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GDF11 knockdown downregulates SMURF1 to inhibit breast cancer progression by activation of p53 and inactivation of ERα signaling

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Breast cancer (BC) is a prevalent neoplasm that occurs in women all over the world. Growth and differentiation factor 11 (GDF11) plays an essential role in cancer progression. This study focused on investigating the biological role and underlying mechanisms of GDF11 in BC. We detected the expression of GDF11 in 27 patients with BC and BC cell lines. Kaplan-Meier plotter was employed to analyze the relationship between GDF11 expression and overall survival (OS) of BC patients. The proliferative, migratory, invasive, and apoptotic abilities of T47D cells were examined. Correlation analysis of GDF11 with Smad ubiquitination regulatory factor 1 (SMURF1) was conducted. The association between GDF11 and the p53 pathway was analyzed by western blot and PFT-α (a p53 inhibitor)-mediated rescue assays. A brief analysis of the role of estrogen receptor alpha (ERα) signaling in BC progression was performed. The results showed that GDF11 was increased in BC tissues and cell lines, and the high expression of GDF11 was associated with the poor OS of BC patients. GDF11 knockdown inhibited the proliferation, migration, and invasion of T47D cells, but promoted cell apoptosis. Meanwhile, the GDF11 knockdown reduced the SMURF1 expression and invoked the p53 pathway activation. SMURF1 overexpression and PFT-α partially blocked the effects of GDF11 knockdown. In addition, GDF11 knockdown and SMURF1 silencing inhibited the activation of the ERα signaling pathway. In summary, GDF11 was involved in the progression of BC by regulating SMURF1-mediated p53 and ERα pathways, opening up a new way for BC treatment.

Key words: breast cancer, Erα, GDF11, p53, SMURF1

Breast cancer (BC) is the leading cause of increasing cancer morbidity and mortality in females [1]. Besides, BC is one of the sex hormone receptor-dependent tumors, and about two-thirds of patients in the clinic are with estrogen receptor alpha-positive ($ER\alpha^+$) BC [2]. Notably, some risk factors, such as genetic susceptibility and hormonal therapy, facilitate the development of BC. Despite the remarkable advances that have been made in the molecular hallmarks of BC in recent years, the early diagnosis of BC remains a challenging problem [3]. The emergence of targeted therapy has optimized the treatment of BC. Therefore, it is necessary to seek new biomarkers and investigate the regulatory mechanism to expand the molecular profile of anti-BC.

Growth and differentiation factor 11 (GDF11), also called bone morphogenetic protein (BMP11), is a member of the Transforming Growth Factor β (TGFβ) family and is widely expressed in human tissues and organs [4, 5]. The binding of GDF11 to the activin type II B receptor triggers the activation of the Smad2/3 pathway, playing an important role in multiple physiological and pathological processes [6]. During spinal cord development, GDF11 was involved in the formation of neurons and glial cells, as well as the differentiation of progenitor cells [7]. GDF11-mediated inhibition of erythrocyte differentiation and maturation contributed to the development of β-thalassemia [8]. The administration of GDF11 had a positive effect on the treatment of cardiac dysfunction [6]. Interestingly, GDF11 has a paradoxical role in cancer biology. In hepatocellular carcinoma, the tumor suppressive property of GDF11 was manifested by reducing cell proliferation, mitogenesis, and invasion [9]. There was a significant association between high expressions of GDF11 and poor prognosis in colorectal cancer patients [10]. Moreover, elevated GDF11 provided benefits for BC chemotherapy [11]. However, the role and regulatory mechanism of GDF11 in BC are still misty.

SMURF1 (Smad Specific E3 Ubiquitin Protein Ligase 1) is a HECT-type E3 ubiquitin ligase and negatively regulates BMP signaling pathway [12]. A previous study showed that SMURF1 silencing promoted the expressions of Smad to maintain bone homeostasis [13]. Besides, the overexpression of SMURF1 accelerated microglia cell apoptosis and inflammatory injury in the lipopolysaccharide (LPS)-induced spinal cord injury model [14]. Furthermore, SMURF1 interacts with abundant substrates to exert oncogenic effects in various cancers. Highly expressed SMURF1 destabilized phosphatase and tensin homolog deleted on chromosome ten (PTEN) to facilitate the tumorigenesis of prostate cancer [15]. The metastasis of cervical cancer is regulated by SMURF1-mediated FOX2 ubiquitination [16]. SMURF1 inhibited ERα protein degradation and thus enhanced the proliferation of BC cells [17].

