Cancer Letters 440–441 (2019) 54–63

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Inhibition of BTF3 sensitizes luminal breast cancer cells to PI3Kα inhibition through the transcriptional regulation of ERα

Jinlei Ding[a,1], Xiaonan Wang[a,1], Yuan Zhang[a], Xiaolin Sang[a], Jingyan Yi[a], Chongya Liu[a], Zundong Liu[a], Min Wang[a], Nan Zhang[a], Yijue Xue[a], Lanlin Shen[a], Wenzhi Zhao[b], Fuwen Luo[c], Pixu Liu[a,d,**], Hailing Cheng[a,*]

[a] Cancer Institute, The Second Hospital of Dalian Medical University, Dalian Key Laboratory of Molecular Targeted Cancer Therapy, Institute of Cancer Stem Cell, Dalian Medical University, Dalian, Liaoning, China
[b] Department of Orthopedics, The Second Hospital of Dalian Medical University, Dalian, Liaoning, China
[c] Department of Acute Abdomen Surgery, The Second Hospital of Dalian Medical University, Dalian, Liaoning, China
[d] College of Pharmacy, Dalian Medical University, Dalian, Liaoning, China

ARTICLE INFO

Keywords:
BTF3
ERα
ER+ breast cancer
PI3Kα inhibition

ABSTRACT

Selective phosphatidylinositol 3 kinase (PI3K) inhibitors are being actively tested in clinical trials for ERα-positive (ER+) breast cancer due to the presence of activating PIK3CA mutations. However, recent studies have revealed that increased ERα transcriptional activity limits the efficacy of PI3K inhibitor monotherapy for ER + breast cancers. Herein, we report the identification of BTF3 as an oncogenic transcription factor that regulates ERα expression in luminal breast cancers. Our TCGA analysis reveals high expression levels of BTF3 in luminal/ER + breast cancer and cell line models harboring ERα overexpression. Concordantly, *BTF3* expression is highly and strongly associated with *ESR1* expression in multiple breast cancer cohorts. We further show that BTF3 promotes the proliferation, survival and migration of ER + breast cancer cells by modulating *ESR1* expression and ERα-dependent transcription. Moreover, BTF3 knockdown sensitizes ER + breast cancer cells to the PI3Kα inhibitor BYL-719 in both *in vitro* and *in vivo* models. Together, our findings highlight a novel role of BTF3 in modulation of ERα-dependent transcriptional activity and its potential as a predictive marker for the response to PI3K-targeted therapy in ER + breast cancer.

1. Introduction

Estrogen receptor alpha (ERα) is a hormone-regulated transcription factor that is present in approximately 70% of breast cancers [1,2]. ERα-positive (ER+) breast cancers often respond well to endocrine therapies that block ERα signaling [2,3]. Adjuvant endocrine therapies, including tamoxifen, as a selective ER modulator, fulvestrant, as an ER downregulator and letrozole as an aromatase inhibitor, have been approved for the adjuvant treatment of ER + breast cancer [2,3]. While endocrine therapies are usually effective both in the adjuvant and metastatic setting, ER + breast cancer frequently relapse after prolonged therapies [3,4], justifying the need for the development of alternative effective treatment strategies.

Activating PIK3CA (encoding the p110α catalytic subunit of the phosphatidylinositol 3 kinase,PI3K) mutations, including hot spot mutations that reside in the catalytic (H1047R) or helical (E542/E545K) domains, are found in more than 30% of ER + breast cancer [1,5,6], representing the most common genomic alterations in this subtype of tumors, which indicates that the PI3K signaling pathway plays an important role in the disease pathogenesis. The high frequency of PIK3CA mutations in luminal/ER + cancers suggests that inhibitors of PI3K kinases or key nodes in this pathway may benefit ER+/PIK3CA mutant breast cancer patients. Intensive efforts have been made to test selective PI3Kα inhibitors in clinical trials in patients with advanced breast tumors harboring PIK3CA alterations [7–9]. While clinical trials of these inhibitors have yielded promising results [7–9], not all the PIK3CA mutation-positive breast cancers respond to these agents, and even those that initially respond often relapse after months of therapy [5,10].

* Corresponding author. Cancer Institute, The Second Hospital of Dalian Medical University, Dalian Key Laboratory of Molecular Targeted Cancer Therapy, Dalian, Liaoning, China.
** Corresponding author. Institute of Cancer Stem Cell, College of Pharmacy, Dalian Medical University, Dalian, Liaoning, China.
E-mail addresses: pixu_liu@dmu.edu.cn (P. Liu), hailingcheng_dmu@163.com (H. Cheng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2018.09.030
Received 7 March 2018; Received in revised form 7 September 2018; Accepted 26 September 2018

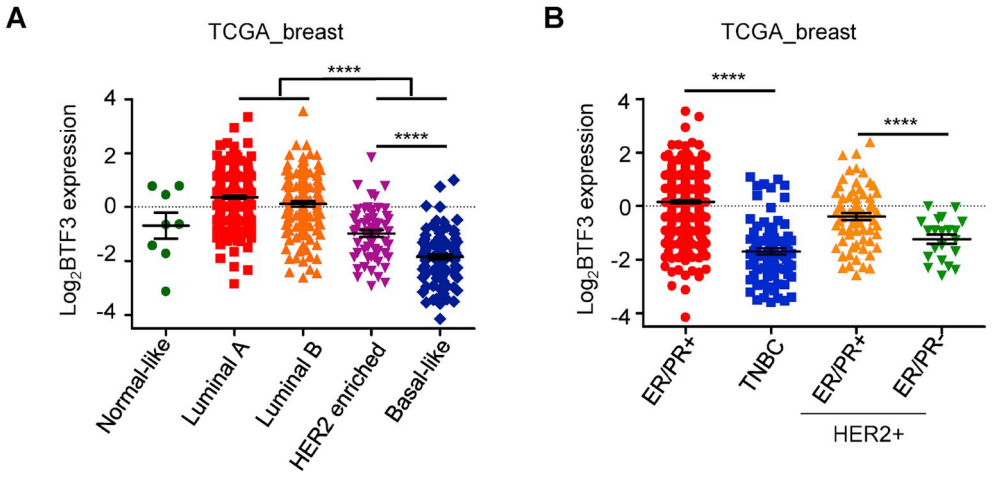

Fig. 1. *BTF3* is overexpressed in luminal/ER positive breast cancer. (A) *BTF3* expression levels in different molecular subtypes of breast cancer. Using the prediction analysis of microarray 50 (PAM50), all the samples were classified into five distinct molecular subtypes (TCGA). (B) *BTF3* expression in the subtypes of breast cancer that is defined by ER/PR and HER2 expression (TCGA). The samples that were HER2+ were classified into ER/PR+ and ER/PR− groups. The black lines in each group indicate the mean ± S.E.M. ****$P < 0.0001$ (Student's *t*-test).

Recent studies have reported that the blockade of ER activity sensitizes luminal/ER + breast tumors to PI3K inhibition, in particular PI3Kα isoform specific inhibitors [11,12]. Indeed, these studies reveal that increased ER transcriptional activity accounts for the limited efficacy of PI3K inhibitors used as monotherapy in breast cancers of the luminal subtype [11,12]. As such, the investigation of the molecular mechanisms that are involved in the modulation of ER expression may open up new avenues for the development of biomarkers that help stratify patients most likely to benefit from PI3Kα targeted therapies.

The current study aims to explore the oncogenic role of basic transcription factor 3 (BTF3) in breast cancer. While little is known about its biological function, BTF3 has been reported to be overexpressed and proposed to function as an oncogene in various tumor types [13–18]. Nevertheless, the correlation of BTF3 expression with breast cancer has not been reported. In the current study, our results show that BTF3 is frequently upregulated in luminal breast cancer and possesses a strong oncogenic function by promoting cell proliferation, survival and migration via the transcriptional regulation of ERα. Furthermore, we test the hypothesis that BTF3 knockdown sensitizes ER + breast cancer cells to PI3Kα inhibition through the transcriptional regulation of ERα.

2. Materials and methods

2.1. Cell culture and reagents

All the cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). The human breast cancer cell lines MCF7, T47D, BT474, AU565, HCC1954, HCC38, BT549 and HCC1187 were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% penicillin streptomycin (Gibco). The MDA-MB-361 and MDA-MB-453 cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Biological Industries) and 1% penicillin streptomycin (Gibco). The SKBR3 cells were maintained in McCoy's 5a Medium Modified (Gibco) supplemented with 10% FBS (Biological Industries) and 1% penicillin streptomycin (Gibco, USA). All the cells were incubated in a humidified atmosphere, with 5% CO_2 at 37 °C. The siRNA reagents were purchased from GenePharma (Suzhou, China). Lipofectamine3000 (Invitrogen, USA) was used to transfect the on-target (siBTF3 #1:GCCGAAGAAGC CUGGGAAUCA; siBTF3 #2: GCAGGCACAAGUGCGCAUUTT) and negative control siRNA oligos (150 pmol per well in 6-well cell culture plates) into the cells according to the manufacturer's suggestions. BYL-719, AZD-6482 and BKM-120 were purchased from Medchemexpress (Shanghai, China).

