Fibroblast growth factor 18 promotes the growth, migration and invasion of MDA-MB-231 cells

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Abstract. Fibroblast growth factor 18 (FGF18) increases cell motility and invasion in colon tumors, and is linked with ovarian and lung tumors. Furthermore, the increased expression of FGF18 mRNA and protein has been associated with poor overall survival in cancer patients. However, its function has not been investigated in breast cancer. In the present study, we demonstrated that FGF18 promoted cell growth and metastasis *in vitro* and stimulated tumor growth in xenograft models *in vivo*. FGF18 mediated the proliferation of MDA-MB-231 cells via the ERK/c-Myc signaling pathway and induced epithelial-to-mesenchymal transition (EMT) factors to promote cancer migration and invasion. The decreased expression of FGF18 was strongly correlated with the loss/reduction of p-ERK, c-Myc, N-cadherin, vimentin and Snail 1 protein in MDA-MB-231 cells. Collectively, our results indicated that FGF18 played an important role in the growth and metastasis of breast cancer via the ERK/c-Myc signaling pathway and EMT, indicating that FGF18 may be a potential molecular treatment target for breast cancer.

Introduction

Breast cancer is the most prevalent cancer in women worldwide and accounts for approximately 25% of all cancer cases among women (1,2). In spite of the significant increase in the survival rates of breast cancer patients during the last decades, the leading cause of cancer-related mortality among women remains breast cancer (3). However, the mechanisms of progression and metastasis of breast cancer are still poorly understood. Hence, further mechanistic explorations are crucial to the discovery of new targeted drug treatments for breast cancer.

The FGF family contains 23 recognized members acting on 5 FGF receptors that are composed of an intracellular domain, a transmembrane region and an extracellular portion (4). Members of the FGF family regulate numerous cellular and physiology processes including cell differentiation, growth, tissue repair, angiogenesis, morphogenesis, inflammation, tumor growth and the development of the embryo and the skeleton (5-8). The FGF family increases the motility, proliferation, invasiveness, and migration of many different cells (9-11). The important role of FGF18 in limb development and skeletal growth, probably through the modulation of osteoclasts, chondrocytes and osteoblasts, has been investigated (12). Furthermore, the expression of FGF18 in colon and ovarian tumors was upregulated, and tumor progression as well as poor overall survival in patients were highly related to the increased expression of FGF18 mRNA and protein (13-15).

The MAPK (mitogen-activated protein kinase) signaling pathway is a highly conserved intracellular pathway that has vital effects in the transmission of signals to the nucleus, where it transcriptionally mediates genes that participate in various cellular processes. The MAPK pathway is also correlated with pancreatic, colon, brain and breast cancer development. It is commonly abnormally activated, increasing proliferation, migration and invasion characteristics through a downstream pathway including extracellular signal-regulated kinase (ERK), PI3K/AKT, p38 and JNK pathways during neoplastic transformation (16,17). The ERK signaling pathway, one of the downstream pathways of MAPK, is involved in cell proliferation, differentiation, and migration (18). FGFs stimulate specific signaling pathways after activating FGFRs, such as ERK, AKT, protein kinase C and phospholipase Cγ. As one of the MYC family of transcription factors, the c-Myc protein has a fundamental effect on cellular transformation, apoptosis and cell cycle progression (19,20).

Epithelial-to-mesenchymal transition (EMT) is a process in which epithelial cells are converted to migratory and invasive cells. EMT activation is closely associated with cancer cell motility and invasiveness (21). A group of EMT-inducing

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Abbreviations: FGF18, fibroblast growth factor 18; ERK, extracellular signal-regulated kinase; EMT, epithelial-to-mesenchymal transition; FGF, fibroblastic growth factors; FGFR, fibroblastic growth factor receptor; MAPK, mitogen activated protein kinase; siRNA, short interfering RNA

Key words: fibroblast growth factor 18, MDA-MB-231, proliferation, migration, invasion, ERK, EMT

transcription factors (EMT-TFs) is functionally activated during the EMT cellular program. Mesenchymal markers, such as N-cadherin, vimentin and Snail 1, activate EMT cellular programs in epithelial cells. N-Cadherin is commonly expressed in mesenchymal cells, however high expression of N-cadherin is associated with increasing motility and invasion in some cancer cells. N-cadherin is not required for EMT development, however it promotes EMT by inducing cell migration abilities (22,23). Vimentin mediates cell migration ability in numerous cell types. In fibroblasts, vimentin filaments bind the nucleus to the plasma membrane and play a vital role in the formation of vimentin-associated matrix adhesions that are dynamically turned over in migrating cells (24). Snail 1, one of the transcription suppressors, has been found to participate in regulating the early development of EMT, and the expression of Snail 1 is highly linked to metastasis in primary human breast tumors (25,26). In the present study, we investigated the role of FGF18 in the growth and metastasis of breast cancer, as well as its possible mechanisms, and discovered a potential targeted therapeutic agent for breast cancer.

Materials and methods

Cell culture and treatment. The human breast cancer cell line (MDA-MB-231) was provided from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and penicillin-streptomycin solution and incubated at 37˚C with 5% CO_2.

siRNA transfection. Lipofectamine™ 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to perform cell transfection, following the manufacturer's instructions. For the FGF18 functional analysis, the MDA-MB-231 cells were transfected with negative control FGF18 (FGF18-NC), and FGF18 siRNA, all obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of the reagent were as follows: NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; siRNA1, 5'-GCAUUGCCUGUGUUUACATT-3'; siRNA2, 5'-GCAAGGAGUGUGUGUUCAUTT-3'; and siRNA3, 5'-GCAAGGAGACGGAAUUCUATT-3'. The transfection complexes of siRNA and Lipofectamine™ 3000 were added into the cells in serum-free medium for 48 h. RT-qPCR and western blot analysis were used to screen whether siRNA had been successfully transfected.

Lentivirus packaging and stable cell lines. FGF18 overexpression lentivirus (FGF O), FGF18 knockdown (FGF18 KO) lentivirus and control check vectors (FGF18 CK) were designed and packaged by GenePharma. MDA-MB-231 cells were transfected with lentivirus/medium at a ratio of 1:50. Stable cell lines were selected by puromycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at the concentration of 5 μg/ml for a two-week period. The lentivirus vectors had enhanced green fluorescent protein (eGFP) which could provide the means to screen the efficiency of transfection using a microscope.

CCK-8 proliferation assay. The proliferation of breast cancer cells was examined by CCK-8 assay (Dojindo Laboratories, Japan). MDA-MB-231 cells were stimulated with 0, 10, 20 and 50 ng/ml FGF18 (MultiSciences Biotech Co., Ltd., Hangzhou, China). ERK inhibitor (10 μmol/ml, FR180204; Selleck Chemicals, Shanghai, China) was added to 96-well cell culture plates. Cells ($2x10^3$) were seeded into each well with 100 μl culture media. The same treatment was used for MDA-MB-231/FGF18-NC, MDA-MB-231/siFGF18 cell lines. Following 24, 48, 72, 96 and 120 h, 100 μl fresh medium was replaced with 10% CCK-8 in each well. Subsequently, the cells were incubated for an additional 2 h at 37˚C. The absorbance was determined at 450 nm wavelength with a microplate reader (Tecan Austria GmbH, Grödig, Austria). All tests were performed in triplicate.

Colony formation assay. For the cells to form colonies, a total of 700 MDA-MB-231 cells with 0, 10, 20 and 50 ng/ml FGF18 or 10 μmol/ml ERK inhibitor were added to 6-well plates and maintained in medium with 10% FBS. The medium was replaced every 4 days. After 2 weeks, the colonies were fixed with methanol and stained with 0.1% crystal violet for 20 min. The visible colonies were manually counted. Each experiment was repeated at least three times independently.

