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Original Paper

FGF18 Enhances Migration and the Epithelial-Mesenchymal Transition in Breast Cancer by Regulating Akt/GSK3β/Β-Catenin Signaling

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Key Words
Fgf18 • Breast cancer • EMT • Akt/GSK3β/β-catenin

Abstract
Background/Aims: Fibroblast growth factors (FGFs) and their high-affinity receptors contribute to autocrine and paracrine growth stimulation in several human malignant tumors, including breast cancer. However, the mechanisms underlying the carcinogenic actions of FGF18 remain unclear. ***Methods:*** The transcription level of FGF18 under the hypoxic condition was detected with quantitative PCR (qPCR). A wound-healing assay was performed to assess the role of *FGF18* in cell migration. A clonogenicity assay was used to determine whether *FGF18* silencing affected cell clonogenicity. Western blotting was performed to investigate Akt/GSK3β/β-catenin pathway protein expression. Binding of β-catenin to the target gene promoter was determined by chromatin immunoprecipitation (ChIP) assays. ***Results:*** FGF18 promoted the epithelial-mesenchymal transition (EMT) and migration in breast cancer cells through activation of the Akt/GSK3β/β-catenin pathway. FGF18 increased Akt-Ser473 and -Thr308 phosphorylation, as well as that of GSK3β-Ser9. FGF18 also enhanced the transcription of proliferation-related genes (CDK2, CCND2, Ki67), metastasis-related genes (TGF-β, MMP-2, MMP-9), and EMT markers (Snail-1, Snail-2, N-cadherin, vimentin, TIMP1). β-catenin bound to the target gene promoter on the ChIP assay. ***Conclusion:*** FGF18 contributes to the migration and EMT of breast cancer cells following activation of the Akt/GSK3β/β-catenin pathway. FGF18 expression may be a potential prognostic therapeutic marker for breast cancer.

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Introduction

Breast cancer, first recognized by the Egyptians more than 3500 years ago [1], is currently the most common form of cancer and the second leading cause of death from cancer among women. An estimated 1 to 1.3 million breast cancer cases are diagnosed annually worldwide. Breast cancers can be divided into molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Moreover, with the development of genetic diagnostic techniques such as cDNA microarrays, a novel classification system for breast cancer has been introduced, with the ER^+ group divided into at least three subgroups [2]. These cancers can be treated with hormone therapy as an effective and significantly less toxic alternative to chemotherapy. Among the subtypes, triple-negative (ER^-, PR^-, and $HER2^-$) breast cancers have a particularly poor prognosis. In addition, the expression of proliferative markers such as Ki-67 is used for further prognostic distinction of molecular subtypes [3].

Over the past few decades, our understanding of breast cancer and its treatment has undergone a metamorphosis, shifting from generally indiscriminate to a more personalized approach based on gene expression analysis and the development of markers that can aid informed diagnostic and treatment decisions [2, 4-12]. Recently, a new panel of markers involving the relative expression of FGF18, BCL2, PRC1, MMP-9, and SERF1A was identified and validated for risk prediction in early breast cancer [8].

FGF18 is a 21.2 kDa glycosylated secretory protein that is highly conserved among humans, mice, and rats. The structure of FGF18 is similar to that of FGF8 and FGF17, which together constitute the FGF8 subfamily [13]. As a mitogenic, chemotactic, and angiogenic factor, FGF18 is essential for the embryonic and postnatal development of multiple tissues, such as bone, cartilage, hair, and vasculature [13-16]. Overexpression of FGF18 has been frequently identified in synovial sarcoma, hepatocellular carcinoma, and colon and ovarian cancers [17-21]. FGF18 is also an informative prognostic and therapeutic biomarker for a subset of ovarian cancers [20, 21].

Despite the apparent importance of FGF18 in the pathogenesis of ovarian and breast cancers, few studies have addressed the mechanistic aspects of FGF18 in tumorigenesis. The purpose of this study was to investigate the role of FGF18 in migration and the epithelial-mesenchymal transition (EMT) and to determine why it preferentially reappears in hormonal cancers.

Materials and Methods

Cell culture

MCF-7, MDA-MB-453, SK-BR-3 and T47D breast cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and cultured in HyClone™ High-Glucose DMEM medium (Thermo, Cat. SH30243.01B) supplemented with 10% fetal bovine serum (Gibco, Cat. 10099-133) and 1% penicillin/streptomycin (Thermo, Cat. 15140122).

TCGA and OncoLnc analyses

The FGF18 mRNA expression and the FGF18 gene copy number of clinical breast cancer tissues were examined with Oncomine web (https://www.oncomine.org/resource/main.html), which the data based on The Cancer Genome Atlas (TCGA). In the Oncomine web, we set the filter as following: Gene: FGF18; Analysis type: Cancer vs. Normal Analysis; Cancer type: Breast cancer. The results were displayed as following: Group by: Cancer and normal type; Fingure type: Box-plot. The detail instructions for use, please refer to the Oncomine Support Community (https://support.oncomine.com/?authToken=1fc8a8a156c3ae215016c1ba3d804575). The survival rates of the patients with FGF18 expression based on website of OncoLnc (http://www.oncolnc.org/). In the OncoLnc web, we set the filter as following: Target gene: FGF18; Cancer type: Breast cancer. The detail instructions for use, please refer to the Guide to OncoLnc: A new TCGA data portal (https://www.lncrnablog.com/guide-to-using-oncolnc-a-new-tcga-data-portal/).

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Wound-healing assays

Wound healing assay was carried out to determine migration of breast cancer cells. In brief, MDA-MB-453 and SK-BR-3 cells were grown to 80% confluences in 6-wells plates. Wounds were created by scraping the cells with a sterile 200 μl pipette tip, then cells were washed with warmed PBS and cultured in complete medium with the different concentration of recombinant FGF-18 (Thermo, Cat. PHG0234) at 37°C. Images were taken at 48 and 96 h after scratching with an Eclipse TS100 microscope (Nikon).The migrating distance was quantified by determining the width between the edges of the injured monolayer of cells.