In this study, we attempted to explore the role of GDF11 on BC cell proliferation, migration, invasion, and apoptosis. Based on tissue data, we performed a preliminary prognostic analysis of the relationship between GDF11 expression and overall survival (OS). Further, we revealed the possible downstream mechanism of GDF11 in association with SMURF1.

Patients and methods

Patient specimens. A total of 27 pairs of tumor and adjacent tissue specimens were obtained from patients who underwent surgery at Tangdu Hospital. None of these patients had ever received preoperative treatment. All tissue specimens were immediately frozen in liquid nitrogen and stored at –80 °C for subsequent experiments.

The study was performed with the approval of the Ethics Committee of Tangdu Hospital, in accordance with the Declaration of Helsinki. And all participants signed informed consents.

Cell culture. A normal human breast epithelial cell line MCF-10A (#CRL-10317) and five human BC cell lines: HCC1937 (#CRL-2336), Hs 606.T (#CRL-7368), MCF7 (#HTB-22), MDA-MB-231 (#HTB-26), and T47D (#HTB-133) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI 1640 medium (Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% L-glutamine (Gibco) at 37 °C with 5% CO_2.

Cell transfection and treatment. T47D cells were seeded in 24-well plates and then cultured to ~70–80% confluence. For transfection, the cells were transfected with short hairpin RNAs (shRNAs) of GDF11 (GDF11 shRNA-1; GDF11 shRNA-2; GDF11 shRNA-3), negative control (NC) shRNA (Genepharma, Shanghai, China), the overexpression plasmid of SMURF1 (pc-SMURF1) constructed from pcDNA3.1 vector (Invitrogen, USA) using Lipofectamine 3000™ (Invitrogen) according to the manufacturer's instructions. Simultaneously, the cells were treated with pifithrin-alpha (PFT-α; p53 inhibitor; Calbiochem, Merck, German) or estradiol (E2; Sigma, St. Louis, MO, USA) according to the protocols of instructions, respectively. The sequences of shRNAs were as follows: GDF11 shRNA-1 (GAGATGTAGAGACAGTGATAG); GDF11 shRNA-2 (CCTGCAGATCTTGCGACTAAA); GDF11 shRNA-3 (GATCGCTGTGGCTGCTCTTAA).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from BC cell lines and tissue specimens using TRIzol reagent (Invitrogen). Reverse transcription was performed according to the standard procedure of the PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Dalian, China). The relative mRNA levels were detected by using SYBR Premix Ex Taq (Takara) and the $2^{-\Delta\Delta Ct}$ method. The normalization standard for data was GAPDH. The primers used in the study were provided: GDF11-F: 5'-CAAGTCGCAGATCTTGAGCA-3'; GDF11-R: 5'-CACTTGCTTGAAGTCGATGC-3'; SMURF1-F: 5'-GAAACCCAATGGCAGAAA-3'; SMURF1-R: 5'-GCAGATGTTGAGGGATGAG-3'; GAPDH-F: 5'-GGGAAGGTGAAGGTCGGAGT-3'; GAPDH-R: 5'-TTGAGGTCAATGAAGGGGTCA-3'.

Gene expression profiling interactive analysis (GEPIA) database. The GEPIA web server (https://doi.org/gepia.cancer-pku.cn/index.html) was developed by Zhang lab of Peking University in 2017 [18], providing normalized analysis of RNA sequencing datasets from TCGA and GTEx databases to characterize gene expression profiles, intergenic relationships and the role of the gene in cancer prognosis. Using GEPIA, the expression of GDF11 (Ensembl ID: ENSG00000135414.9) in breast invasive carcinoma (BRCA) tissues and normal tissues was examined.