Wound healing scratch assay. MDA-MB-231 cells with 0, 10, 20 or 50 ng/ml FGF18 were seeded in a 6-well cluster plate ($2x10^6$ cells/well) with 2 ml of complete DMEM. The same treatment was used for MDA-MB-231/FGF18-NC, and MDA-MB-231/siFGF18 cell lines. A clean and uniform scratch was made in the single layer of cells with a sterile pipette at 24 h. The assay was performed three times. Images were captured from each well every 24 h to determine the width of the wound.

Invasion and migration assays. Cell migration and invasion assays were performed using a Tanswell chamber (8 mm, 24-well format; Corning; Sigma-Aldrich) that was coated with or without 150 μl of ice-cold diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in DMEM basal medium and incubated overnight at 37˚C. MDA-MB-231 cells with 0, 10, 20 or 50 ng/ml FGF18 at a density of $2x10^4$ (migration)/$5x10^4$ (invasion) were seeded on the upper chamber in serum-free medium. The lower chamber was filled with 600 μl of 10% FBS-supplemented medium; 20 h later, the reduced cells were removed by a cotton swab in the top chamber, and then were fixed with methanol. Crystal violet (1%) was used to stain cells outside the inserts for 20 min. The same treatment was used for MDA-MB-231/FGF18-NC and MDA-MB-231/siFGF18 cell lines. A light microscope (Olympus Corp., Tokyo, Japan) was used to count the number of invading cells on the membrane and the results were calculated as the means ± SD. The assay was repeated three times.

Flow cytometry analysis. Cells were collected after stimulating with 0, 10, 20 or 50 ng/ml FGF18 for two days and resuspended in cold PBS, and then stained with a cell cycle staining kit (MultiSciences Biotech Co., Ltd.) according to the manufacturer's protocol. The same treatment was used for the MDA-MB231/FGF18-NC and MDA-MB231/siFGF18

cell lines. Data were analyzed using flow cytometry (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with TRIzol reagent (Takara Bio, Inc., Otsu, Japan) and cDNA was synthesized using PrimeScript RT reagent (Takara Bio) following the manufacturer's protocol. The PCR program used for amplification was as follows: i) 94˚C for 30 sec; ii) 94˚C for 30 sec; iii) 55˚C for 30 sec; iv) 72˚C for 1 min and ; v) 72˚C for 10 min. Steps ii) through iv) were repeated 35 times for β-actin and other genes. The following PCR primers were used: FGF18 forward sequence, 5'-GGACATGTGCAGGCT GGGCTA-3' and reverse, 5'-GTAGAATTCCGTCTCCTT GCCCTT-3'. All PCR reactions were performed using the fluorescent SYBR Green I methodology. Quantitative RT-PCR (qRT-PCR) was performed on StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with FastStart Universal SYBR-Green Master (Roche Diagnostics, Basel, Switzerland) following the manufacturer's protocol. The relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. All of the proteins were extracted using Total Protein Extraction kits (KeyGen Biotech, Nanjing, China) following the manufacturer's protocol. SDS-PAGE (5X; Beyotime Institute of Biotechnology, Shanghai, China) was used to form a complex with the extracted protein for storage in -20˚C. Equal amounts of protein were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (EMD Millipore Corporation, Billerica, MA, USA). The membranes were first stained to confirm the uniform transfer of all samples and then incubated in the blocking solution (5 ml of skimmed milk powder dissolving in 45 ml of TBST) for 2 h at room temperature. Membranes were then incubated overnight at 4˚C with diluted (1:1,000) primary antibodies against FGF18 (cat. no. ab169615; Abcam, Cambridge, MA, USA) ERK (cat. no. 4695; Cell Signaling Technology, Inc., Danvers, MA, USA), p-ERK (cat. no. 4370), c-Myc (cat. no. 13987), N-cadherin (cat. no. 14215), vimentin (cat. no. 5741), Snail 1 (cat. no. 3879) and GAPDH (cat. no. 51332; all form Cell Signaling Technology, Inc., Danvers, MA, USA), followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (1:1,000; cat. no. sc-2004; Santa Cruz Biotechnology, CA, USA) and anti-mouse IgG secondary antibodies (1:1,000; cat. no. sc-2005; Santa Cruz Biotechnology, CA, USA) for 2 h. Following washing with TBST, the immune-reactive proteins were detected with a Bio-Rad Western Blotting Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Tumor xenograft mouse model. The animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health and were in accordance with the approved guidelines and the experimental protocols of Nanjing Medical University. All twenty BALB/c nude female mice used in this experiment were purchased from The Model Animal Research Center of Nanjing University (Nanjing, China). We divided the mice into four groups: i) FGF18 control check (FGF18 CK); ii) FGF18 overexpression (FGF18 O); iii) FGF18 knockdown (FGF18 KO); and iv) ERK inhibitor group. Subsequently, each 5-week-old mouse was subcutaneously injected with MDA-MB-231 cells ($2x10^6$) suspended in 200 μl of PBS. The ERK inhibitor group was injected with FR180204 at the dose of 20 mg/kg/day, administered intraperitoneally. The tumor was visible at 2 weeks after injection. Tumor sizes and body weight were monitored every 3 days. After 29 days, the nude mice were sacrificed by cervical dislocation and the tumor volume was calculated following the formula: $Ixb^2x0.5$ (I and b are the largest perpendicular lengths of the tumor). The longest tumor exhibited by a single subcutaneous had been indicated, and none of the mice presented multiple tumors. The tumor tissues were frozen immediately at -80˚C for further study.

Statistical analysis. Each experiment was repeated three times and data are presented as the mean ± standard error. Data were analyzed using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used to determine the difference among at least three groups, while a t-test was used to analyze differences between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FGF18 improves the proliferation of MDA-MB-231 cells. To explore the effects of FGF18 on MDA-MB-231 cells, we used a cell proliferation assay with various FGF18 concentrations and exposure times. Fig. 1A displays that MDA-MB-231 cell proliferation was stimulated by FGF18. However, the proliferation did not differ with different concentrations of FGF18. Colony formation assays (Fig. 1B and C) revealed the proliferative promotion ability of FGF18, and the number of colonies formed in MDA-MB-231 cells with the number of colonies being markedly higher with 50 ng/ml FGF18 than with lower doses. These results revealed that FGF18 has an important effect in increasing the proliferation of breast cancer cells.

FGF18 regulates the cell cycle progression of MDA-MB-231 cells. To further investigate how FGF18 stimulated MDA-MB-231 cell proliferation, the role of FGF18 on cell cycle kinetics was analyzed to explore how FGF18 mediated the cell cycle. We found that the percentage of G_0/G_1 phase increased and the percentage of S phase decreased from FGF18 stimulation relative to the untreated MDA-MB231 cells (Fig. 1D and E). These results suggest that FGF18 promotes the proliferation of breast cancer cells by increasing the percentage of cells in G_0/G_1 phase.