Small interfering RNA (siRNA) assay

siRNA targeting human FGF18 (NCBI Reference Sequence: NM_003862.2) and scrambled small interfering RNA (si-scrambled) were obtained from Sangon Biotech, Shanghai, China. A 19-nucleotide (nt), FGF18-specific, double-stranded siRNA (siFGF18) sequence: 5'- CCTGCACTTGCCTGTGTTT-3'; scramble siRNA (siSCR) as the control, 5'- CCTTCACGTGTCGTGCTTT-3'. siRNAs were transfected into MDA-MB453 cells at 30 nM with the Lipofectamine RNAiMAX from Thermo Fisher Inc (Cat. 13778030) according to the manufacturer's instructions. The cells were incubated for at least 24 h before the analyses were carried out.

Clonogenicity assay

Forty-eight hours after transfection (described as siRNA), cells were plated at a low density in a DMEM medium containing 10% fetal bovine serum (FBS). After 10 days in culture, the cancer cells formed colonies, which were stained with methyl violet. Colony numbers were quantified by counting colonies that contained more than 25 cells observed under a light microscope. The numbers of clones were determined in at least three dishes per group. The statistical analyses for p values were obtained using SPSS18.0 software (SPSS, Inc., Chicago, IL, USA).

RNA isolation and quantitative reverse-transcriptase PCR (qPCR)

Total RNAs were isolated using the RNeasy Mini Kit (QIAGEN, Cat. 74104). 1 μg total RNAs were reversely transcribed into cDNAs using a Super Script® First-Strand Synthesis System for RT-PCR Kit (Thermo, Cat. 11904018). qPCR was performed with SYBR® Select Master Mix (Thermo, Cat. 4472918) using StepOne Plus real-time PCR system (Applied Biosystems). Results were normalized to those obtained with β-Actin mRNA and presented as fold vs. control. Each experiment was performed in triplicate. Human Primers:

β-Actin, F:5'-GCACAGAGCCTCGCCTT-3', R:5'-GTTGTCGACGACGAGCG-3';
FGF8, F:5'-ATCGTGGAGACGGACACCTT-3', R:5'-GCGATCAGCTTCCCCTTCTT-3';
FGF17, F:5' -GCTGCTGATTCTCTGCTGTCAA-3', R:5'-GCTCAGCTGGTCGGTCATG-3';
FGF18, F:5'-TGCTTCCAGGTACAGGTGCT-3', R:5'-GCTGCTTACGGCTCACATCG-3';
CDK2, F:5'-ACACGCTGCTGGATGTCATTC-3', R:5'-CCTGGAGCAGCTGGAACAG-3';
CCND2, F:5'-TGGGGAAGTTGAAGTGGAAC-3', R:5'-ATCATCGACGGTGGGTACAT-3';
Ki67, F:5'-TTCGCAAGCGCATAACCCA-3', R:5'-AACCGTGTCACAGTGCCAAA-3';
TGF-β, F:5'-GTACCTGAACCCGTGTTGCT-3', R:5'-CAACTCCGGTGACATCAAAA-3';
MMP-2, F:5'-TGACTTTCTTGGATCGGGTCG-3', R:5'-AAGCACCACATCAGATGACTG-3';
MMP-9, F:5'-CCCGGACCAAGGATACAGTTT-3', R:5'-GTGCCGGATGCCATTCAC-3';
Snail-1, F:5'-CCCAATCGGAAGCCTAACTA-3', R:5'- GGACAGAGTCCCAGATGAGC-3';
Snail-2, F:5'-CCTTCCTGGTCAAGAAGCAT-3', R:5'- CACAGTGATGGGGCTGTATG-3';
E-Cadherin, F:5'-TCATGAGTGTCCCCCGGTAT-3', R:5'- CGGAACCGCTTCCTTCATAG-3';
N-Cadherin, F:5'-CTGGATCGCGAGCAGATAGC-3', R:5'- CCCATTCCAAACCTGGTGTAA-3';
Vimentin, F:5'-GGCTCAGATTCAGGAACAGC-3', R:5'- AGCCTCAGAGAGGTCAGCAA-3';
TIMP-1, F:5'-GGGACACCAGAAGTCAACCA-3', R:5'-GTTGTGGGACCTGTGGAAGT-3'.

Western blotting assay

2-5 $\times 10^6$ cells were lysed with 1 ml of lysis buffer (50 mM Tris-HCl, pH7.2, 1% NP-40, 150 mM NaCl, 50 mM β-Glycerophosphate disodium salt hydrate, 5 mM DL-Dithiothreitol, 0.1 mM PMSF) plus protease inhibitors (Sigma-Aldrich) for 30 min on ice. After centrifugation at 10000 rpm for 15 min at 4°C, the lysates were boiled with 5 × sample buffer, then separated by SDS-PAGE and transferred to PVDF membrane.

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Immunoblotting was performed as described [22] and visualized by chemiluminescence (Bio-Rad, Series No. ChemiDoc XRS+). Antibodies used for immunoblotting were as follows: FGF18 (Abcam, Cat. Ab86571), Akt (Santa Cruz, Cat. sc-8312), p-Akt Ser473 (Santa Cruz, Cat. sc-7985-R), p-Akt Thr308 (Santa Cruz, Cat. sc-16646-R), p-GSK3β Ser9 (Cell Signaling, Cat. #9323), p-GSK3β Tyr216 (Santa Cruz, Cat. sc-135653), GSK3β (Santa Cruz, Cat. sc-9166), β-catenin (Cell Signaling, Cat. #8480), Actin (Santa Cruz, Cat. sc-1616-R) and anti–rabbit IgG antibodies conjugated to horseradish peroxidase (Abcam, Cat. ab6721).