2.2. Plasmids and stable cell line generation

To construct pWZL-ERα and pWZL-BTF3, human full-length ESR1 and BTF3 were cloned into the pWZL-Blast vector, respectively. The MCF7 cells stably expressing ERα or BTF3 were generated by retroviral infection followed by selection with Blasticidin (Invitrogen, USA).

2.3. Immunoblotting and antibodies

The cells were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors as described [19]. The cell lysates were cleared by centrifugation, and the proteins were resolved by SDS-PAGE and were then transferred to Nitrocellulose Blotting membranes (GE Healthcare life Science, Germany). The blots were probed with the following antibodies: anti-BTF3 (Abcam, USA), anti-ERα (Cell signaling technology, CST, USA), anti-cleaved PARP (CST), anti-pAKT S473 (CST), anti-AKT (H-136) (Santa Cruz Biotechnology, USA), and anti-Vinculin (Sigma Aldrich, USA).

2.4. Proliferation assay

The Cell Counting Kit - 8 (CCK-8) assay (Dojindo Molecular Technologies, Japan) was carried out according to the manufacturer's guidelines and as previously described [20]. The optical density (OD) was measured at 450 nm by a xMark Microplate Spectrophotometer (Bio-Rad, USA). The IC50s were calculated from the sigmoidal dose-response curves utilizing Prism.

2.5. Clonogenic survival assay

The cells were seeded in 12-well plates and were cultured overnight. Fresh media was replaced every 3 days. At the end point, the cells were fixed with methanol that contained crystal violet (Sigma) and were dissolved with 10% glacial acetic acid later. The optical density (OD) was measured at 590 nm by a xMark Microplate Spectrophotometer (Bio-Rad).

2.6. Cell cycle analysis by flow cytometry

The cells were serum starved for 24 h before complete medium was added. After 12 h, BTF3 expression was knocked down by siRNA for an additional 48 h. Single-cell suspensions were prepared after the cells were harvested by trypsinization. After centrifugation, the cells were fixed by adding 70% ethanol (−20 °C) dropwise while vortexing. The cells were then stained with a Phosphate buffered saline (PBS) solution containing propidium iodide (50 μg/ml, Sigma), 100 μg/ml DNase-free RNase A (Sigma). After 30 min of incubation, the samples were washed

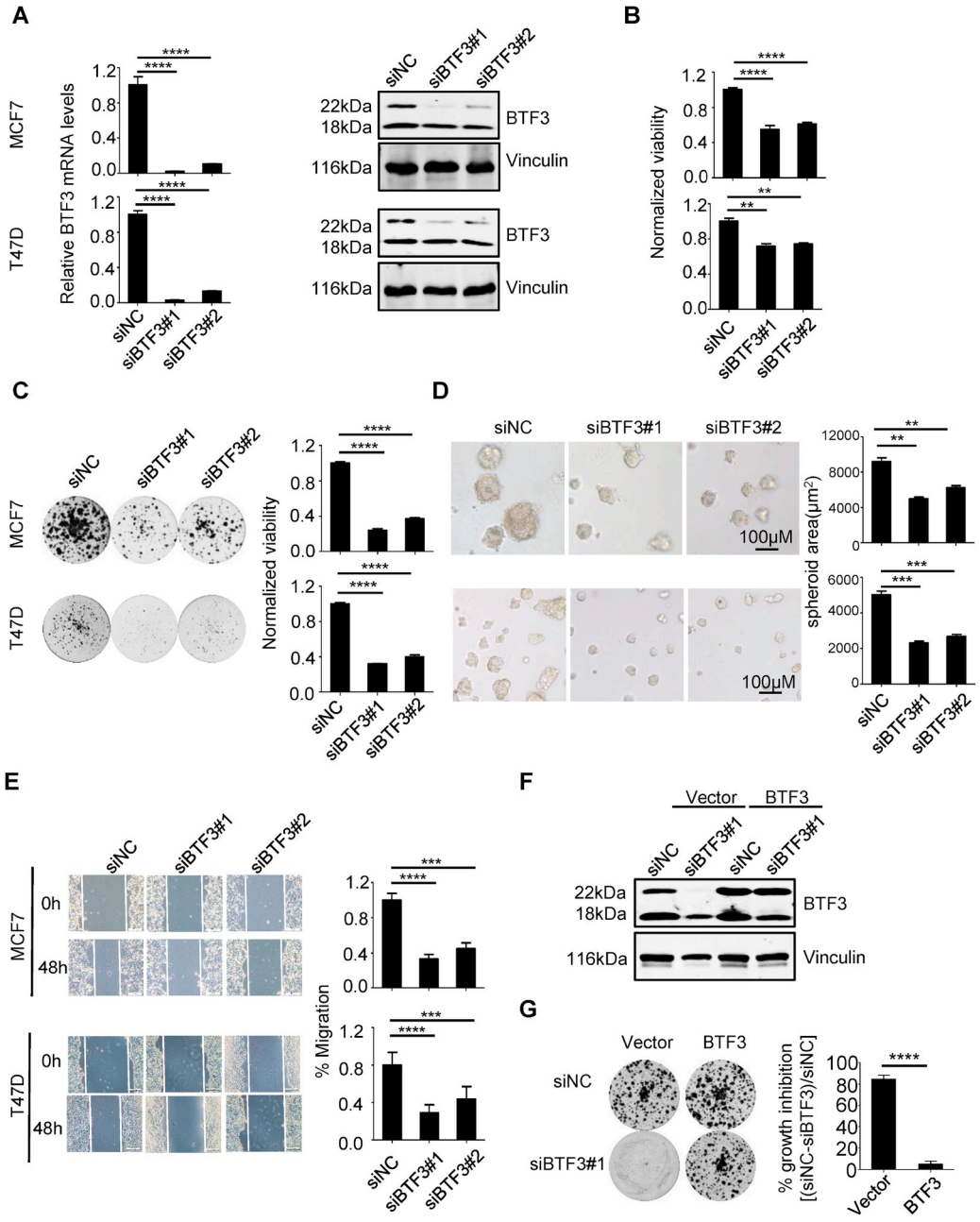

Fig. 2. BTF3 knockdown inhibits the growth and migration of ER positive breast cancer cells. (A) *BTF3* mRNA levels in the breast cancer cells, as indicated, were determined by a quantitative RT-PCR analysis (left panels). *β-actin* was used as the internal control. BTF3 protein abundance in the breast cancer cells, as indicated, was determined by an immunoblotting analysis (right panels). Vinculin was used as the loading control. (B) Cell viability was measured by a CCK8 assay in cells that were transiently transfected with siBTF3 or siNC for three days. Note: siBTF3 #1 targets a specific sequence in the 3'UTR of *BTF3*; siBTF3#2 targets a specific sequence in the open reading frame of *BTF3*. (C) Breast cancer cells transiently transfected with siBTF3 or siNC were cultured for 14 days and were then crystal violet stained. Fresh media was replaced every three days. (D) Breast cancer cells, as indicated, were cultured in 3D Matrigel for 10 days. Representative images of the 3D spheroid growth are shown. Quantification of 3D spheroid area was shown. Scale bar, 100 μm. (E) The cell migration potential of the breast cancers, as indicated, was determined using a wound-healing assay. The images of the wound areas are shown at 0 and 48 h. (F) The protein abundance of BTF3 was determined by an immunoblotting analysis in the siNC- or siBTF3-transfected breast cancer cells with or without the ectopic expression of BTF3. Vinculin was used as the loading control. (G) The breast cancer cells, as indicated, were cultured for 14 days and were then crystal violet stained. Fresh media was replaced every three days. The error bars represent the mean ± S.D. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and resuspended in PBS with 0.5% FBS. The analysis was performed on a BD FACSCanto™ II (BD Biosciences, USA).

2.7. 3-Dimensional spheroid assay

The breast cancer cells were seeded on plates with 50% precoated Matrigel (BD Biosciences, San Jose, CA, USA) plus 50% medium without serum. The cells were cultured in media supplemented with 2% FBS and 2% Matrigel that was replaced every 3 days. Three independent experiments were conducted. The 3D spheroid areas were quantified using ImageJ software.