FGF18 mediates the promotion of cancer cell proliferation via the ERK/c-Myc signaling pathway in MDA-MB231 cells. We demonstrated that FGF18 significantly stimulated the proliferation of MDA-MB231 cells, however the underlying mechanisms were unclear. Therefore, a western blot analysis was used to investigate the effects of FGF18 on signal transduction in the MAPK pathway. We incubated the MDA-MB231 cells with 0, 10, 20 and 50 ng/ml FGF18 for 48 h, and collected the total protein lysates. These protein lysates were subjected to western blotting with p-ERK1/2 and ERK1/2 antibodies. The level of p-ERK1/2 increased with FGF18 stimulation (Fig. 2A and B), and the expression of c-Myc rose significantly relative to the untreated control

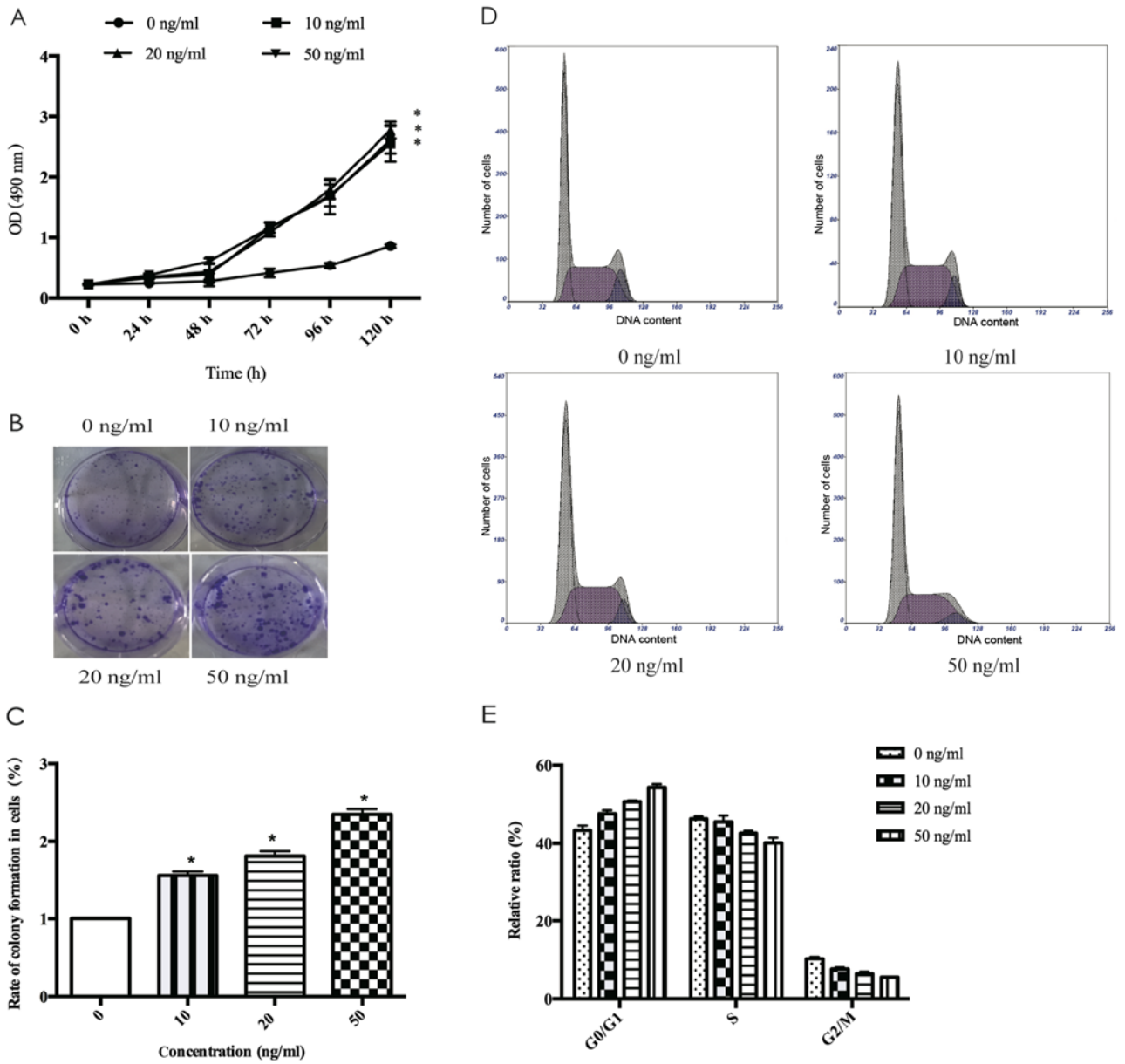

Figure 1. Effect of FGF18 on the regulation of the proliferation and cell cycle of MDA-MB-231 cells. (A) The CCK-8 assay revealed that FGF18 promoted MDA-MB-231 cell proliferation. (B and C) The colony-forming ability of cells treated with FGF18 compared with NC. The colony number was normalized to cells without any other treatment, which was set to 100%. (D and E) FGF18 increased the percent of G_0/G_1 phase and decreased the percent of S phase of MDA-MB-231 cells. The cell cycle of MDA-MB-231 cells was detected using flow cytometry. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05. FGF18, fibroblast growth factor 18.

MDA-MB231 cells (Fig. 2C and D). To further confirm the effects on the ERK pathway, we used the specific inhibitor FR180204 (ERK inhibitor) and found that at a concentration of 10 μmol/ml, FR180204 could significantly inhibit the proliferation of MDA-MB231 cells with the stimulation of FGF18 (Fig. 2E). Furthermore, c-Myc protein was also changed by the inhibitors (Fig. 2F-G). These results indicated that FGF18 promoted the proliferation of MDA-MB231 cells via modulations of the ERK/c-Myc signaling pathway.

FGF18 promotes the migration and invasion of MDA-MB-231 cells and regulates EMT-inducing transcription factors. The wound healing scratch assays revealed that after treatment with 10, 20 and 50 ng/ml FGF18 for 24 h, the migration distance of MDA-MB-231 cells significantly increased (Fig. 3A and B). Furthermore, Transwell assays also confirmed our findings on the effect of FGF18 on the migration enhancement of MDA-MB-231 cells (Fig. 3C and D). High concentration of FGF18 significantly enhanced the invasiveness of the MDA-MB-231 cells (Fig. 3E and F). Furthermore, the protein expression levels of N-cadherin, vimentin and Snail 1 increased after stimulation with FGF18 in a dose-dependent manner, particularly at the concentration of 50 ng/ml (Fig. 3G and H). These results indicated that FGF18 promoted the invasion and migration of MDA-MB-231 cells by increasing EMT-inducing transcription factors.

FGF18 siRNA inhibits cell proliferation, migration and invasion of MDA-MB-231 cells. RT-qPCR indicated that siFGF18 significantly reduced the expression of FGF18 in MDA-MB-231