Chromatin immunoprecipitation(ChIP) and quantitative ChIP (qChIP)

qChIP assay was performed as reported previously [22, 23]. Briefly, 2×10^7 MDA-MB-453 cells were collected by centrifugation at 2000 g for 5 min at room temperature and cross-linked in PBS containing 1% formaldehyde for 10 min on ice. The reaction was stopped by adding glycine to a final concentration of 0.125 M. ChIP assay was performed by incubation of the chromatin with 20 μL rabbit mAb β-catenin antibody (Cell Signaling, Cat. #8480) or normal rabbit IgG (Cell Signaling, Cat. #2729). Enrichment of the ChIP sample over input was evaluated by qPCR with specific primers in the promoter region of target genes. Three technical replicates were performed. Fold enrichment was calculated by the formula: $2^{-CTsample/2-Ctinput}$. qChIP primers for target gene promoter and location relative to TSS (Transcript Start Site, TSS):
CDK2 (-541 to -463bp), F: 5'-GGTCACTCTCATCTTGAT-3', R: 5'-AACTGTTGTGTCTGGTAT-3'.
CCND2 (-396 to -518bp), F: 5'- GTATGCGAGGTTGCTATT, R:5'-TTAAGATCCAGGAATGTAGG-3'.
Ki67 (-306 to -381bp), F: 5'-AATCTTCTGGCAATGAGTAATGT-3', R: 5'-ATAACCCGTCCTGCTATCC-3'.
TGF-β (-508 to -618bp), F: 5'-TTCCCTATCTGTAAATTGG-3', R: 5'-GTAAGAATTGCTCTCCTT-3'.
MMP-2 (-254 to -356bp), F: 5'-CAAGTTAAGGCTTACACATT-3', R: 5'-GGATGAACAGAGATGGAA-3'.
MMP-9 (-931 to -1006bp), F: 5'-TGTATCCTTGACCTTCTT-3', R: 5'-CATTCCTGTAATCTTAGCA-3'.
N-Cadherin (-793 to -872bp), F: 5'-CACCATTCTTCATCTCCATTAG-3', R: 5'-ATAACTTGGGCAGGAACTT-3'.
Snail-1 (-753 to -891bp), F: 5'-CCTTGATAATTCTTCACT-3', R: 5'-GACGAAGTAAACAGATAA-3'.
Snail-2 (-332 to -421bp), F: 5'-GCAATCTTCCAGTTCTTCC-3', R: 5'-TACTCATGTCACCGTGTTAG-3'.
Vimentin (-861 to -966bp), F: 5'-GTCCTGTAATTCCACCTT-3', R: 5'-GAGCGTATTCTGACTTCT-3'.
TIMP1 (-140 to -277bp), F: 5'- ATAGCAATTAGGAATCAGTATAG-3', R: 5'- TTCTCAACATTGGCATTAG-3'.

Statistical Analysis

All data are expressed as mean ± standard deviation at least three independent experiments. Two-tailed *p*-value less than 0.05 was viewed as statistically significant (displayed as *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$). All analyses were performed using SPSS 19.0 software (Statistical Package for Social Studies, Chicago, IL, USA).

Results

FGF18 expression and its relationship with breast cancer patient survival

To clarify the relationship between FGF18 expression and breast cancer incidence, we examined FGF18 expression and gene copy number via the Oncomine website (https://www.oncomine.org/resource/main.html) [24], which is based on The Cancer Genome Atlas database (https://cancergenome.nih.gov/). FGF18 mRNA levels (n=582) and gene copy number (n=229) were significantly increased in breast cancer samples compared with normal breast tissues (Fig. 1A-B). Furthermore, analysis of FGF18 protein content using the Human Protein Atlas database (http://www.proteinatlas.org/) revealed that FGF18 tended to be highly expressed in breast cancer samples compared with normal tissue (Fig. 1C).

Next, we analyzed the survival rates of patients with high (n=150) and low (n=150) levels of FGF18 expression based on the OncoLnc website (http://www.oncolnc.org/) [25]. As shown in Fig. 1D, a higher expression of FGF18 was associated with worse breast cancer patient survival. Altogether, the high expression of FGF18 appears to be a biomarker of poor prognosis, which indicates that FGF18 may play a significant role in the development of breast cancer.