2.8. Quantitative RT-PCR analysis

The total RNA was extracted using the Nucleozol Reagent (Macherey-Nagel, Germany), and the reverse transcription was performed using a reverse-transcription (RT)—PCR Kit PrimeScript™ RT reagent Kit (Takara, Japan) according to the manufacturer's instructions. For the quantitative real-time PCR (qPCR), the complementary DNA (cDNA) was amplified using a SYBR Green PCR Kit (Applied Biosystems, USA) and an ABI StepOnePlus Real-Time PCR Systems. The DNA sequencing and primer synthesis were performed by Sangon Biotech (Shanghai, China) Co. Ltd. The relative expression was normalized to *β-actin* expression and was calculated by the 2ΔΔCt method.

β-actin

5'CATGTACGTTGCTATCCAGGC3' (Forward). 5'CTCCTTAATGTCACGCACGAT3' (Reverse).

BTF3

5'GCCAGTCTCCTTAAACTAGTCAG3' (Forward).
5'TTTCACCATTACAGGCCATGCT3' (Reverse).

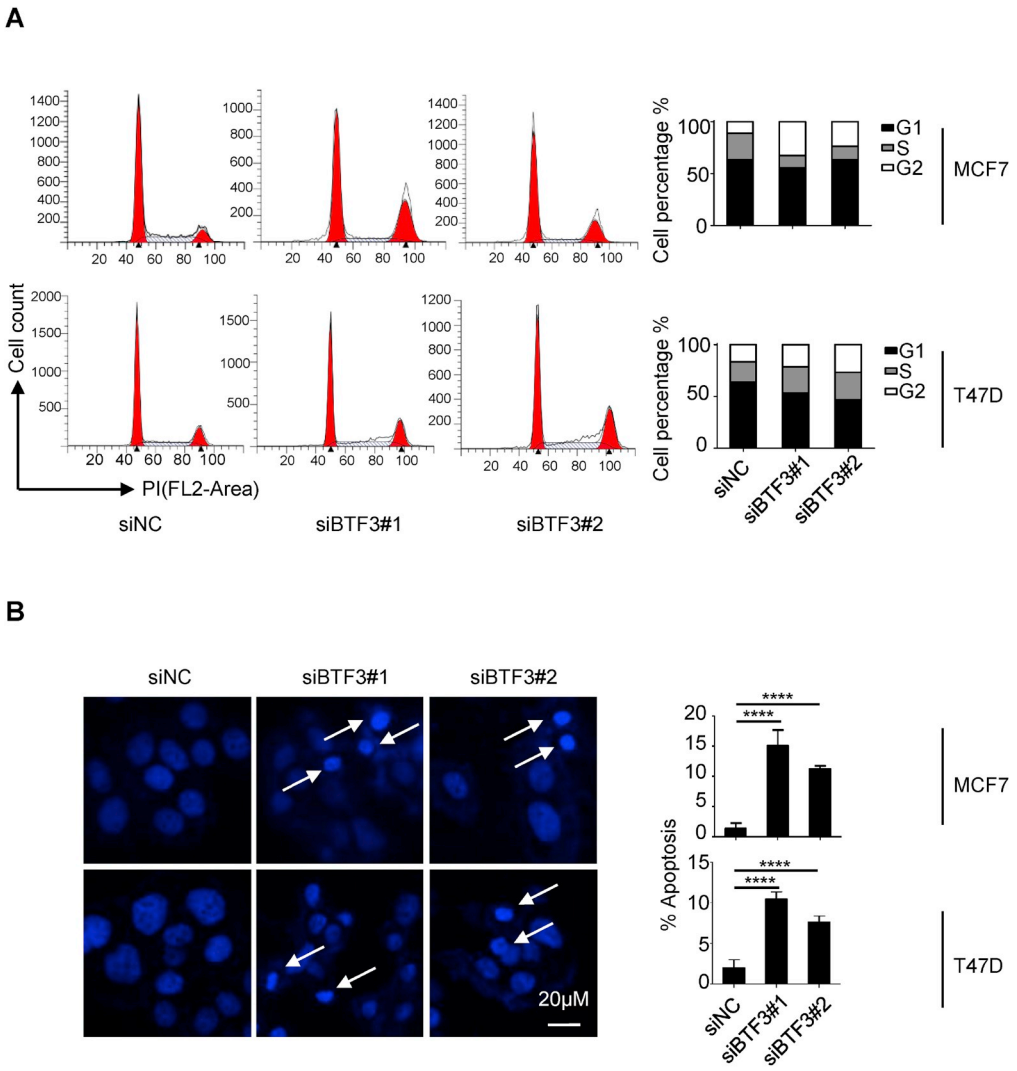

Fig. 3. BTF3 promotes the G2/M transition and blocks cell apoptosis in ER positive breast cancer cells. (A) A flow cytometry analysis was used to compare the DNA content between the siNC- or siBTF3-transfected breast cancer cells, as indicated. (B) Apoptosis was quantified by DAPI staining the siNC- or siBTF3-transfected breast cancer cells, as indicated. Scale bar, 20 μm ****$P < 0.0001$ (Student's *t*-test).

ESR1

5‘GAAAGGTGGGATACGAAAAGACC3‘ (Forward).
5′GCTGTTCTTCTTAGAGCGTTTGA3′ (Reverse).

PGR

5‘GGCATGGTCCTTGGAGGT3‘ (Forward). 5′CCACTGGCTGTGGGA GAG3′ (Reverse).

GREB1

5‘GTGGTAGCCGAGTGGACAAT3‘ (Forward).
5′ATTTGTTTCCAGCCCTCCTT3′ (Reverse).

IGFBP4

5′AACTTCCACCCCAAGCAGT3′ (Forward).
5′GGTCCACACACCAGCACTT3′ (Reverse).

2.9. Wound-healing assay

A wound-healing assay was used to evaluate cell migration as previously described [21]. Briefly, the cells were seeded in 24-well plates and were grown until a confluent state. and then, the cells were scratched using sterile tips. After 48 h, the cell monolayer was rinsed twice with PBS to remove the cell debris. Fresh culture medium was added. The mean width of each scratch was measured using Image Pro Plus (Media Cybernetics, USA).

2.10. Apoptosis assay

The cells were plated at 40% confluence, allowed to grow overnight and then subjected to the indicated inhibitors or siRNAs. To quantify apoptosis, the cells were then fixed for DAPI staining as previously described [22]. The images were captured with a Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.11. Clinical data analysis

The gene expression data were downloaded from TCGA or other published databases (http://www.ncbi.nlm.nih.gov/geo/). GSE7390 was for Desmedt_breast [23] and GSE2034 was for Wang_breast [24]. In the dot plot graphs, each dot indicates an individual sample, with the results expressed as the mean ± S.E.M.

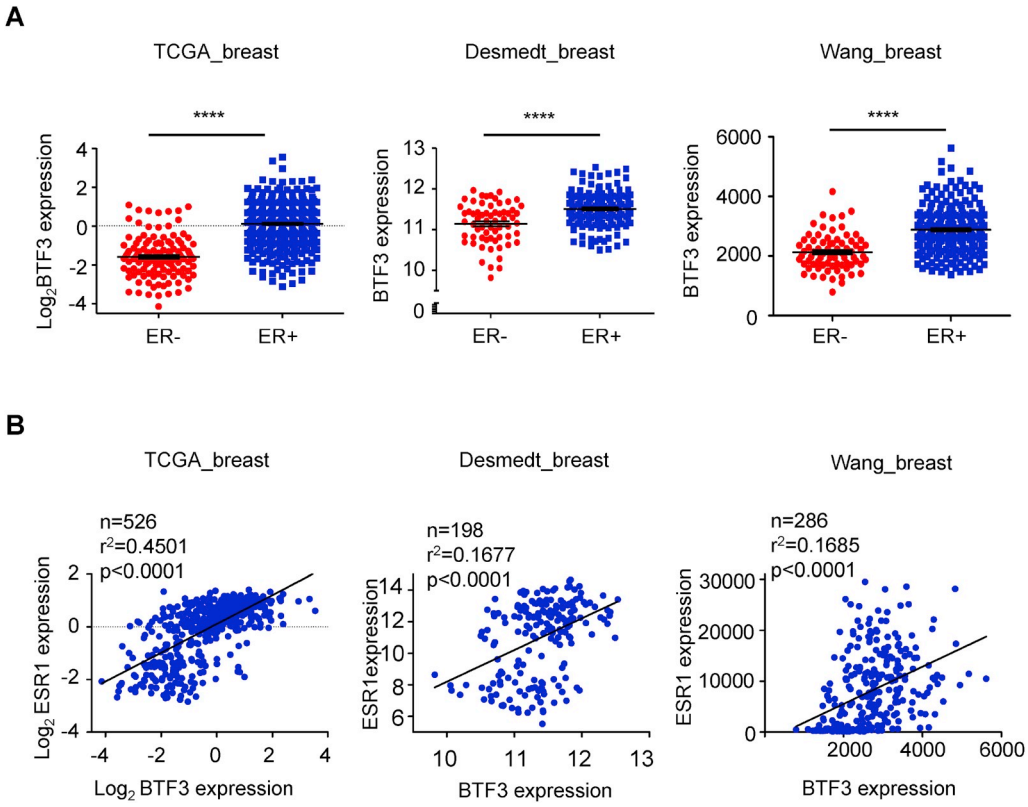

Fig. 4. *BTF3* and *ESR1* expression are tightly correlated. (A) The ER + breast tumors had higher *BTF3* expression levels than the ER-ones. The black lines in each group indicate the mean ± S.E.M. (Student's *t*-test) (B) The *BTF3* expression positively correlates with that of *ESR1*. Each circle represents an individual sample of human breast carcinoma (n = 526 for TCGA dataset; n = 198 for Bittner_dataset; n = 286 for Wang_breast). The correlation analysis was performed by GraphPad Prism. The linear regression Pearson's correlation coefficient (R^2) and its *P* value are indicated. ****$P < 0.0001$.