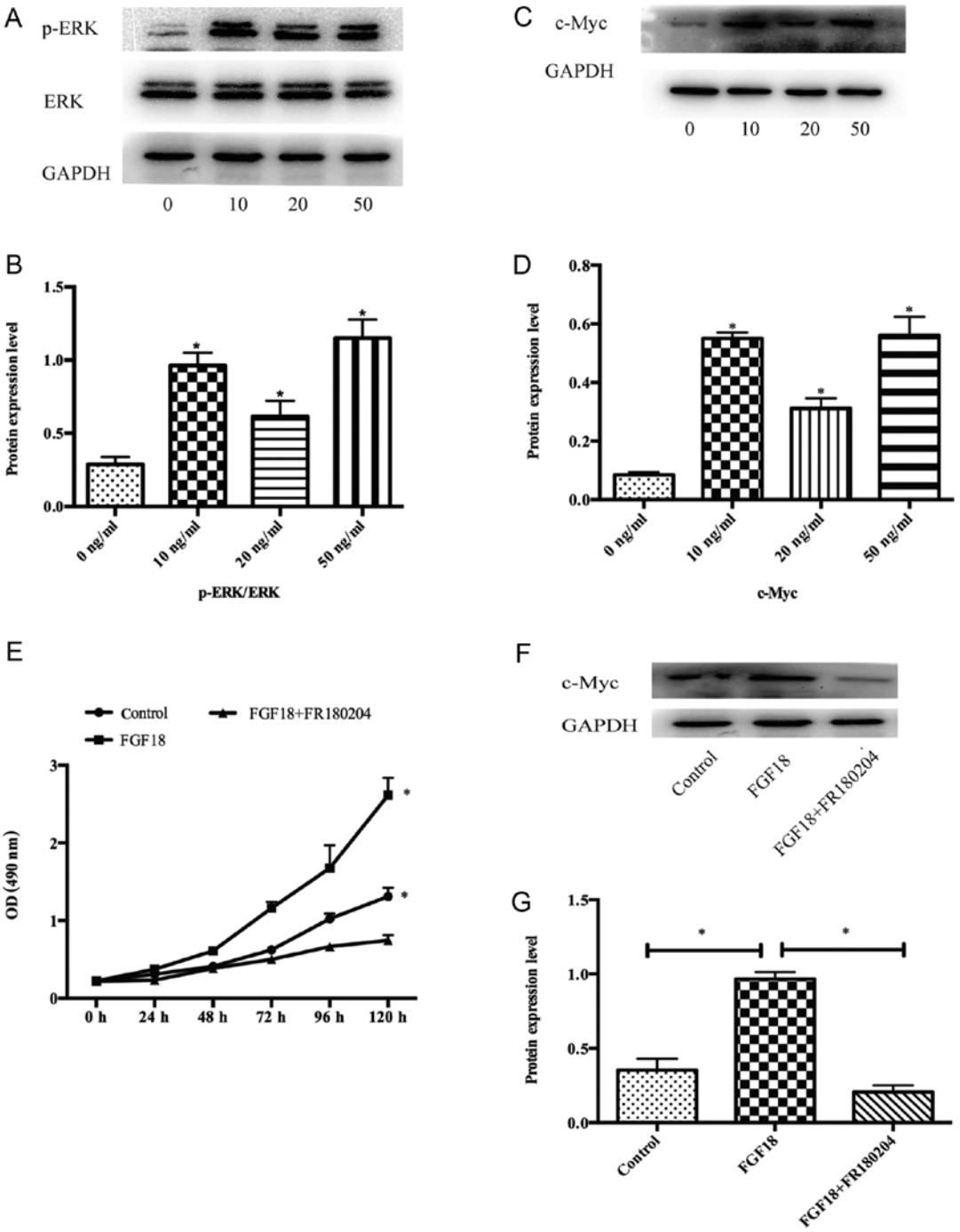

Figure 2. Effect of FGF18 on the expression levels of ERK, p-ERK and c-Myc in MDA-MB-231 cells. (A and B) FGF18 induced the activation of the ERK pathways in MDA-MB-231 cells. The expression of ERK and p-ERK was evaluated by western blotting. The relative protein expression level of p-ERK was quantified in MDA-MB-231 cells. Each assay was performed in triplicate. (C and D) c-Myc protein levels were determined using western blotting and quantification is illustrated by a bar chart. (E) The CCK-8 assay revealed that 10 μmol/l ERK inhibitor (FR180204) inhibited the proliferation of MDA-MB-231 cells treated with 50 ng/ml FGF18. (F and G) The effect of FGF18 and FGF18+FR180204 on the expression levels of c-Myc in MDA-MB-231 cells. Data are presented as the mean ± standard deviation; *P<0.05 vs. the control. FGF18, fibroblast growth factor 18.

cells compared with the control group (Fig. 4A). In addition, the results of the western blot analyses were consistent with the mRNA data, demonstrating that the FGF18 was successfully knocked down using siRNA (Fig. 4B and C). Following cell transfection, we evaluated the cell proliferation of MDA-MB-231 cells. The OD values were significantly reduced in the siFGF18 group in comparison with the control group (Fig. 5A), and colony formation assays revealed that the number of colonies of MDA-MB-231 cells with siFGF18 decreased (Fig. 5B and C), indicating that siFGF18 inhibited cell proliferation. The proportion of MDA-MB-231 cells in the G_0/G_1 phase also decreased (Fig. 5D and E). The expression of c-Myc protein decreased in the siFGF18 group, with a 58% protein inhibition rate. The level of p-ERK protein was obviously reduced in the siFGF18 group in comparison to the control group (Fig. 5F and G). The wound healing and Transwell assays

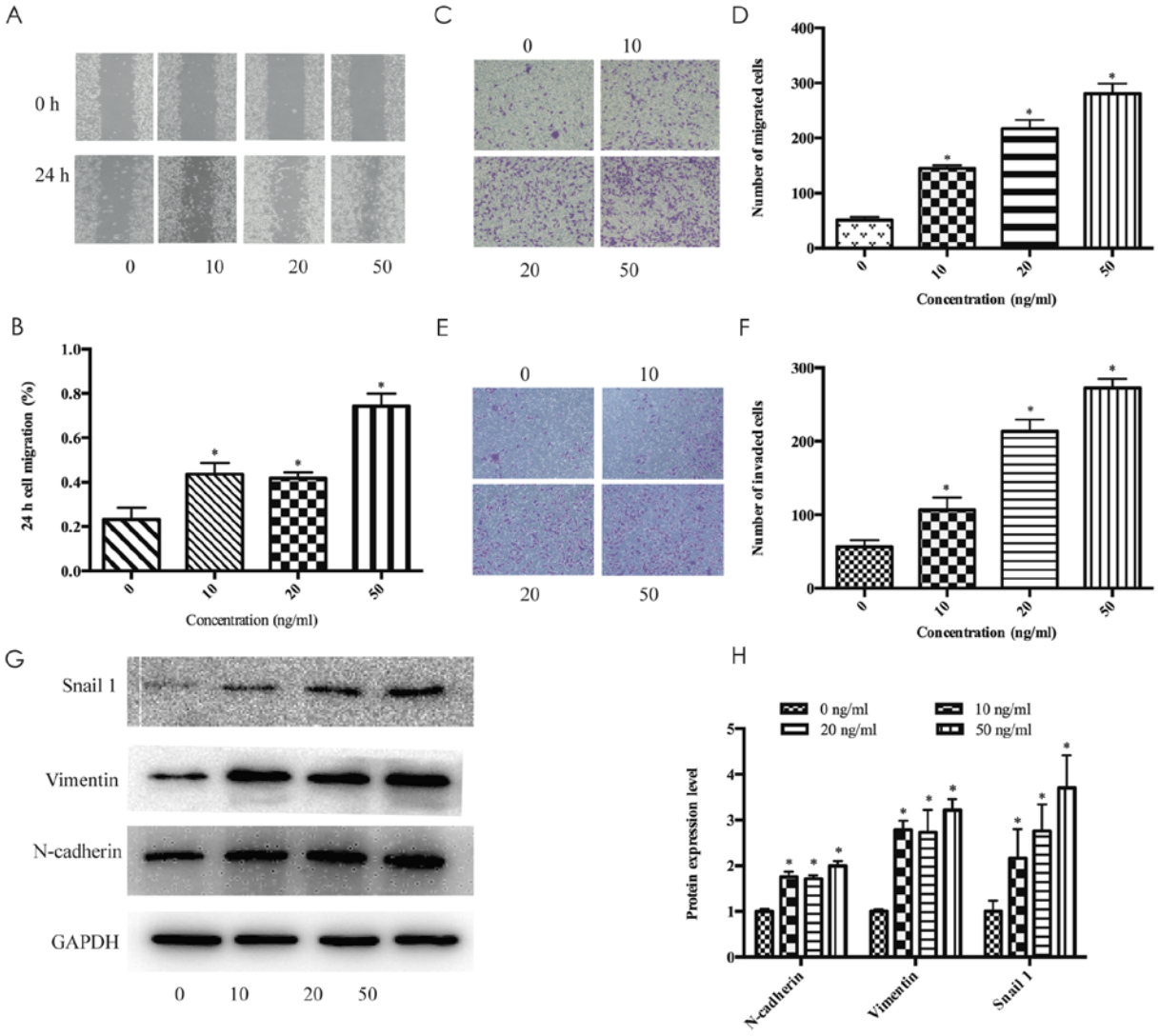

Figure 3. Effect of FGF18 on the regulation of migration and invasion of MDA-MB-231 cells. (A and B) Wound healing assay was performed on MDA-MB-231 cells with 0, 10, 20 and 50 ng/ml FGF18. Quantitative results are shown. Magnification, x40. (C-F) Migration and invasion analysis through the Transwell assay. Numbers of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown. Magnification, x100. (G and H) The effect of FGF18 on EMT-inducing transcription factors was determined by detecting protein levels. FGF18 induced the activation of N-cadherin, vimentin and Snail 1 in MDA-MB-231 cells. The expression of N-cadherin, vimentin and Snail 1 was evaluated by western blotting and quantification is illustrated by a bar chart. Each assay is performed in triplicates. Data are presented as the mean ± standard deviation; *P<0.05 vs. the control. EMT, epithelial-to-mesenchymal transition; FGF18, fibroblast growth factor 18.