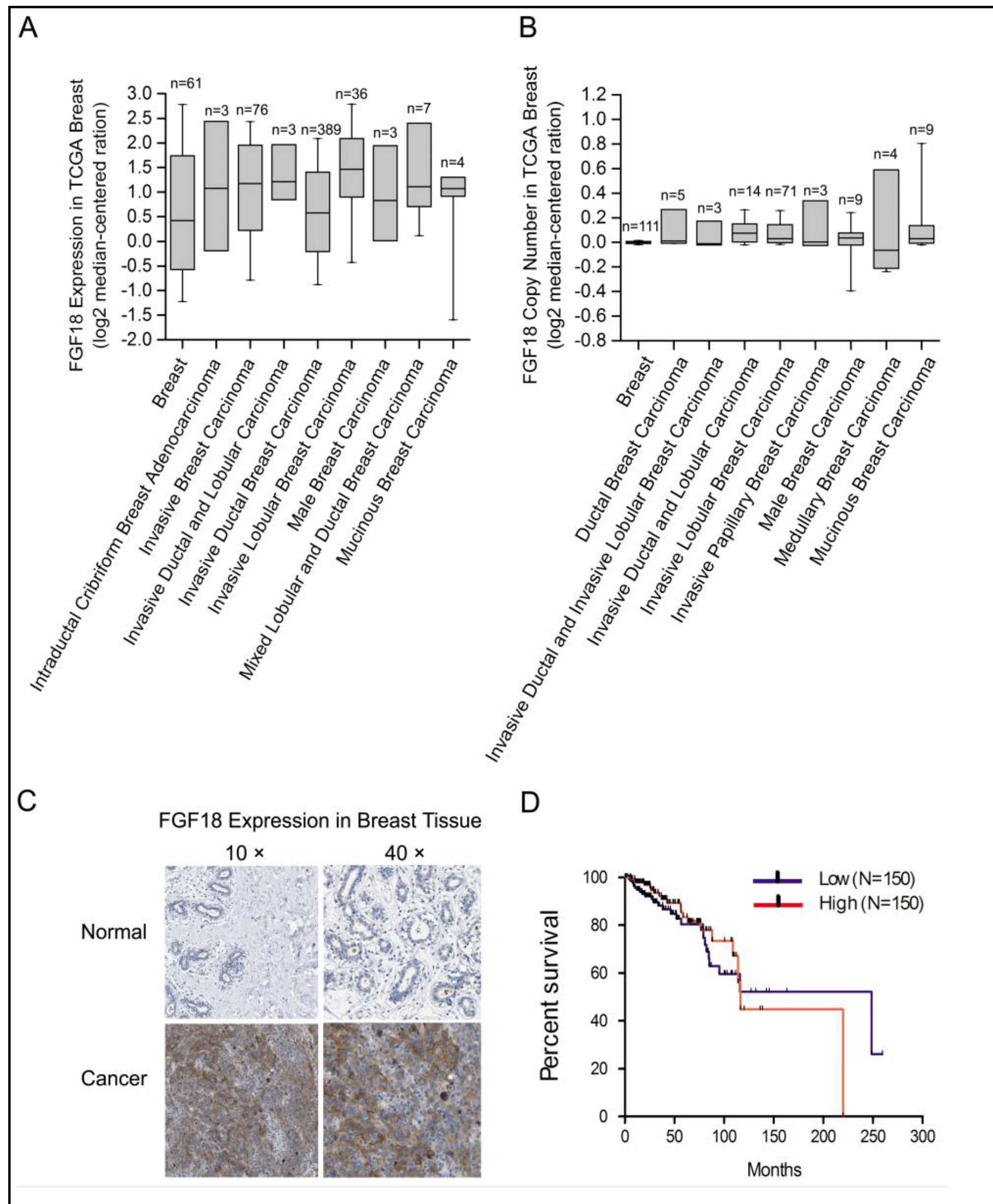

Fig. 1. FGF18 expression in breast cancer tissues and its relationship with the survival of patients with breast cancer. (A) FGF18 expression in different breast cancers (n=582) based on TCGA breast cancer database. Normal breast tissues (n=61), intraductal cribriform breast adenocarcinoma (n=3), invasive breast carcinoma (n=76), invasive ductal and lobular carcinoma (n=3), invasive ductal breast carcinoma (n=389), invasive lobular breast carcinoma (n=36), male breast carcinoma (n=3), mixed lobular and ductal breast carcinoma (n=7), mucinous breast carcinoma (n=4). (B) FGF18 gene copy number in different breast cancers (n=229) based on TCGA breast cancer database. Normal breast tissues (n=111), ductal breast carcinoma (n=5), invasive ductal and invasive lobular breast carcinoma (n=3), invasive ductal and lobular carcinoma (n=14), invasive lobular breast carcinoma (n=71), invasive papillary breast carcinoma (n=3), male breast carcinoma (n=9), medullary breast carcinoma (n=4), mucinous breast carcinoma (n=9). (C) FGF18 expression in breast cancer by immunohistochemistry (IHC) staining. (D) The overall survival rates of breast cancer patients with high (n=150) and low level (n=150) of FGF18.

Upregulation of FGF8 family genes in breast cancer cell lines under hypoxic conditions

Rapidly growing tumors such as breast cancer often suffer from an inadequate blood supply. Hypoxia is one of the inducers of breast cancer development that causes breast cell damage via increased reactive oxygen species (ROS) production [26]. Therefore, we wondered whether a lack of oxygen leads to the upregulation of the FGF8 subfamily in breast carcinoma. When the breast cancer cell lines MCF-7, MDA-MB-453, SK-BR-3, and T47D were cultured with the hypoxia-mimetic drug deferoxamine mesylate for 48 h, the transcript levels of FGF8, FGF17, and FGF18 were increased up to 20-fold within 48 h above the already considerable expression levels in control cultures (Fig. 2). These results demonstrated that hypoxia could induce the expression of the FGF8 subfamily.

Effects of FGF18 on cell migration in MDA-MB-453 and SK-BR-3 cells

To investigate whether FGF18 expression increases cancer cell motility, a wound-healing assay was performed in MDA-MB-453 and SK-BR-3 cells treated with 10 ng/ml and 20 ng/ml FGF18. FGF18-treated cells were able to migrate more rapidly, with the migration distance dependent on the FGF18 concentration (Fig. 3A–D). We also observed that MDA-MB-453 cells were more sensitive to FGF18 stimulation than SK-BR-3 cells, and therefore chose the MDA-MB-453 cell line for further analyses.

FGF18 silencing reduces clone formation in MDA-MB-453 cells

To assess the role of FGF18 in the malignant behavior of MDA-MB-453 cells, the expression of FGF18 was knocked down by small interfering RNA (siRNA). As shown in Fig. 3E, FGF18 siRNA significantly decreased both the protein and mRNA expression levels of FGF18 compared with scrambled siRNA. More importantly, knockdown of FGF18 also markedly decreased the clonal formation ability of MDA-MB-453 cells (Fig. 3F).