2.12. Establishment of tumor xenografts and in vivo treatments

One week prior to the tumor cell injection, 6-week-old female nude mice were implanted with 0.72 mg of 90-d release 17-estradiol pellets (Innovative Research). The MCF7 cells (6×10^6), in a PBS/Matrigel mixture, were injected orthotopically into the mammary fat pads. The drug treatment started when the tumor xenografts reached approximately 100 mm^3. BYL-719 was dissolved in 0.5% methylcellulose/0.5%Tween 80 and was administered once daily via oral gavage at 25 mg/kg. Both negative control siRNA and human *BTF3* siRNA (#1) were purified by high-performance liquid chromatography and were purchased from GenePharma (Suzhou, China). An intratumoral injection of siRNAs was conducted at four time points (as indicated in Fig. 8A), negative control siRNA or siRNA targeting *BTF3* was given at 20 μg per tumor each time using GP-siRNA-Mate plus (GenePharma). The tumor volumes were measured every other day with calipers and were calculated according to the following formula: tumor volume = (length × $width^2$)/2. All the animal experiments were carried out in accordance with the approval of the Animal Research Committee of Dalian Medical University.

2.13. Statistical analysis

The unpaired two-sided *t*-tests and the one-way ANOVA, with Tukey's multiple comparison tests, were performed using GraphPad Prism software for the analysis of the data obtained in the *in vitro* experiments and the *in vivo* animal studies, respectively. *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. BTF3 is commonly overexpressed in luminal breast tumors

When we analyzed *BTF3* expression in the breast cancer data set of the Cancer Genome Atlas with PAM50 classification [25] (TCGA), we found that higher expression levels of *BTF3* were strongly associated with the luminal subtype of breast cancers (Fig. 1A). In contrast, *BTF3* expression was significantly lower in the basal-like breast cancer samples than in the HER2-enriched breast tissues. We further analyzed the status of *BTF3* expression in the TCGA dataset with an alternative categorization of the breast cancer subtypes that is routinely used in the clinic for diagnosis [26,27]. *BTF3* expression was significantly higher in the ER/PR + subtype than in the TNBC. It was also notable that even within the HER2+ subtype, the ER/PR + tumors had a much higher *BTF3* expression than the ER/PR-tumors (Fig. 1B). Together, these data indicated that *BTF3* expression was significantly elevated in the luminal breast tumors.

3.2. BTF3 has a strong oncogenic ability in luminal breast cancer

We next determined if BTF3 played an oncogenic role in luminal breast cancer. We first confirmed that BTF3 knockdown by siRNA resulted in the downregulation of BTF3 mRNA and protein in MCF7 and T47D luminal breast cancer cells, respectively (Fig. 2A). We next showed that BTF3 knockdown significantly reduced the growth of MCF7 and T47D cells cultured in monolayer for 3 days and 14 days, respectively (Fig. 2B–C). To further examine the oncogenic role of BTF3 in luminal breast cancer, we assessed the effect of BTF3 on the growth of breast cancer cells cultured in 3D Matrigel, which is a condition that closely mimics the natural microenvironment. Indeed, BTF3 knockdown markedly attenuated the 3D spheroid growth of the MCF7 and T47D cells (Fig. 2D). To examine if BTF3 affected cellular migration, we conducted the wound-healing assays. We found that the gap between the scratched area was larger in the BTF3 knockdown group than in the control group 48 h postwounding (Fig. 2E). Importantly, restoring BTF3 expression rescued the growth inhibitory effect caused by the BTF3 knockdown (Fig. 2F–G). Together, these results suggest that BTF3 has strong oncogenic ability in luminal breast cancer cells.

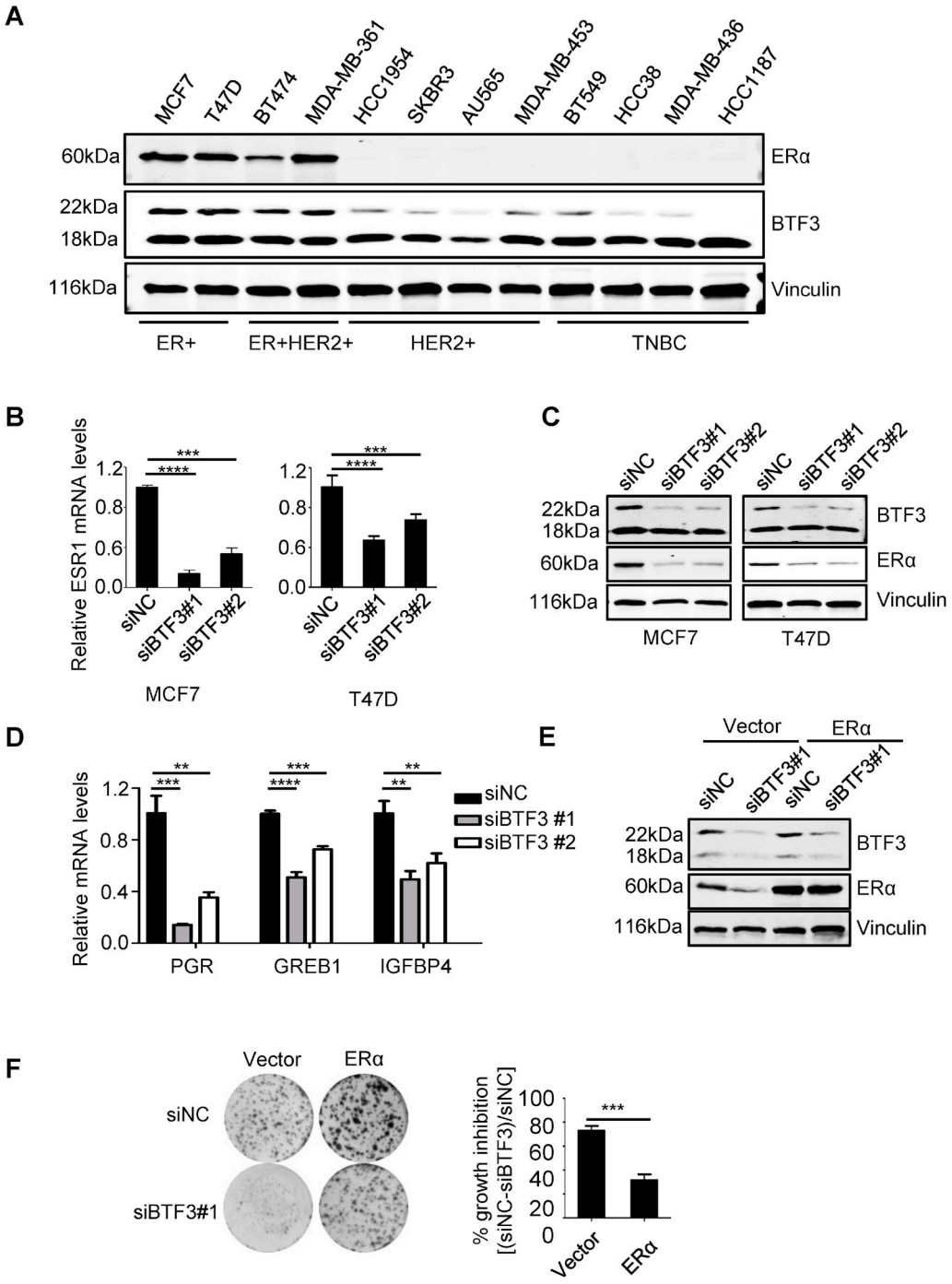

Fig. 5. BTF3 knockdown inhibits the expression and function of ERα. (A) Cell lysates prepared from the indicated breast cancer cell lines were immunoblotted with the BTF3 and ERα antibodies. Vinculin was used as the loading control. (B) The *ESR1* mRNA levels were determined by a quantitative RT-PCR analysis in the siNC- or siBTF3-transfected breast cancer cells, as indicated. (C) The protein abundance of BTF3 and ERα was determined by an immunoblotting analysis in the siNC- or siBTF3-transfected breast cancer cells, as indicated. Vinculin was used as a loading control. (D) *PGR*, *GREB1* and *IGFBP4* mRNA levels were determined by a quantitative RT-PCR analysis in the siNC- or siBTF3-transfected breast cancer cells, as indicated. The mean ± S.D. of three independent experiments is shown. (E) The protein abundance of BTF3 and ERα was determined by an immunoblotting analysis in the siNC- or siBTF3-transfected overexpressing breast cancer cells with or without the ectopic expression of ERα. Vinculin was used as the loading control. (F) The breast cancer cells, as indicated, were cultured for 10 days and were then crystal violet stained. Fresh media was replaced every three days. The error bars represent the mean ± S.D. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student's *t*-test).