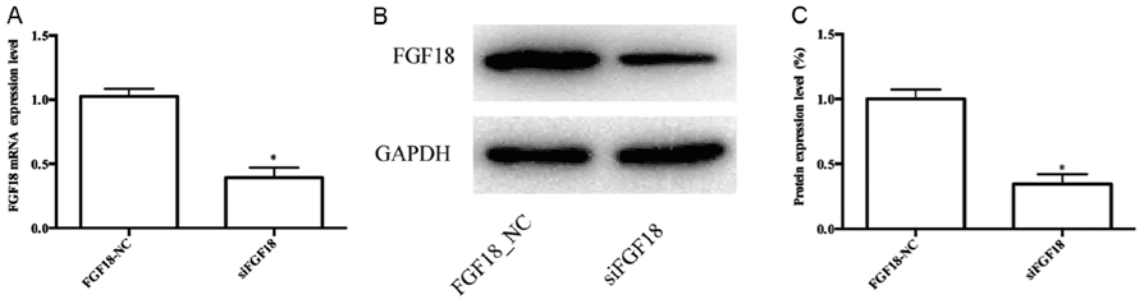

Figure 4. FGF18 siRNA inhibits the expression of FGF18. MDA-MB-231 cells were cultured in 6-well plates and were transfected with FGF18 siRNA. (A) Reverse transcription-quantitative polymerase chain reaction analysis of the expression of FGF18 mRNA. (B and C) Western blot analysis of the FGF18 protein expression was performed 48 h following transfection. *P<0.05 vs. FGF18-NC. siRNA, short interfering RNA; FGF18, fibroblast growth factor 18.

also revealed that the migration ability in the siFGF18 group decreased in comparison with the control group (Fig. 6A-D). Additionally, the invasion ability of shFGF18-transfected MDA-MB-231 cells was reduced significantly (Fig. 6E and F).

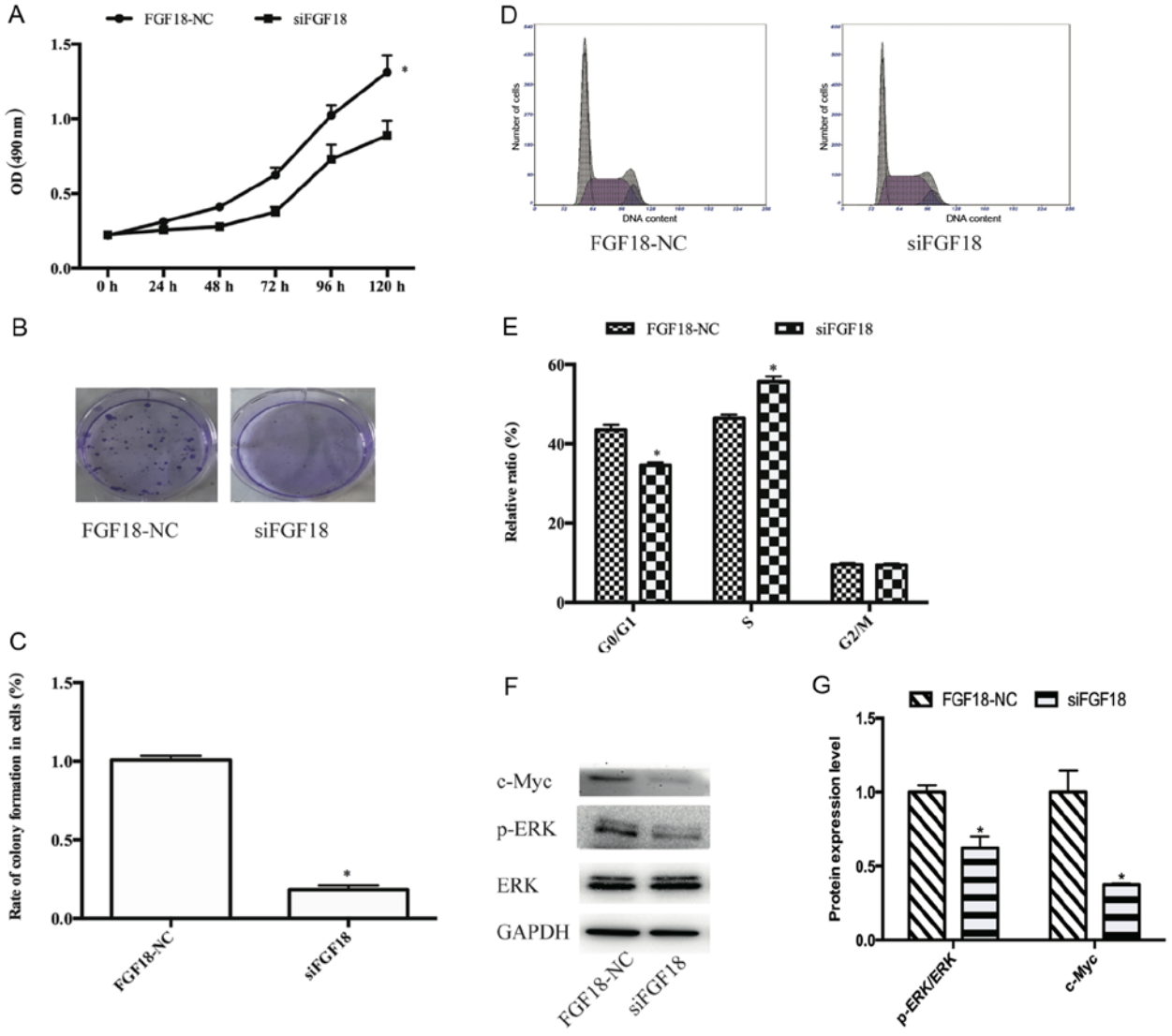

Figure 5. Effect of siFGF18 on the regulation of proliferation of MDA-MB-231 cells. (A) The CCK-8 assay revealed that siFGF18 inhibited MDA-MB-231 cell proliferation. (B and C) The colony-forming ability of siFGF18-transfected MDA-MB-231 cells compared with FGF18-NC. The colony number was normalized to cells without any other treatment, which was set to 100%. (D and E) The cell cycle of MDA-MB-231 cells was detected using flow cytometry. (F and G) ERK, p-ERK and c-Myc protein levels were determined using western blotting and quantification is illustrated by a bar chart. Data are presented as the mean ± standard deviation from three independent experiments; *P<0.05. FGF18, fibroblast growth factor 18; siFGF18, short interfering RNA against fibroblast growth factor 18.

The expression of N-cadherin, vimentin and Snail 1 proteins decreased significantly following siFGF18, with 55, 46 and 39% protein inhibition rate respectively (Fig. 6G and H). These results indicated that FGF18 siRNA suppressed cell proliferation through the ERK/c-Myc signaling pathway, and reduced the migration and invasion abilities of MDA-MB-231 cells by mediating EMT-inducing transcription factors.

