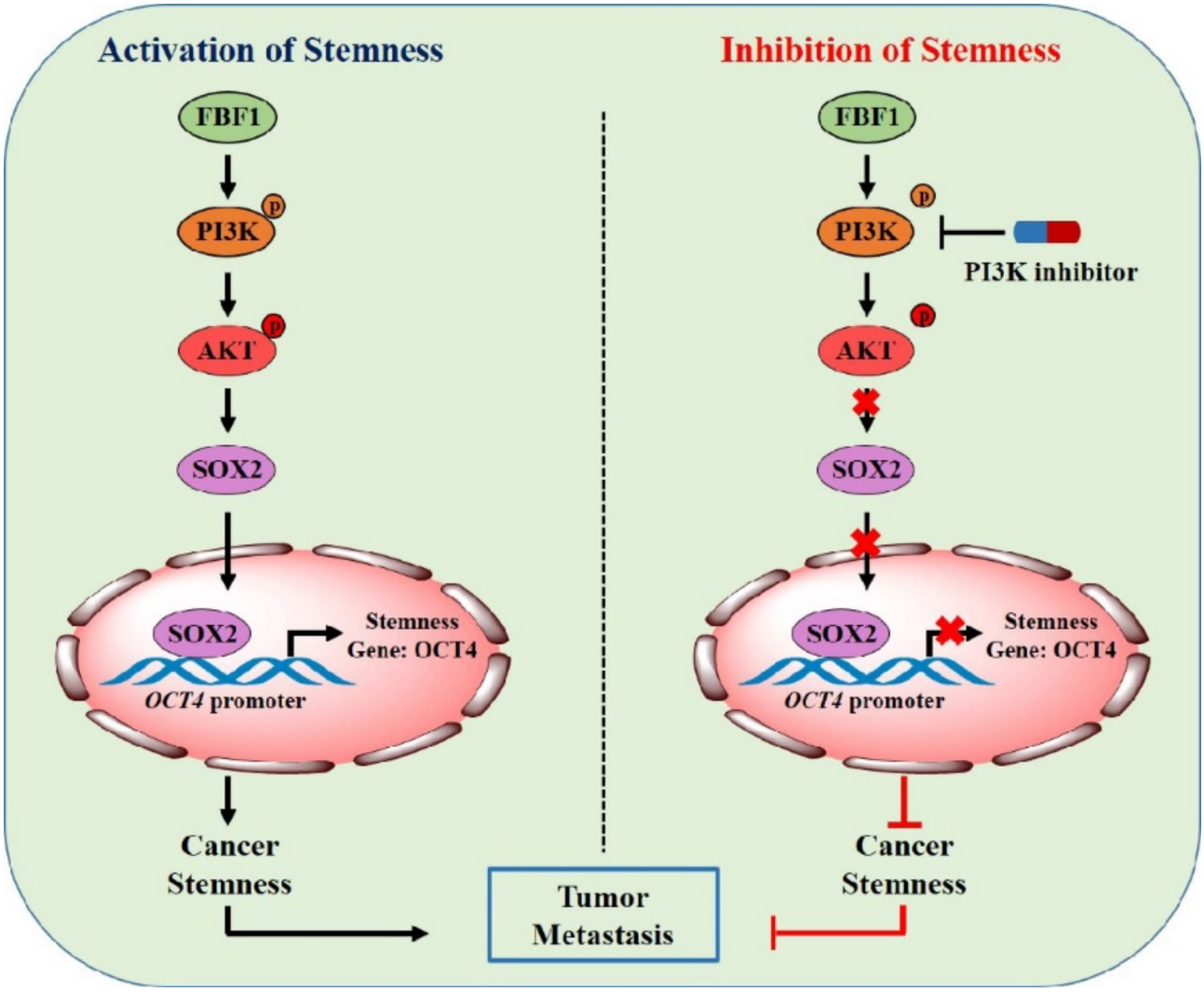

Fig. 8 Proposed model of FBF1 in breast cancer CSCs-like identity maintenance

together, we confirmed that FBF1 boosted CSCs characteristics through PI3K/AKT signaling.

However, how does FBF1 regulate PI3K and further activate the PI3K/AKT phosphorylation cascade, as well as what is the mechanism by which PI3K/AKT signaling mediate CSCs features. In order to elucidate these questions, we conducted immunoprecipitation assay and the result indicated that FBF1 binds PI3K and followed by PI3K phosphorylation, which then activates PI3K/AKT pathway. AKT reportedly plays a pivotal role in regulating SOX2 activity. It directly phosphorylates mouse SOX2, thereby stabilizing SOX2 in the ESCs [48]. Moreover, TROY supports hepatocellular carcinoma stemness through activating AKT/TBX3/SOX2 axis [49]. In our study, we found new mechanism by which AKT regulates SOX2, we conducted immunoprecipitation by means of anti-p-AKT antibody and immunoblotting using anti-SOX2 antibody in T47D cells. We verified that p-AKT could interact with SOX2, elevates SOX2 and OCT4 activity, and thus maintains breast cancer stemness.

Conclusions

In summary, we identified that FBF1 drives stem cell-like properties in breast cancer cells and in a xenograft mouse model. Furthermore, exploration of the underlying mechanism indicated that FBF1 binds PI3K which then activates PI3K-AKT phosphorylation cascades. And the activated p-AKT maintains cancer cell stemness by interacting with SOX2 and enhancing its activity. Moreover, PI3K inhibitors abolished FBF1-mediated signaling pathway and suppressed breast cancer stemness *in vitro* and *in vivo*. These findings collectively demonstrated that FBF1 promotes CSCs-like identities via PI3K/AKT/SOX2 axis. Thus, targeting FBF1 represents an attractive therapeutic strategy to anti-cancer therapy aimed

at suppressing tumor progression by preventing CSCs characteristics.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04194-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Author contributions

HW designed the project and supervised the process of the study. CG, SL, JL, YM and AL performed the experiments, and revised the manuscript. CG, SL, JL, YL and HL analyzed the data. CG and AL drafted the manuscript. YL and HL provided support with experimental techniques. All authors read and approved the final version of the manuscript.

Funding

This project was supported by the National Natural Science Foundation of China (Grant No. 81802967), the Key Scientific and Technological Project of Henan Province (Grant No. 232102311013), the Program for Ph.D. Starting Research Funding from Xinxiang Medical University (Grant No. 505247) and the 111 Project (No. D20036).

Data availability

Data will be made available on request. The RNA sequencing data reported in this paper have been deposited in Sequence Read Archive (Accession number: PRJNA961214).

Declarations

Ethics approval and consent to participate

Mice were used according to federal guidelines, and all animal procedures were approved by the Animal Ethical and Welfare Committee of Xinxiang Medical University (Project title: The mechanism of FBF1 promotes stemness and metastasis of breast cancer cell; approval number: XYLL-20190026; date of approval: January 2nd, 2019).

Consent for publication

All authors confirm their consent for publication.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Received: 7 March 2024 / Accepted: 29 January 2025
Published online: 23 February 2025

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