3.3. BTF3 is involved in the regulation of the G2/M transition and cell survival in luminal breast cancer cells

As BTF3 promotes cell growth, we next assessed its effect on cell cycle progression. The flow cytometry data analysis revealed that the percentage of cells in the G2/M phase was significantly higher in siBTF3-transfected MCF7 cells than in siNC transfected cells 48 h after serum stimulation (Fig. 3A). Similar results were observed in the T47D cells. We also examined the effect of BTF3 on cell survival and found that BTF3 knockdown induced apoptotic cell death, which was characterized by cytoplasmic shrinkage, nuclear fragmentation and chromatin condensation [22] (Fig. 3B). Together, these results suggest a role for BTF3 in the regulation of G2/M transition and cell survival in luminal breast cancer cells.

3.4. BTF3 regulates ERα expression in luminal breast cancer

In line with the observation that *BTF3* is predominantly overexpressed in luminal breast tumors (Fig. 1), an analysis of multiple large-sized breast cancer databases revealed that *BTF3* expression was substantially elevated in the ER + cohorts when compared to the ER- cohorts (Fig. 4A). We also identified a highly significant correlation between *BTF3* and *ESR1* expression in these cohorts (Fig. 4B). Interestingly, the immunoblotting analysis revealed that BTF3 was only overexpressed in breast cancer cell lines with a high abundance of ERα (Fig. 5A). Together, these results prompted us to further investigate the relationship between BTF3 and ERα.

Given that BTF3 was previously reported as a transcription factor [28–30] and its strong correlation with ERα expression, as shown in our

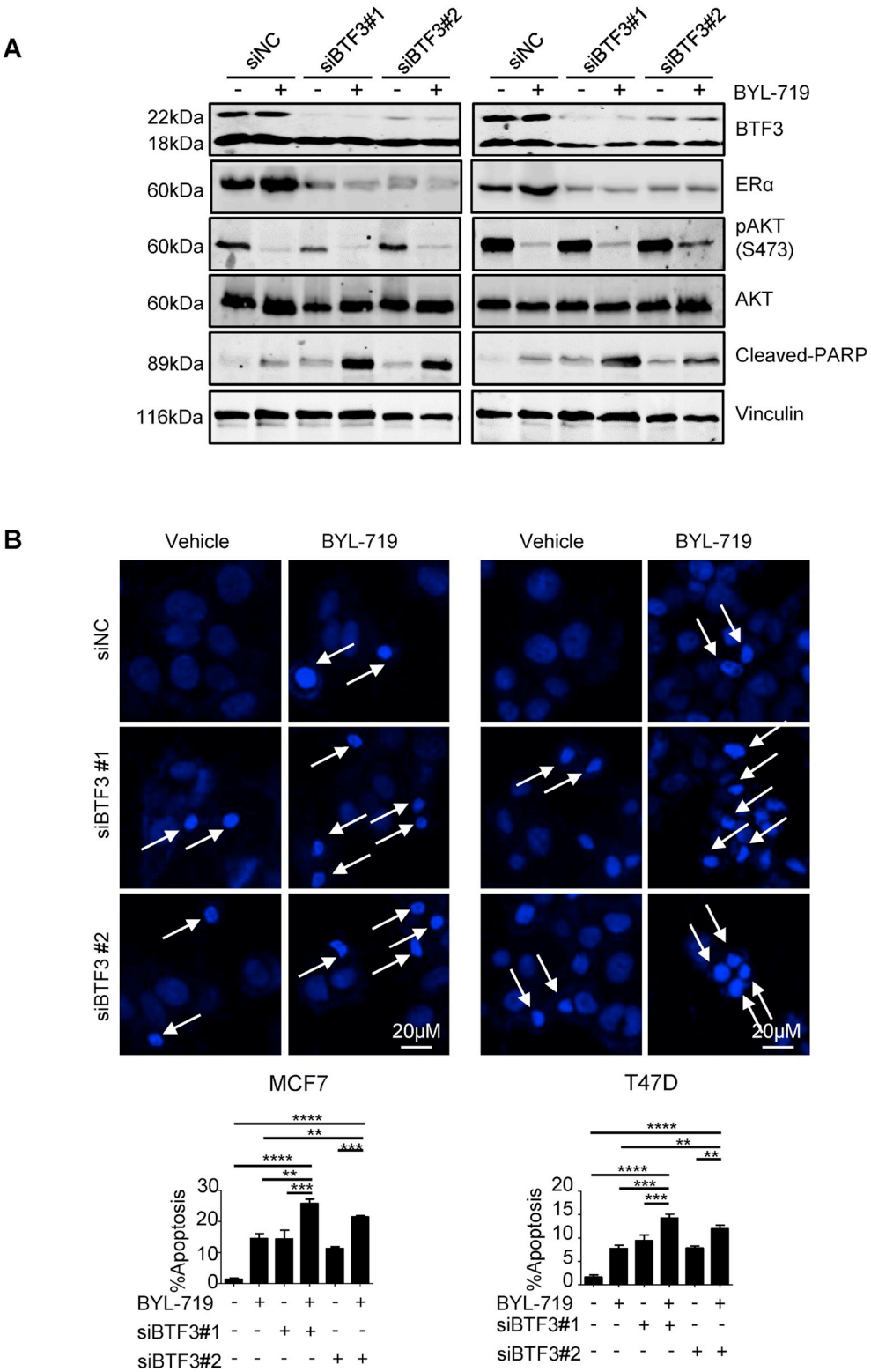

Fig. 6. BTF3 knockdown induces apoptosis in ER positive breast cancer cells upon BYL-719 treatment. (A) The siNC- or siBTF3-transfected breast cancer cells, as indicated, were treated with BYL-719 and were then subjected to an immunoblotting analysis. (B) Apoptosis was quantified by DAPI staining the siNC- or siBTF3-transfected breast cancer cells, as indicated. The error bars represent the mean ± S.D. Scale bar, 20 μm $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student's *t*-test).

study (Figs. 4 and 5A), we tested the hypothesis that BTF3 regulates ERα expression. Indeed, BTF3 knockdown led to a substantially reduced expression of ERα at the mRNA and protein levels (Fig. 5B–C). Furthermore, we showed that BTF3 knockdown also led to a reduced expression of the ERα-mediated transcriptional targets, *PGR*, *GREB1* and *IGFBP4* [31] (Fig. 5D). We next examined if BTF3 promoted the growth of luminal breast cancer cells in an ERα-dependent manner. For this, we ectopically expressed ERα in siBTF3 or siNC transfected MCF7