Efficacy of FGF18 xenograft models of MDA-MB-231 cells. Xenograft models to investigate tumor growth promotion via FGF18 *in vivo*, using RT-qPCR revealed that the expression of FGF18 mRNA in the FGF18 O group transfected with lentivirus (the overexpression group) increased in comparison with the FGF18 CK group (control check) and the expression of FGF18 in the FGF18 KO (knockdown) group decreased compared with the FGF18 CK group (Fig. 7A). The results of the expression of FGF18 protein examined by western blot analysis were consistent with the mRNA data. Furthermore, the expression of FGF18 in tumor tissues was also examined by RT-qPCR and western blot analysis, and the findings were still consistent with the *in vitro* results (Fig. 7D-F). As above-mentioned, the model of overexpression and knockdown of FGF18 could be successfully built following lentivirus transfection. As displayed in Fig. 7G and H, the xenograft tumor sizes of the FGF18 O group were significantly larger than in the other groups, and the tumor sizes of the FGF18 KO group were markedly smaller than the other groups. In addition, the FGF18 O group was regarded as the control of ERK inhibitor and the FGF18 O+ERK inhibitor group had markedly smaller tumor sizes compared with the FGF18 O group (Fig. 7G and H). These results indicated that FGF18 promoted MDA-MB-231 cell growth *in vivo*, and ERK inhibitor could significantly prevent the growth of MDA-MB-231 cells in response to the FGF18 stimulation.

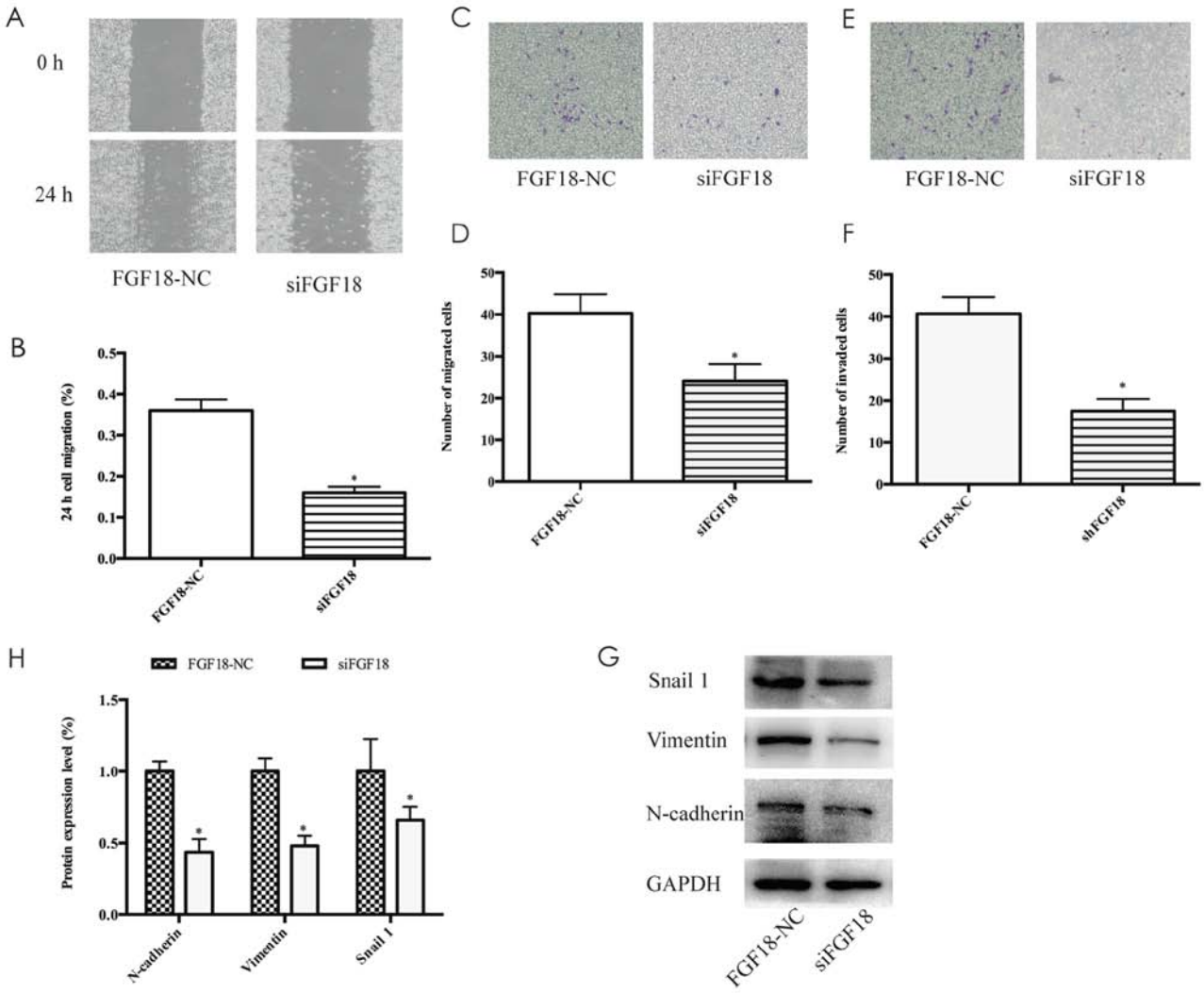

Figure 6. Effect of siFGF18 on the regulation of migration and invasion of MDA-MB-231 cells. (A and B) Wound healing assay was performed on MDA-MB-231 cells transfected with siRNA and negative control. Quantitative results are shown. Magnification, x40. (C-F) Migration and invasion evaluation using the Transwell assay. Migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown. (G-H) The effect of siFGF18 on EMT-inducing transcription factors was evaluated by detecting the protein levels. Each assay was performed in triplicates. Data are presented as the mean ± standard deviation; *P<0.05 vs. the control. EMT, epithelial-to-mesenchymal transition. siFGF18, short interfering RNA against fibroblast growth factor 18.

Discussion

Research has demonstrated that FGF and FGFR family have an important relationship with the progression of breast cancer. FGF1 could have an important role in human breast cancer growth and patients with high levels of bFGFR may have a more favorable prognosis (27). FGF□2/FGFR interaction led to complex signal transduction pathways and to the activation of a 'proangiogenic phenotype' in the endothelium, which regulated the proliferation, migration and survival of breast cancer cells (28). The growth of lung and colon cancer cells were induced by the high expression of FGF18 (29,30) and FGF18 increased ovarian tumor growth and metastasis (14). In the present study we observed that FGF18 promoted the growth and metastasis of MDA-MB-231 cells. To the best of our knowledge, this study is the first to report the effects of FGF18 in breast cancer cells.

MAPKs (JNK1/2, ERK1/2 and p38) participate in the growth and metastasis of many tumors, such as breast, lung, ovarian, colorectal and prostate cancer (31-33). Similarly, the ERK signaling pathway may mediate factors related to the and poor prognosis and progression in breast cancer (34). The *c-Myc* proto-oncogene is a senior administrator of the cell, helping to allocate resources and direct proliferation, apoptosis, differentiation and growth (35). A recent study also revealed that the c-*Myc* gene was involved in most aspects of the cellular function, such as the growth, replication, apoptosis, differentiation and metabolism in breast cancer (36). FR180204 (specific inhibitor for ERK) was used to identify whether ERK was involved in the progression of MDA-MB-231 cells. We found that the proliferation in response to FGF18 was reduced with the inhibition of ERK, and the expression of the target gene c-Myc decreased. These investigations indicated that the activation of ERK induced the proliferation of MDA-MB-231 cells by increasing the expression of the target gene c-Myc. In addition, *in vivo*, the tumor sizes of mice in the FGF18 O+ERK inhibition group were similar to the tumor sizes of the FGF18-NC group. These findings indicated that