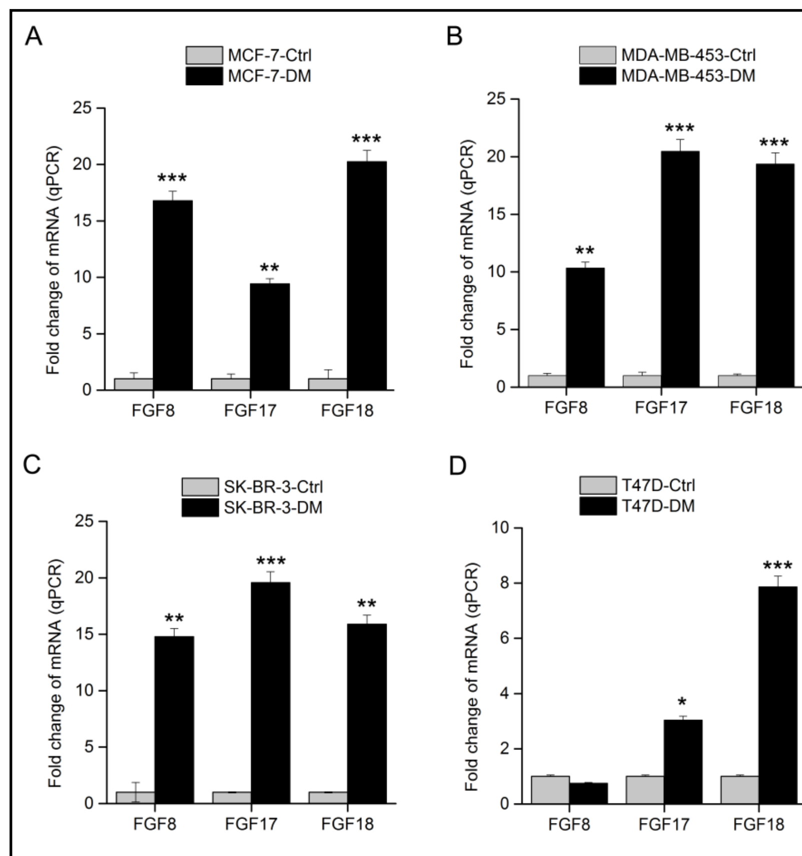

Fig. 2. The expression profile of fibroblast growth factor 8 subfamily (FGF8, FGF17, FGF18) under the induced hypoxic condition. Breast cancer cells were treated with 100μM deferoxamine mesylate for 48 h. Cells were harvested for total RNA extraction. Expressions of FGF8, FGF17, and FGF18 mRNA were determined by qPCR. (A) MCF-7 cells. (B) MDA-MB-453 cells. (C) SK-BR-3 cells. (D) T47D cells. DM: deferoxamine mesylate. The β-Actin RNA was used as an internal control for all qPCR analysis. The quantified data from three independent experiments are shown as means and standard errors. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

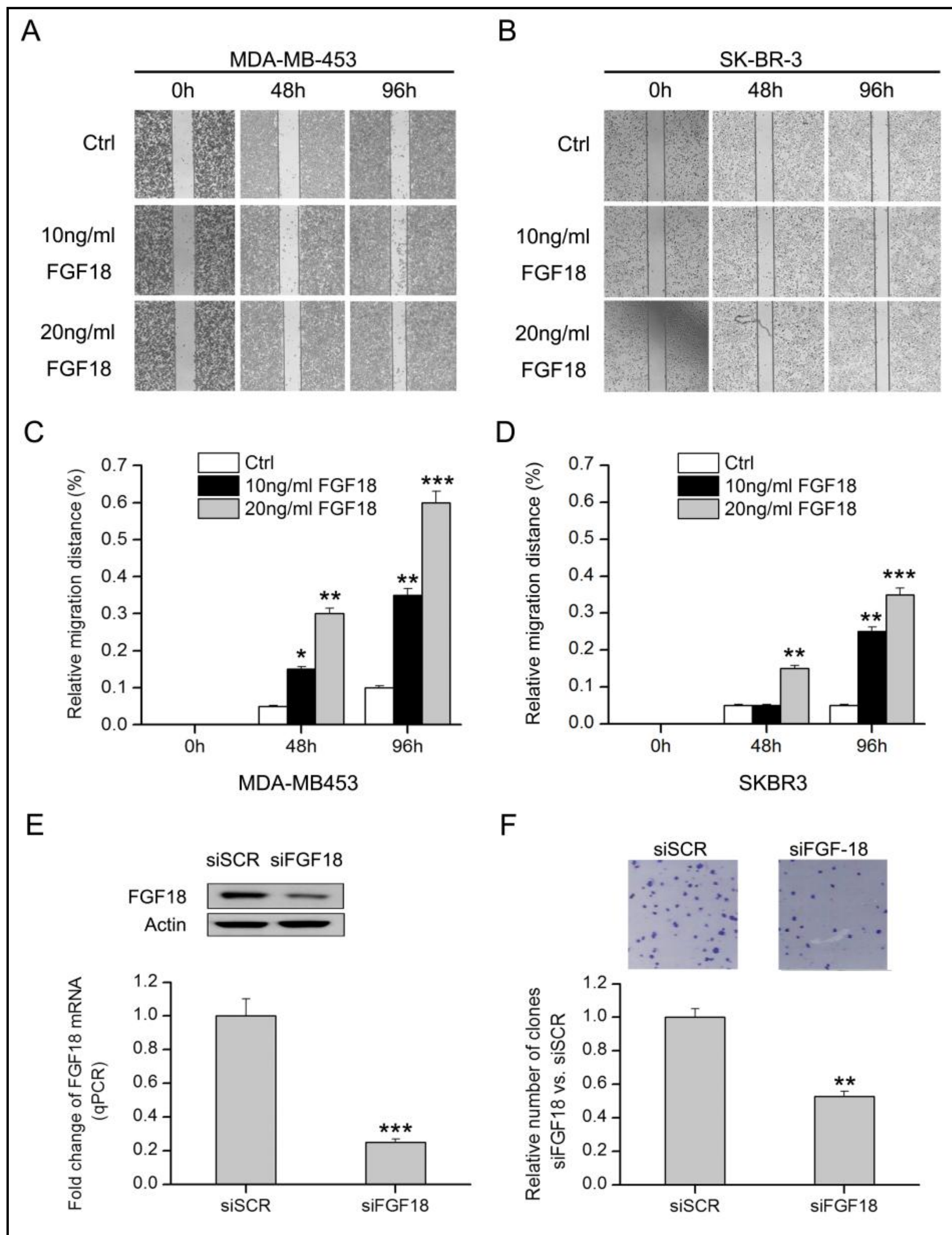

Fig. 3. Effect of FGF18 on migration of MDA-MB-453 and SK-BR-3 cells. Breast cancer cells were treated with 10 ng/ml or 20 ng/ml FGF18 for 0, 48 and 96 h. (A) MDA-MB-453 cells; (B) SK-BR-3 cells, magnifcation, 40×. (C-D) The relative distance plot according to the data from A and B. (E) The impact of FGF18-siRNA on the expression level of the gene was determined by Western blotting and qRT-PCR. (F) Representative clones of MDA-MB-453 cells formed after the treatment with siSCR or siFGF18. Results are shown as means ± SD from three experiments in triplicates. *p<0.05, **p<0.01, ***p<0.001.