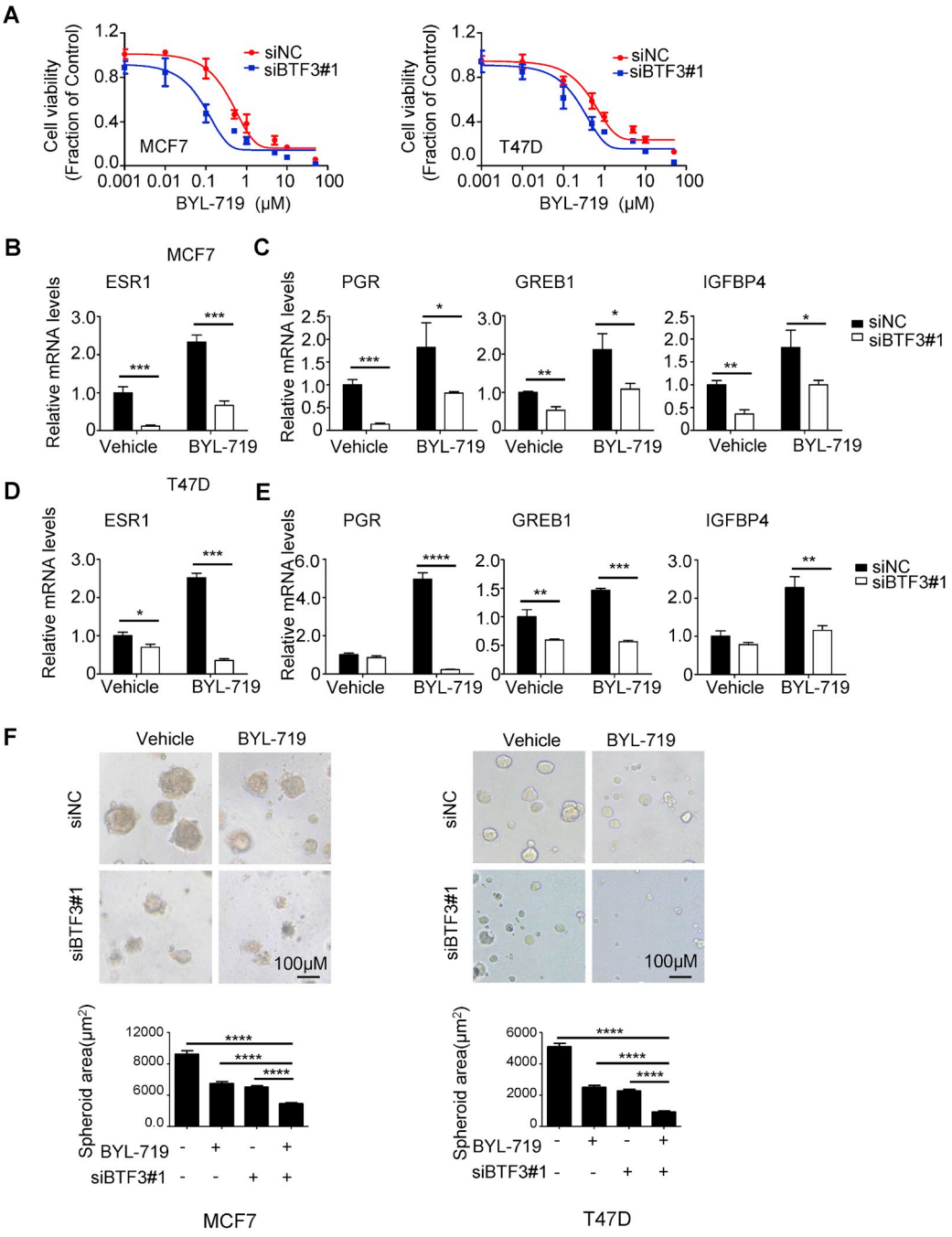

Fig. 7. BTF3 knockdown sensitizes ER positive breast cancer cells to BYL-719 treatment. (A) The cell viability was measured by a CCK8 assay using the siNC- or siBTF3-transfected breast cancer cells treated with BYL-719 for 72 h. The error bars represent the mean ± S.D. (B–E) The quantitative RT-PCR analysis of the *ESR1*,*PGR*, *GREB1* and *IGFBP4* mRNAs (B—C, MCF7 cells; D-E, T47D cells) was conducted in the siNC- or siBTF3-transfected breast cancer cells treated, as indicated, for 24 h. BYL-719, 2 μM. The error bars represent the mean ± S.D. (F) The siNC- or siBTF3-transfected breast cancer cells were cultured in 3D Matrigel with or without drug treatment for 10 days. BYL-719, 0.5 μM. Representative images of the 3D spheroid growth are shown. Quantification of 3D spheroid area was shown. The error bars represent the mean ± S.D. Scale bar, 100 μm $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student's *t*-test).

cells (Fig. 5E). Our results showed that the ectopic expression of ERα, at least in part, rescued the growth inhibitory effect caused by BTF3 silencing (Fig. 5F). Together, these data suggest that BTF3 contributes to the growth of luminal breast cancer cells, at least in part, through the transcriptional regulation of ERα.

3.5. BTF3 expression affects the response of luminal breast cancer cells to BYL-719 treatment

PIK3CA activating mutations are most frequently found in luminal/ER + breast tumors [1,5], justifying further investigating the pharmacological inhibition of PI3Kα isoform for the treatment of this disease. Recent studies reveal that the blockade of PI3K signaling results in the induction of ER-dependent transcriptional activity [11,12]. Since our data pointed to a role of BTF3 in the regulation of ERα transcription, we next tested if BTF3 affected the response of luminal breast cancer cells to the PI3Kα selective inhibitor BYL-719 treatment. As expected, BYL-719 nearly completely abolished phosphorylated AKT (pAKT) levels in the MCF7 and T47D cells, which was indicative of the blockade of PI3K/AKT signaling as a result of PI3Kα inhibition (Fig. 6A). Similar to the previous reports [11,12], BYL-719 treatment did lead to a striking increase in ERα protein expression. Interestingly, while either BYL-719 or, siBTF3 alone, as single-agents resulted in a moderate increase in the apoptotic signal as determined by a western blot analysis of cleaved PARP protein abundance, the combination of BTF3 knockdown and BYL-719 induced more substantial apoptosis (Fig. 6A–B). These results promoted us to further examine if BTF3 blockade sensitized ER + breast cancer cells to PI3Kα inhibition.

Consistent with our finding on the role of BTF3 in the regulation of ERα expression, BTF3 knockdown reduced the overall cell viability in the BYL-719-treated MCF7 cells and, to a lesser extent, in the T47D cells (Fig. 7A). Furthermore, while the BYL-719 treatment resulted in a

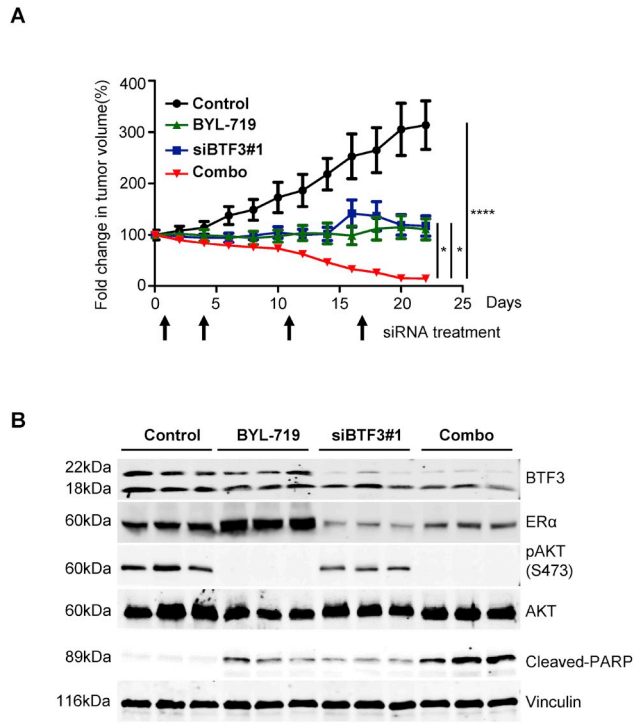

Fig. 8. Combination of BYL-719 and siBTF3 *in vivo* induces prolonged responses. (A) The MCF7 xenografts were treated with control, siBTF3, BYL-719 or the combination, at the indicated schedule. The graph shows the fold change in tumor volume, with respect to the initial treatment, at day 0. Control (Vehicle + siNC), n = 8; siBTF3 (Vehicle + siBTF3), n = 12; BYL-719 (BYL-719 + siNC), n = 11; Combo (BYL-719 + siBTF3), n = 12. siNC or siBTF3#1 was given at 20 μg per tumor each time; BYL-719, 25 mg/kg/day. $^{*}P < 0.05$ and $^{****}P < 0.0001$ by a one-way ANOVA, with Tukey's multiple comparison tests. The error bars represent the mean ± S.E.M. (B) The MCF7 xenograft tumor-bearing mice were treated as in (A) for 5 days and were sacrificed 3.5 h after the last dose. The tumor lysates were harvested, and the protein abundance was determined by an immunoblotting analysis.

remarkable elevation of *ESR1* mRNA expression in both the MCF7 and T47D cells [11,12], BTF3 knockdown substantially attenuated the induced expression of *ESR1* as well as the ERα -mediated transcriptional targets (Fig. 7B–E), at least partially explaining the observation that BTF3 knockdown sensitized the luminal breast cancer cells to the BYL-719 treatment (Fig. 7A). Finally, we confirmed that the combined use of siBTF3 and BYL-719 resulted in a significantly attenuated 3D spheroid growth of the MCF7 and T47D cells (Fig. 7F). siBTF3, in combination with the pan-PI3K inhibitor BKM-120, but not the PI3Kβ inhibitor AZD-6482, yielded similar observations (Supplementary Fig. 1). Together, these results suggest that BTF3 knockdown may sensitize the response of luminal/ER + breast cancer cells to PI3Kα inhibition by BYL-719 treatment.