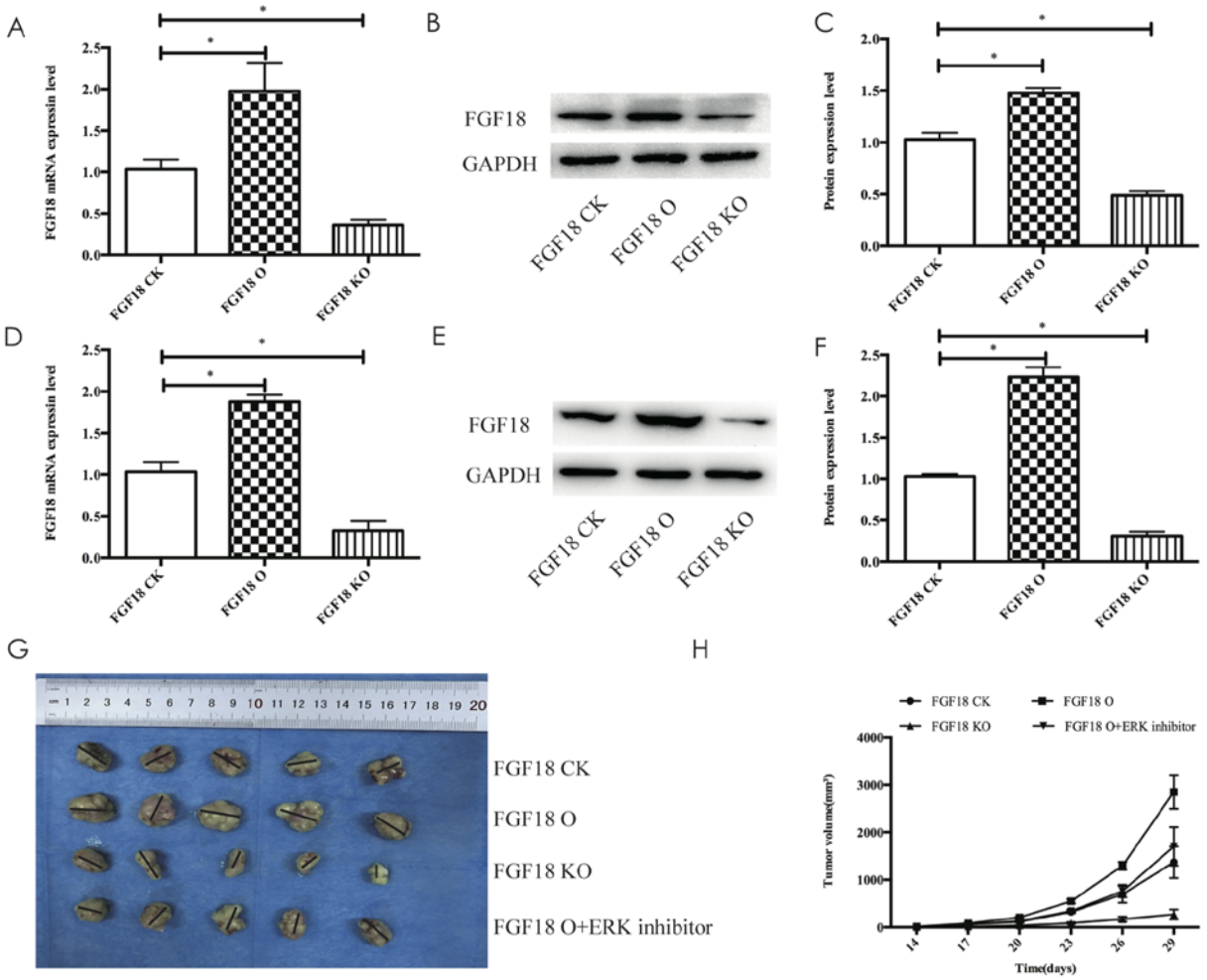

Figure 7. FGF18 promotes MDA-MB-231 cell tumorigenesis *in vivo*. (A) Reverse transcription-quantitative polymerase chain reaction analysis of FGF18 mRNA expression. (B and C) Western blot analysis of FGF18 protein expression was performed 48 h following transfection. (D) Reverse transcription-quantitative polymerase chain reaction analysis of FGF18 mRNA expression. (E and F) Western blot analysis of FGF18 protein expression was performed in tumor tissues. (G and H) The graphs represent the growth of tumors 29 days after inoculation. The weight and volume of tumors were calculated. Data are presented as the mean ± standard deviation. *P<0.05 vs. FGF18 CK. FGF18, fibroblast growth factor 18.

the ERK/c-Myc signaling pathway was activated by FGF18 in the progression of breast cancer. For this reason, we infered that the ERK/c-Myc signaling pathway may induce proliferative signals in breast cancer cells.

EMT plays an important role in the acquisition of migration and invasion capabilities by improving mesenchymal phenotypes and motility (37). FGF18 mediates Wnt-dependent stimulation of CD44-positive human colorectal adenoma cells (30) and the Wnt signaling pathway is involved in the progression of EMT (38,39). We observed that FGF18 increased the expression of EMT-inducing transcription factors N-cadherin, vimentin and Snail 1, indicating that FGF18 may induce the progression of EMT in breast cancer cells and then promote the migration and invasion capabilities of MDA-MB-231 cells. However, EMT progression can be induced through several other signaling pathways including TGF-β and Notch (40,41). The underlying mechanism of EMT-inducing factors mediated by FGF18 has not been investigated. Therefore, further studies exploring the mechanisms of migration and invasion in MDA-MB-231 cells should be undertaken.

Furthermore, it was confirmed that the transfection of siFGF18 could suppress the expression of FGF18 gene and reduce the effects of growth and metastasis of MDA-MB-231 cells. The expression of ERK, c-Myc, N-cadherin, vimentin and Snail 1 in human MDA-MB-231 cells was detected by western blot analysis following siRNA-FGF18 transfection. These results indicated that the use of siFGF18 can be a potential treatment for breast cancer.

However, in the preliminary experiment of this study, we observed that the effect of FGF18 only functioned in the MDA-MB-231 cells compared with several other cell lines (SUM1315MO2, SKBR3 and MCF 7). All of these results is not mentioned in the present study. The ERK signaling pathway may be involved in these differences. Our future study would be to explore the underlying molecular mechanisms of the above-mentioned phenomenon. Using only one cell line was a limitation of the present study, and a greater number of cell lines would further support our conclusions.

In conclusion, the present study revealed that through the ERK/c-Myc signaling pathway and EMT transition, FGF18 had a significant effect on the growth and metastasis of breast