Effects of FGF18 on the expression of proliferation-, migration-, and EMT-related genes in MDA-MB-453 cells

To explore FGF18-mediated downstream events, we isolated total RNA from MDA-MB-453 cells treated with 10 ng/ml FGF18 and performed qRT-PCR to assess the expression levels of target genes related to proliferation, migration, and the EMT. Proliferation-related genes, including CDK2, CCND2, and Ki67, were significantly upregulated in MDA-MB-453 cells exposed to 10 ng/ml FGF18 (Fig. 4A). The expression levels of TGF-β, MMP-2, and MMP-9, which are involved in tumor migration activities, were notably increased after FGF18 treatment (Fig. 4B). The EMT markers, including Snail-1, Snail-2, N-cadherin, vimentin, and TIMP-1, were also significantly upregulated in response to FGF18 stimulation. In contrast, the expression of E-cadherin was significantly decreased in MDA-MB-453 cells after treatment with FGF18 (Fig. 4C). E-cadherin is one of the epithelial molecular markers that is always downregulated during EMT progression [27, 28].

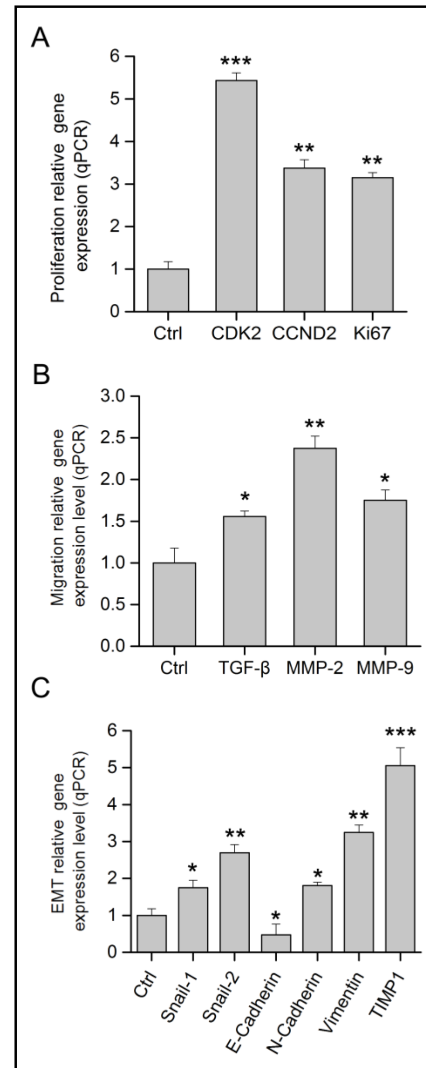

Fig. 4. Gene expression profile following FGF18 treatment. MDA-MB-453 cells were treated with 10 ng/ml FGF18 for 48h, and then CDK2, CCND2, Ki67, TGF-β, MMP-2, MMP-9, Snail-1, Snail-2, N-Cadherin, Vimentin, TIMP1 mRNA expressions were measured by qPCR. (A) Proliferation related gene expression: CDK2, CCND2, Ki67. (B) Migration related gene expression: TGF-β, MMP-2, MMP-9. (C) EMT related gene expression: Snail-1,Snail-2, N-Cadherin, Vimentin, TIMP1. Results are shown as means ± SD from three experiments in triplicates. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Effects of FGF18 on Akt/GSK3β/β-catenin signaling in MDA-MB-453 cells

To further explore whether FGF18 promotes carcinogenesis in breast cancer by activating the Akt pathway, we performed western blotting. As shown in Fig. 5, the expression levels of phospho-Akt-Ser473 (p-Akt-Ser473) and phospho-Akt Thr308 (p-Akt-Thr308) were increased after FGF18 treatment in a dose-dependent manner. Both phosphorylation sites are indicative of Akt activation. We next tested GSK3β expression, which is a main downstream target of Akt activation. FGF18 treatment was followed by phosphorylation of Ser9 (p-GSK3β-Ser9), but not Tyr216 (p-GSK3β-Tyr216). The total β-catenin level was also found to be upregulated in the FGF18-treated cells, suggesting a downstream link to the changes in EMT-related gene expression. Overall, our findings indicate that FGF18 regulates the genes related to breast carcinogenesis via Akt/GSK3β-mediated β-catenin signaling (Fig. 5).

To further ascertain how β-catenin regulates proliferation-, migration-, and EMT-related genes, we performed a chromatin immunoprecipitation assay (ChIP) and quantitative ChIP with β-catenin antibody in MDA-MB-453 cells. As shown in Fig. 6, ChIP analysis revealed that β-catenin bound to the promoters of CDK2, CCND2, and Ki67, as well as several migration (TGF-β, MMP-2, MMP-9)- and EMT (Snail-1, Snail-2, N-cadherin, vimentin, and TIMP1)-related genes.