3.6. BTF3 knockdown sensitizes estrogen receptor (ER)-positive breast cancer cells to BYL-719 in vivo

The fact that PI3Kα inhibitors induce a robust compensatory increase in the expression of ERα and its transcriptional targets has limited the therapeutic efficacy in estrogen receptor-positive breast cancer [11,12]. We next used siRNA-mediated BTF3 knockdown to ask whether the inhibition of BTF3 abrogates the induction of ERα expression and enhances the anti-tumor efficacy of the PI3Kα inhibitor BYL-719 in the well-established ER+/PIK3CA mutant MCF7 breast tumor xenograft model. Single-agent BYL-719 led to only tumor stasis and resulted in the induction of ERα expression, despite the suppression of pAKT (Fig. 8A–B). Meanwhile, treatment with the siRNA targeting BTF3 also resulted in tumor stasis but with a significant decrease in ERα expression, which was consistent with our *in vitro* finding that BTF3 may play an oncogenic role by regulating ERα expression (Fig. 8A–B). Moreover, silencing BTF3, combined with the BYL-719 treatment, resulted in dramatic tumor regressions (Fig. 8A). Concordantly, the tumors in the mice treated with the combination experienced suppression of both pAKT and ERα and the induction of apoptosis (Fig. 8B). Together, these results suggest that BTF3 silencing sensitizes ER + breast cancer cells to PI3Kα inhibition through the suppression of ERα expression *in vivo*.

4. Discussion

Up to 70% of breast cancers are luminal-like subtypes that are estrogen receptor positive [1,2]. Compared to other subtypes, luminal/ER + breast cancers are typically associated with a favorable prognosis [32]. However, patients with luminal-like breast cancer typically relapse following long-term endocrine therapies or years after the therapies end. Activating PIK3CA mutations are most often seen in luminal breast cancer, and the recent clinical development of PI3Kα inhibitors in this disease has yielded promising results [1,6,33]. Nevertheless, adaptive resistance or recurrence inevitably occurs due to the induction of ERα expression during the course of the PI3K-targeted therapy [11,12]. ERα is a master driver for the transcription of genes involved in tumor growth and survival in luminal breast cancer, and thus, the activation of ERα upon PI3K inhibition limits the efficacy of PI3Kα inhibitors in this disease [11,12,34]. In the current study, we reported that BTF3 played an oncogenic role in luminal/ER + breast cancer through the transcriptional regulation of ERα and may serve as a potential marker to predict the response to PI3Kα inhibition in this disease.

While BTF3 overexpression has been associated with a number of other cancer types, including colorectal [17], pancreatic [13,15], prostate [16], gastric [18] and brain cancers [14], our study shows that high BTF3 expression seems to be unique to luminal breast cancer. Nevertheless, it remains to be understood regarding the mechanism underlying high levels of BTF3 expression in this particular subtype of breast cancer and other human malignancies. Through a comprehensive analysis of databases with multiple cohorts of breast cancer, we found BTF3 to be significantly overexpressed in luminal breast cancer compared to normal-like or other subtypes of breast cancer. Strikingly, in the multiple independent breast cancer cohorts analyzed, the expression levels of BTF3 and ERα demonstrated a substantial, significant positive correlation across all the luminal-subtype breast cancer samples and cancer cell lines examined. These observations led us to consider whether BTF3 overexpression transcriptionally upregulates ERα expression. This indeed proved to be the case, and we found that BTF3 knockdown led to a concordant decrease in ERα mRNA and protein in luminal breast cancer cell lines with a high BTF3 expression and, consequently, a reduction in cell proliferation, survival and migration. Consistent with the dependency of luminal breast cancer cells on ERα-mediated signaling [34], BTF3 knockdown induced apoptosis in the MCF7 and T47D ER + breast cancer cells. In line with our hypothesis, BTF3 knockdown also led to the downregulation of ERα-mediated transcription. In addition, the ectopic expression of ERα partially rescued the growth inhibitory effect caused by BTF3 knockdown. Together, our data suggest that the oncogenic role of BTF3 in ER + positive breast cancer is, at least in part, through the transcriptional regulation of ERα targets.

In the current study, we speculate that BTF3 may serve as a biomarker of resistance to PI3K monotherapy in ER + breast cancers harboring PIK3CA alterations. Our findings highlight the importance of the transcriptional factor BTF3 in ER + breast cancers and its potential as a therapeutic target. While a previous study indicated that BTF3 may cooperate with ERα to regulate the transcription of ER target genes [35], interestingly, for the first time, our study reported that BTF3 was critical to ERα expression, thus adding an additional layer of

complexity to the pathogenesis of ER + breast cancer. Indeed, the impact of BTF3 expression on the response to targeted therapies in ER + breast cancer cells, as we found in this study, may expand to other regulators of ER transcription. Our additional data indicate that BTF3 knockdown also results in the decreased proliferation of ER-negative breast cancer cells that retain a low but discernible BTF3 protein abundance (Supplementary Fig. 2). Future studies will be conducted to explore the alternative mechanisms underlying the oncogenic role of BTF3 in the ER-negative subtype of breast cancer.

In summary, our findings highlight the importance of the transcriptional factor BTF3 in luminal/ER + breast cancers and its potential as a marker to predict the response to PI3Kα inhibition in ER + breast cancer. The discovery of the role of BTF3 in luminal/ER + breast cancer provides a rationale for targeting regulators of ERα transcription-based combination therapies with PI3K inhibitors in ER + breast cancer.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [No. 81472447 and 81672575 to H Cheng; No. 81372853 and 81572586 to P Liu], the Liaoning Provincial Climbing Scholars Supporting Program of China [H Cheng, P Liu], the Liaoning Provincial Science and Technology Program for Oversea Talents [H Cheng], the Provincial Natural Science Foundation of Liaoning [No. 2014023002 to P Liu, 2015020316 to F Luo] and the Liaoning Provincial Key Basic Research Program for Colleges and Universities [No. LZ2017002 to H Cheng].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.09.030.