cancer cells, demonstrating that FGF18 provided a potential target for the effective treatment of breast cancer. Further studies of breast cancer, exploring the link between FGF18 and the survival, relapse and metastasis of patients are required.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZYY and LQL conceived and designed the study. ZYY and LQL performed the experiments. ZYY wrote the paper. ZYY and LQL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Nanjing Medical University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Greenlee H, DuPont-Reyes MJ, Balneaves LG, Carlson LE, Cohen MR, Deng G, Johnson JA, Mumber M, Seely D, Zick SM, *et al*: Clinical practice guidelines on the evidence-based use of integrative therapies during and after breast cancer treatment. CA Cancer J Clin 67: 194-232, 2017.
2. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
3. Polychemotherapy for early breast cancer: An overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 352: 930-942, 1998.
4. Antoine M, Wirz W, Tag CG, Mavituna M, Emans N, Korff T, Stoldt V, Gressner AM and Kiefer P: Expression pattern of fibroblast growth factors (FGFs), their receptors and antagonists in primary endothelial cells and vascular smooth muscle cells. Growth Factors 23: 87-95, 2005.
5. Diez Del Corral R and Morales AV: The Multiple roles of FGF signaling in the developing spinal cord. Front Cell Dev Biol 5: 58, 2017.
6. Majidinia M, Sadeghpour A and Yousefi B: The roles of signaling pathways in bone repair and regeneration. J Cell Physiol 233: 2937-2948, 2018.
7. Presta M, Chiodelli P, Giacomini A, Rusnati M and Ronca R: Fibroblast growth factors (FGFs) in cancer: FGF traps as a new therapeutic approach. Pharmacol Ther 179: 171-187, 2017.
8. Itoh N and Ornitz DM: Evolution of the Fgf and Fgfr gene families. Trends Genet 20: 563-569, 2004.
9. Elo T, Lindfors PH, Lan Q, Voutilainen M, Trela E, Ohlsson C, Huh SH, Ornitz DM, Poutanen M, Howard BA, *et al*: Ectodysplasin target gene Fgf20 regulates mammary bud growth and ductal invasion and branching during puberty. Sci Rep 7: 5049, 2017.
10. Feng S, Wang J, Zhang Y, Creighton CJ and Ittmann M: FGF23 promotes prostate cancer progression. Oncotarget 6: 17291-17301, 2015.
11. Wu XL, Gu MM, Huang L, Liu XS, Zhang HX, Ding XY, Xu JQ, Cui B, Wang L, Lu SY, *et al*: Multiple synostoses syndrome is due to a missense mutation in exon 2 of *FGF9* gene. Am J Hum Genet 85: 53-63, 2009.
12. Ohbayashi N, Shibayama M, Kurotaki Y, Imanishi M, Fujimori T, Itoh N and Takada S: FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev 16: 870-879, 2002.
13. Sonvilla G, Allerstorfer S, Stättner S, Karner J, Klimpfinger M, Fischer H, Grasl-Kraupp B, Holzmann K, Berger W, Wrba F, *et al*: FGF18 in colorectal tumour cells: Autocrine and paracrine effects. Carcinogenesis 29: 15-24, 2008.
14. Wei W, Mok SC, Oliva E, Kim SH, Mohapatra G and Birrer MJ: FGF18 as a prognostic and therapeutic biomarker in ovarian cancer. J Clin Invest 123: 4435-4448, 2013.
15. Shimokawa T, Furukawa Y, Sakai M, Li M, Miwa N, Lin YM and Nakamura Y: Involvement of the *FGF18* gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. Cancer Res 63: 6116-6120, 2003.
16. MacCorkle RA and Tan TH: Mitogen-activated protein kinases in cell-cycle control. Cell Biochem Biophys 43: 451-461, 2005.
17. Martin GS: Cell signaling and cancer. Cancer Cell 4: 167-174, 2003.
18. Kohno M and Pouyssegur J: Targeting the ERK signaling pathway in cancer therapy. Ann Med 38: 200-211, 2006.
19. Baudino TA, McKay C, Pendeville-Samain H, Nilsson JA, Maclean KH, White EL, Davis AC, Ihle JN and Cleveland JL: c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 16: 2530-2543, 2002.
20. Liao DJ and Dickson RB: c-Myc in breast cancer. Endocr Relat Cancer 7: 143-164, 2000.
21. Neelakantan D, Zhou H, Oliphant MU, Zhang X, Simon LM, Henke DM, Shaw CA, Wu MF, Hilsenbeck SG, White LD, *et al*: EMT cells increase breast cancer metastasis via paracrine GLI activation in neighbouring tumour cells. Nat Commun 8: 15773, 2017.
22. Hazan RB, Phillips GR, Qiao RF, Norton L and Aaronson SA: Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 148: 779-790, 2000.
23. Maeda M, Johnson KR and Wheelock MJ: Cadherin switching: Essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. J Cell Sci 118: 873-887, 2005.
24. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB and Ivaska J: Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 30: 1436-1448, 2011.
25. Canesin G, Cuevas EP, Santos V, López-Menéndez C, Moreno-Bueno G, Huang Y, Csiszar K, Portillo F, Peinado H, Lyden D and Cano A: Lysyl oxidase-like 2 (LOXL2) and E47 EMT factor: Novel partners in E-cadherin repression and early metastasis colonization. Oncogene 34: 951-964, 2015.
26. Neal CL, Henderson V, Smith BN, McKeithen D, Graham T, Vo BT and Odero-Marah VA: Snail transcription factor negatively regulates maspin tumor suppressor in human prostate cancer cells. BMC Cancer 12: 336, 2012.

27. Blanckaert VD, Hebbar M, Louchez MM, Vilain MO, Schelling ME and Peyrat JP: Basic fibroblast growth factor receptors and their prognostic value in human breast cancer. Clin Cancer Res 4: 2939-2947, 1998.
28. Cai Y, Zhang J, Lao X, Jiang H, Yu Y, Deng Y, Zhong J, Liang Y, Xiong L and Deng N: Construction of a disulfide-stabilized diabody against fibroblast growth factor-2 and the inhibition activity in targeting breast cancer. Cancer Sci 107: 1141-1150, 2016.
29. Chen T, Gong W, Tian H, Wang H, Chu S, Ma J, Yang H, Cheng J, Liu M, Li X and Jiang C: Fibroblast growth factor 18 promotes proliferation and migration of H460 cells via the ERK and p38 signaling pathways. Oncol Rep 37: 1235-1242, 2017.
30. Koneczny I, Schulenburg A, Hudec X, Knöfler M, Holzmann K, Piazza G, Reynolds R, Valent P and Marian B: Autocrine fibroblast growth factor 18 signaling mediates Wnt-dependent stimulation of CD44-positive human colorectal adenoma cells. Mol Carcinog 54: 789-799, 2015.
31. Yang XL, Liu KY, Lin FJ, Shi HM and Ou ZL: CCL28 promotes breast cancer growth and metastasis through MAPK-mediated cellular anti-apoptosis and pro-metastasis. Oncol Rep 38: 1393-1401, 2017.
32. Hrustanovic G, Olivas V, Pazarentzos E, Tulpule A, Asthana S, Blakely CM, Okimoto RA, Lin L, Neel DS, Sabnis A, *et al*: RAS-MAPK dependence underlies a rational polytherapy strategy in EML4-ALK-positive lung cancer. Nat Med 21: 1038-1047, 2015.
33. Urosevic J, Garcia-Albéniz X, Planet E, Real S, Céspedes MV, Guiu M, Fernandez E, Bellmunt A, Gawrzak S, Pavlovic M, *et al*: Colon cancer cells colonize the lung from established liver metastases through p38 MAPK signalling and PTHLH. Nat Cell Biol 16: 685-694, 2014.
34. Whyte J, Bergin O, Bianchi A, McNally S and Martin F: Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. Breast Cancer Res 11: 209, 2009.
35. Wilde BR and Ayer DE: Interactions between Myc and MondoA transcription factors in metabolism and tumourigenesis. Br J Cancer 113: 1529-1533, 2015.
36. Fallah Y, Brundage J, Allegakoen P and Shajahan-Haq AN: MYC-driven pathways in breast cancer subtypes. Biomolecules 7: E53, 2017.
37. Nieto MA, Huang RY, Jackson RA and Thiery JP: EMT: 2016. Cell 166: 21-45, 2016.
38. Nelson WJ and Nusse R: Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303: 1483-1487, 2004.
39. Wang JJ, Li ZF, Li XJ, Han Z, Zhang L and Liu ZJ: Effects of microRNA-136 on melanoma cell proliferation, apoptosis and epithelial mesenchymal transition by targeting PMEL through the Wnt signaling pathway. Biosci Rep 37: BSR20170743, 2017.
40. Derynck R, Muthusamy BP and Saeteurn KY: Signaling pathway cooperation in TGF-β-induced epithelial-mesenchymal transition. Curr Opin Cell Biol 31: 56-66, 2014.
41. Huber MA, Kraut N and Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17: 548-558, 2005.