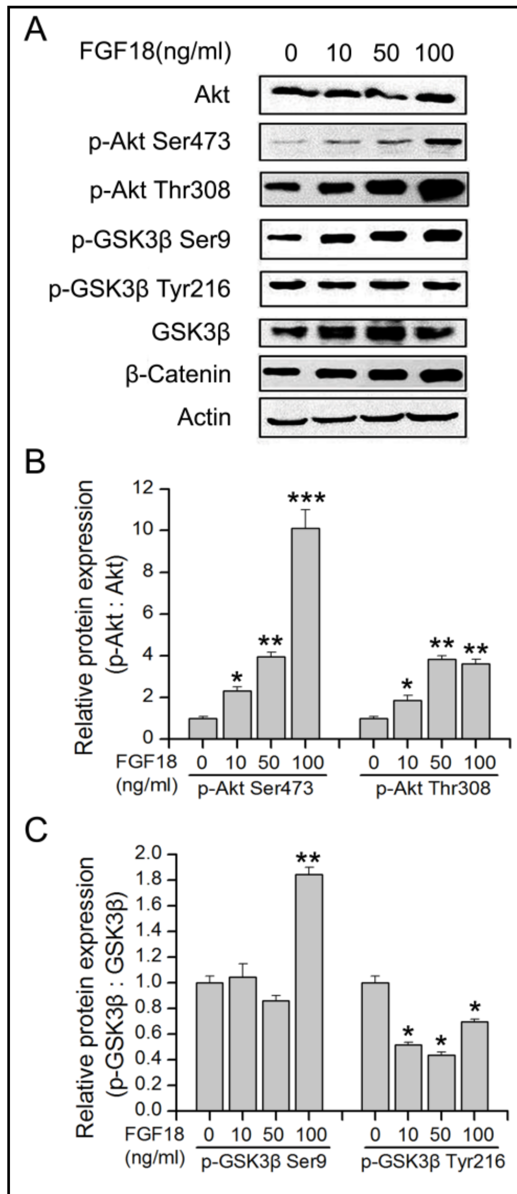

Fig. 5. The effect of FGF18 on Akt/GSK3β/β-Catenin signaling pathway. (A) MDA-MB-453 cells were incubated with 10, 50, 100 ng/ml FGF18 for 48h, and then harvested for total protein extraction. Protein samples were separated on 12.5% sodium dodecyl sulfate gels for immunoblotting. The blots shown in the figure represent averages of three independent experiments (mean ± SD). Image J software (version 1.48, NIH, USA) was used to make the quantitative analysis of phosphorylation of Akt-Ser473 and -Thr308 (B), GSK3β-Ser9 and GSK3β-Tyr216 (C).

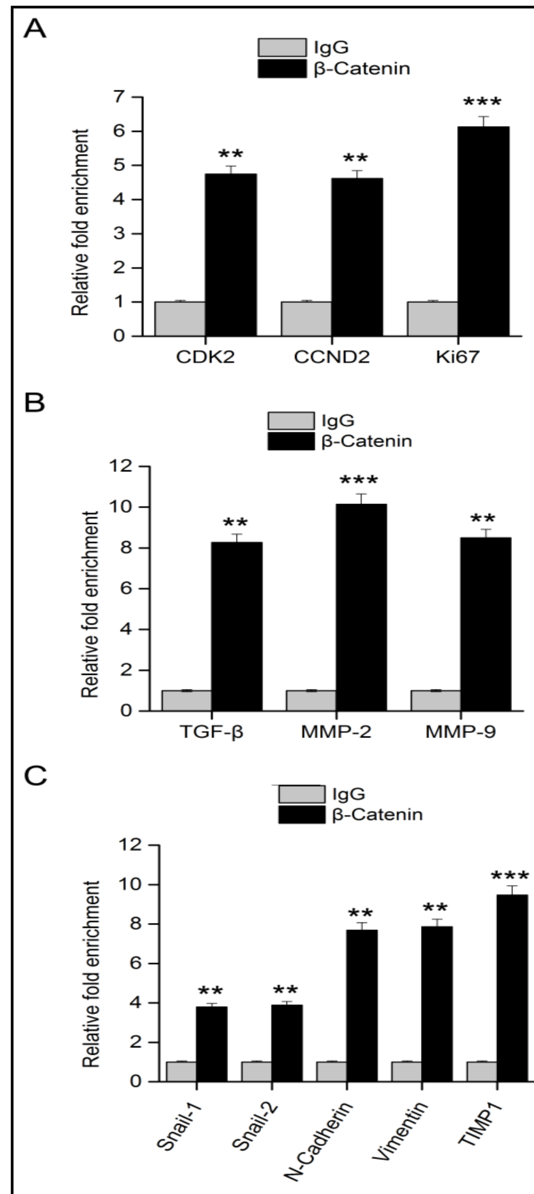

Fig. 6. FGF18 upregulated target genes expression via Akt/GSK3β/β-Catenin signaling pathway. Chromatin immunoprecipitation (ChIP) was performed with β-Catenin antibody in MDA-MB-453 cells. qChIP assay was used to detect the β-Catenin binding to the upstream/promoter regions of target genes. (A) Proliferation related gene promoters: CDK2, CCND2, Ki67. (B) Migration related gene promoters: TGF-β, MMP-2, MMP-9. (C) EMT related gene promoters: Snail-1, Snail-2, N-Cadherin, Vimentin, TIMP1. Results are shown as means ± SD of three experiments in triplicates.

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Discussion

FGF18 is overexpressed in several types of tumors and has also been considered a potential prognostic and therapeutic biomarker [17-21]. FGF18 promotes the development and progression of hepatocellular malignancies, colorectal cancers, and synovial sarcomas by stimulating tumor cell growth or survival [17-19]. As a secreted protein, FGF18 controls migration, invasion, and tumorigenicity in ovarian cancer cells and, by increasing the production of oncogenic cytokines and chemokines, affecting the tumor microenvironment [20, 21]. Because ovarian cancer is strongly associated with the basal-like subtype, a subtype regrouping of triple-negative breast cancer, we believe that FGF18 may also promote breast carcinogenesis [2, 29].

All mammalian FGF ligands activate one or more of four distinct cell-surface receptor tyrosine kinases (FGFR1–FGFR4). FGFR4, also called FGFR3c, is one of the receptors for FGF18 [21] and is implicated in the function of FGF18 as a mesenchymal growth factor [30, 31]. In colorectal cancer, FGFR4 acts as an oncogene and FGF18 is upregulated as a β-catenin target gene [18, 32]. The therapeutic potential of FGFR inhibitors is presently under investigation in clinical trials in breast cancer patients [33].