References

[1] Comprehensive molecular portraits of human breast tumours, Nature 490 (2012) 61–70.
[2] N.C. Turner, P. Neven, S. Loibl, F. Andre, Advances in the treatment of advanced oestrogen-receptor-positive breast cancer, Lancet (London, England) 389 (2017) 2403–2414.
[3] E.A. Musgrove, R.L. Sutherland, Biological determinants of endocrine resistance in breast cancer, Nat. Rev. Canc. 9 (2009) 631–643.
[4] R. Jeselsohn, G. Buchwalter, C. De Angelis, M. Brown, R. Schiff, ESR1 mutations-a mechanism for acquired endocrine resistance in breast cancer, Nat. Rev. Clin. Oncol. 12 (2015) 573–583.
[5] P. Liu, H. Cheng, T.M. Roberts, J.J. Zhao, Targeting the phosphoinositide 3-kinase pathway in cancer, Nat. Rev. Drug Discov. 8 (2009) 627–644.
[6] Y. Samuels, Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S.M. Powell, G.J. Riggins, J.K. Willson, S. Markowitz, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, High frequency of mutations of the PIK3CA gene in human cancers, Science (New York, N.Y.) 304 (2004) 554.
[7] D. Juric, J. Rodon, J. Tabernero, F. Janku, H.A. Burris, J.H.M. Schellens, M.R. Middleton, J. Berlin, M. Schuler, M. Gil-Martin, H.S. Rugo, R. Seggewiss-Bernhardt, A. Huang, D. Bootle, D. Demanse, L. Blumenstein, C. Coughlin, C. Quadt, J. Baselga, Phosphatidylinositol 3-kinase alpha-selective inhibition with alpelisib (BYL719) in PIK3CA-altered solid tumors: results from the first-in-human study, J. Clin. Oncol.: Offic. J. Am. Soc. Clin. Oncol. (2018) JCO2017727107.
[8] C. Massacesi, E. Di Tomaso, P. Urban, C. Germa, C. Quadt, L. Trandafir, P. Aimone, N. Fretault, B. Dharan, R. Tavorath, S. Hirawat, PI3K inhibitors as new cancer therapeutics: implications for clinical trial design, OncoTargets Ther. 9 (2016) 203–210.
[9] I.A. Mayer, V.G. Abramson, L. Formisano, J.M. Balko, M.V. Estrada, M.E. Sanders, D. Juric, D. Solit, M.F. Berger, H.H. Won, Y. Li, L.C. Cantley, E. Winer, C.L. Arteaga, A phase Ib study of alpelisib (BYL719), a PI3Kα-specific inhibitor, with letrozole in ER+/HER2-Negative metastatic breast cancer, Clin. Canc. Res.: Offic. J. Am. Assoc. Canc. Res. 23 (2017) 26–34.
[10] L.M. Thorpe, H. Yuzugullu, J.J. Zhao, PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting, Nat. Rev. Canc. 15 (2015) 7–24.
[11] A. Bosch, Z. Li, A. Bergamaschi, H. Ellis, E. Toska, A. Prat, J.J. Tao, D.E. Spratt, N.T. Viola-Villegas, P. Castel, G. Minuesa, N. Morse, J. Rodon, Y. Ibrahim, J. Cortes, J. Perez-Garcia, P. Galvan, J. Grueso, M. Guzman, J.A. Katzenellenbogen, M. Kharas, J.S. Lewis, M. Dickler, V. Serra, N. Rosen, S. Chandarlapaty, M. Scaltriti, J. Baselga, PI3K inhibition results in enhanced estrogen receptor function and dependence in hormone receptor-positive breast cancer, Sci. Transl. Med. 7 (2015) 283ra251.
[12] E. Toska, H.U. Osmanbeyoglu, P. Castel, C. Chan, R.C. Hendrickson, M. Elkabets, M.N. Dickler, M. Scaltriti, C.S. Leslie, S.A. Armstrong, J. Baselga, PI3K pathway regulates ER-dependent transcription in breast cancer through the epigenetic regulator KMT2D, Science (New York, N.Y.) 355 (2017) 1324–1330.
[13] G. Kusumawidjaja, H. Kayed, N. Giese, A. Bauer, M. Erkan, T. Giese, J.D. Hoheise, H. Friess, J. Kleeff, Basic transcription factor 3 (BTF3) regulates transcription of tumor-associated genes in pancreatic cancer cells, Cancer Biol. Ther. 6 (2007) 367–376.
[14] F. Odreman, M. Vindigni, M.L. Gonzales, B. Niccolini, G. Candiano, B. Zanotti, M. Skrap, S. Pizzolitto, G. Stanta, A. Vindigni, Proteomic studies on low- and high-grade human brain astrocytomas, J. Proteome Res. 4 (2005) 698–708.
[15] B. Zheng, J. Peng, A. Mollayup, A. Bakri, L. Guo, J. Zheng, H. Xu, Construction of a prognostic prediction system for pancreatic ductal adenocarcinoma to investigate the key prognostic genes, Mol. Med. Rep. 17 (2018) 216–224.
[16] A.J. Symes, M. Eilertsen, M. Millar, J. Nariculam, A. Freeman, M. Notara, M.R. Feneley, H.R. Patel, J.R. Masters, A. Ahmed, Quantitative analysis of BTF3, HINT1, NDRG1 and ODC1 protein over-expression in human prostate cancer tissue, PLoS One 8 (2013) e84295.
[17] C.J. Wang, H. Franbergh-Karlson, D.W. Wang, G. Arbman, H. Zhang, X.F. Sun, Clinicopathological significance of BTF3 expression in colorectal cancer, Tumour Biol.: J. Int. Soc. Oncodevelopmental Biol. Med. 34 (2013) 2141–2146.
[18] Q. Liu, J.P. Zhou, B. Li, Z.C. Huang, H.Y. Dong, G.Y. Li, K. Zhou, S.L. Nie, Basic transcription factor 3 is involved in gastric cancer development and progression, World J. Gastroenterol. 19 (2013) 4495–4503.
[19] H. Cheng, P. Liu, F. Zhang, E. Xu, L. Symonds, C.E. Ohlson, R.T. Bronson, S.M. Maira, E. Di Tomaso, J. Li, A.P. Myers, L.C. Cantley, G.B. Mills, J.J. Zhao, A genetic mouse model of invasive endometrial cancer driven by concurrent loss of Pten and Lkb1 Is highly responsive to mTOR inhibition, Cancer Res. 74 (2014) 15–23.
[20] D. Wang, C. Li, Y. Zhang, M. Wang, N. Jiang, L. Xiang, T. Li, T.M. Roberts, J.J. Zhao, H. Cheng, P. Liu, Combined inhibition of PI3K and PARP is effective in the treatment of ovarian cancer cells with wild-type PIK3CA genes, Gynecol. Oncol. 142 (2016) 548–556.
[21] D. Wang, M. Wang, N. Jiang, Y. Zhang, X. Bian, X. Wang, T.M. Roberts, J.J. Zhao, P. Liu, H. Cheng, Effective use of PI3K inhibitor BKM120 and PARP inhibitor Olaparib to treat PIK3CA mutant ovarian cancer, Oncotarget 7 (2016) 13153–13166.
[22] V. Nogueira, Y. Park, C.C. Chen, P.Z. Xu, M.L. Chen, I. Tonic, T. Unterman, N. Hay, Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis, Cancer Cell 14 (2008) 458–470.
[23] C. Desmedt, F. Piette, S. Loi, Y. Wang, F. Lallemand, B. Haibe-Kains, G. Viale, M. Delorenzi, Y. Zhang, M.S. d'Assignies, J. Bergh, R. Lidereau, P. Ellis, A.L. Harris, J.G. Klijn, J.A. Foekens, F. Cardoso, M.J. Piccart, M. Buyse, C. Sotiriou, Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series, Clin. Canc. Res.: Offic. J. Am. Assoc. Canc. Res. 13 (2007) 3207–3214.
[24] Y. Wang, J.G. Klijn, Y. Zhang, A.M. Sieuwerts, M.P. Look, F. Yang, D. Talantov, M. Timmermans, M.E. Meijer-van Gelder, J. Yu, T. Jatkoe, E.M. Berns, D. Atkins, J.A. Foekens, Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer, Lancet (London, England) 365 (2005) 671–679.
[25] J.S. Parker, M. Mullins, M.C. Cheang, S. Leung, D. Voduc, T. Vickery, S. Davies, C. Fauron, X. He, Z. Hu, J.F. Quackenbush, I.J. Stijleman, J. Palazzo, J.S. Marron, A.B. Nobel, E. Mardis, T.O. Nielsen, M.J. Ellis, C.M. Perou, P.S. Bernard, Supervised risk predictor of breast cancer based on intrinsic subtypes, J. Clin. Oncol.: Offic. J. Am. Soc. Clin. Oncol. 27 (2009) 1160–1167.
[26] M. Gnant, N. Harbeck, C. Thomssen, St. Gallen 2011: summary of the consensus discussion, Breast care (Basel, Switzerland) 6 (2011) 136–141.
[27] M. Gnant, N. Harbeck, C. Thomssen, St Gallen/vienna 2017: a brief summary of the consensus discussion about escalation and de-escalation of primary breast cancer treatment, Breast care (Basel, Switzerland) 12 (2017) 102–107.
[28] B. Cavallini, J. Huet, J.L. Plassat, A. Sentenac, J.M. Egly, P. Chambon, A yeast activity can substitute for the HeLa cell TATA box factor, Nature 334 (1988) 77–80.
[29] X.M. Zheng, D. Black, P. Chambon, J.M. Egly, Sequencing and expression of complementary DNA for the general transcription factor BTF3, Nature 344 (1990) 556–559.
[30] X.M. Zheng, V. Moncollin, J.M. Egly, P. Chambon, A general transcription factor forms a stable complex with RNA polymerase B (II), Cell 50 (1987) 361–368.
[31] J.S. Carroll, C.A. Meyer, J. Song, W. Li, T.R. Geistlinger, J. Eeckhoute, A.S. Brodsky, E.K. Keeton, K.C. Fertuck, G.F. Hall, Q. Wang, S. Bekiranov, V. Sementchenko, E.A. Fox, P.A. Silver, T.R. Gingeras, X.S. Liu, M. Brown, Genome-wide analysis of estrogen receptor binding sites, Nat. Genet. 38 (2006) 1289–1297.
[32] C. Sotiriou, S.Y. Neo, L.M. McShane, E.L. Korn, P.M. Long, A. Jazaeri, P. Martiat, S.B. Fox, A.L. Harris, E.T. Liu, Breast cancer classification and prognosis based on gene expression profiles from a population-based study, Proc. Natl. Acad. Sci. U. S. A 100 (2003) 10393–10398.
[33] S.J. Isakoff, J.A. Engelman, H.Y. Irie, J. Luo, S.M. Brachmann, R.V. Pearline, L.C. Cantley, J.S. Brugge, Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells, Cancer Res. 65 (2005) 10992–11000.
[34] C. Thomas, J.A. Gustafsson, The different roles of ER subtypes in cancer biology and therapy, Nat. Rev. Canc. 11 (2011) 597–608.
[35] C.D. Green, P.D. Thompson, P.G. Johnston, M.K. El-Tanani, Interaction between transcription factor, basal transcription factor 3, and the NH2-terminal domain of human estrogen receptor alpha, Mol. Canc. Res.: MCR 5 (2007) 1191–1200.