β-catenin is capable of enhancing FGF18 transcription because the promoter of human FGF18 harbors T cell factor/lymphoid enhancer-binding factor binding sites [34]. In our ChIP studies, we also found that β-catenin binds to the promoters of the proliferation, migration, and EMT genes being studied. The expression of FGF18, in combination with other markers, also predicts the risk of breast cancer [35]. However, although the importance of FGF18 is well established, the mechanisms underlying the involvement of FGF18 in breast cancer development and progression remain largely elusive. Here, we addressed this question using breast cancer cell lines *in vitro*.

In this study, we found pronounced overexpression of the FGF8 subfamily in human breast cancer cell lines under hypoxic conditions. We further found that FGF18 could increase the motility of cells in a time- and dose-dependent manner, as determined by tracking the distance traveled in a wound-healing assay. In agreement with the wound-healing results, genes involved in migration, including TGF-β, MMP-2, and MMP-9, were overexpressed in MDA-MB-453 cells after FGF18 treatment.

The EMT is a process that involves transition to the mesenchymal state and causes cells to gain migratory and invasive properties, key events in cancer metastasis [36-39]. Our results showed that the expression levels of cancer metastasis-related genes, such as Snail-1, Snail-2, N-cadherin, vimentin, and TIMP-1, were markedly increased after FGF18 treatment. In contrast, E-cadherin, an epithelial marker, was downregulated. These results suggest that FGF18 may induce the EMT and contribute to malignization through inhibition of E-cadherin expression and stimulation of EMT-related genes. In addition, we also concluded that the application of exogenous FGF18 could increase the expression of proliferation-related genes, such as CDK2, CCND2, and Ki67.

Akt/GSK3β signal pathway plays an important role in the development of tumors [40]. Our data indicate that FGF18 may promote breast carcinogenesis through the Akt/GSK3β/β-catenin pathway. FGF18 induced the phosphorylation of Akt at Ser473 and Thr308 sites and inactivated GSK3β by elevating Ser9 modification, but not Tyr216. Based on the present data and the evidence available in the literature, we have outlined a hypothetical model to explain our findings (Fig. 7). In this model, FGF18 upregulates Akt phosphorylation at Ser473 and Thr308 sites and then triggers GSK3β phosphorylation at Ser9 (p-GSK3β-Ser9, an inactive form of GSK3β), but not Tyr216 (p-GSK3β-Tyr216, an active form of GSK3β that mainly mediates the degradation of GSK3β). As a result, the inactive GSK3β leads to β-catenin accumulation in the cytoplasm and its translocation to the nucleus, where β-catenin triggers the expression of multiple genes, such as CDK2, CCND2, Ki67, TGF-β, MMP-2, MMP-9, Snail-1, Snail-2, and N-cadherin, responsible for cell survival, migration, and the EMT [41].

The FGF18-mediated signaling in the breast cancer cell lines used in our study further confirms the utility of FGF18 as a reliable biomarker for cancer, as previously reported in

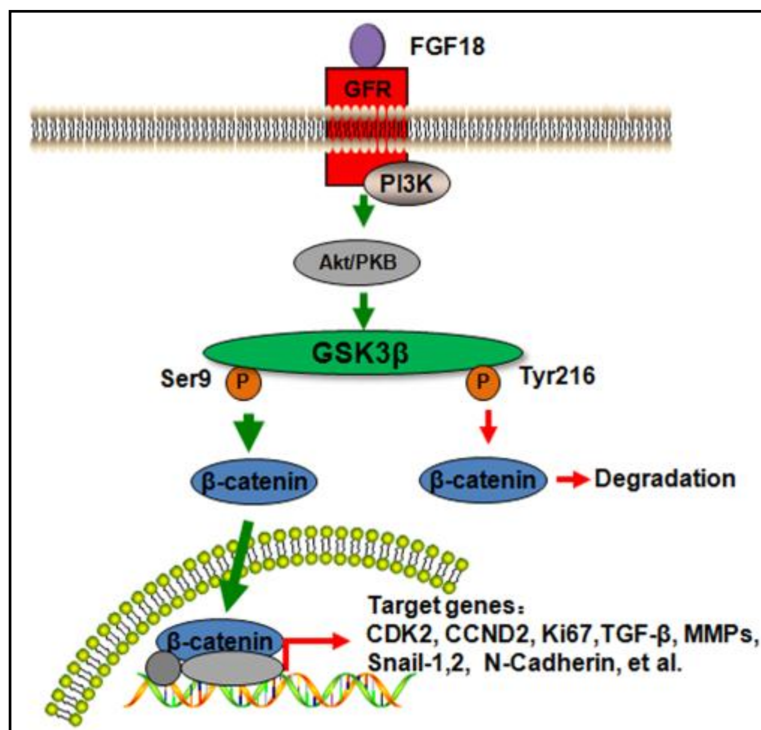

Fig. 7. Schematic representation of FGF18-mediated Akt/GSK3β/β-Catenin signaling in breast cancer cells. FGF18 could promote carcinogenesis of breast cancer via the Akt/GSK3β/β-Catenin signaling pathway. The inactive β-Catenin modulates target gene expression through re-locating into the nucleus and binding to the promoters of proliferation-, migration- and EMT-related genes.

other systems [42-44]. However, the present study was limited to HER⁺ breast cancer cell lines (MDA-MB-453, SKBR3) and not triple-negative breast cancercell lines. Thus, our results have to be confirmed in triple-negative breast cancer cells and *in vivo*.

Conclusion

In summary, our data demonstrate that FGF18 exerts oncogenic effects in HER⁺ breast cancer cells by promoting cell proliferation and migration *in vitro*. This makes FGF18 a promising candidate target for basic study or clinical research in non-triple-negative breast cancers.

Disclosure Statement

The authors declare that no conflict of interest exist..

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