Kaplan-Meier plotter. Kaplan-Meier plotter (https://doi.org/kmplot.com/analysis/) was a statistical tool for cancer survival analysis of data from TGGA, GEO, and EGA databases. The correlation between GDF11 expressions and OS was investigated in the BC patients with 5-year follow-up after the operation. According to the quartiles, BC patients with GDF11 expression above 75% were classified as the high GDF11 expression group, and those below 25% were classified as the low GDF11 expression group. The BC patient samples from an online database (Affy ID: 216860_s_at) were sorted into two cohorts based on the median of GDF11 expression. Kaplan-Meier plotter was employed to evaluate the prognostic value of GDF11 expression for the OS of BC patients. The Kaplan-Meier survival curve was exported using GraphPad Prism 9 (San Diego, CA, USA).

Western blot. BC cell lines and tissue specimens were lysed using RIPA buffer (Beyotime, Shanghai, China) to obtain total proteins. After protein quantification with a BCA protein assay kit (Beyotime), the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate the target bands. Then the proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk for 1 h. After that, the membranes underwent antibody incubation.

The primary antibodies against GDF11 (1:500; ab234647, Abcam, Cambridge, UK), SMURF1 (1:1000; ab57573, Abcam), β-actin (1:1000; ab8227, Abcam), MDM2 (1:1000; ab260074, Abcam), p53 (1:1000; ab245685, Abcam), p21 (1: 1000; ab227443, Abcam), and ERα (1:1000; AE905, Beyotime) were incubated with the membranes overnight at 4 °C. Goat anti-rabbit IgG H&L (HRP) (1:10000, ab205718, Abcam) was used as the secondary antibody. The protein bands were exposed using a BeyoECL Plus Kit (Beyotime). β-actin served as an internal control for normalized analysis.

3-(4,5)-dimethylthiazo(-z-yl)-3,5-diphenytetrazolium bromide (MTT) assay. T47D cells were inoculated into 96-well plates at a density of 2×10^3 cells/well. After 24 h, 48 h, 72 h of transfection, the cells in each well were treated with 20 μl MTT solution (Beyotime) for 4 h. The crystals in cells were fully dissolved with 100 μl DMSO solution on a shaker for 15 min. Finally, the cell viability was measured by evaluating the absorbance at 570 nm using a microplate Reader (BioRad, Shanghai, China).

Colony formation assay. T47D cells were seeded into 6-well plates at a density of 500 cells/well and incubated for 7 d after transfection. Then the cells were fixed with 4% paraformaldehyde for 20 min and stained with 1% crystal violet (Beyotime). The images were photographed under a microscope (100×; Leica, Germany). The number of colonies was counted and analyzed.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Cell apoptosis was assessed using a One-step TUNEL Apoptosis Detection Kit (Beyotime). Briefly, T47D cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton-X 100 for 5 min. After washing, the cells were treated with 50 μl TUNEL solution at 37 °C in the dark for 1 h. Finally, the cell apoptosis rate was detected by a flow cytometer (Beckman Coulter, CA, USA).

Wound healing assay. When the transfected T47D cells reached 90% confluence in 6-well plates, a 200 μl sterile pipet tip was used to make a scratch on the surface of the cell monolayer. The migrated T47D cells were visualized at 0 h, 24 h, and 48 h under a microscope (100×; Leica, Germany). The migration rate of T47D cells was evaluating the degree of wound healing.

Transwell assay. Serum-free cultured T47D cells were added to the upper Transwell chamber pre-covered with 30 μg Matrigel (BD Biosciences, New Jersey, USA). And the lower chamber was supplemented with the medium containing 20% fetal bovine serum (FBS, Gibco). After incubation for 24 h, cells in the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet for 15 min. At last, a microscope (100×; Leica, Germany) was employed to calculate the number of invaded cells in five random fields.

Luciferase reporter assay. The shRNAs of GDF11 and SMURF1 were constructed by Genepharma (Shanghai, China). We cloned the human E2F Transcription Factor 1 (E2F1) promoter and estrogen-response element (ERE) into the pGL3-Basic reporter plasmids (Promega, Madison, WI, USA) carrying the luciferase genes, and named them as E2F1-luc and ERE-luc. Human ERα expression plasmid was constructed by inserting ERα encoding region into the pCMV empty vector (Clontech, Takara, Japan). T47D cells were co-transfected with the shRNAs and constructed plasmids using a Lipofectamine 3000™ (Invitrogen). After 48 h, the activity of luciferase was determined using a Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturer.

Statistical analysis. All data from at least three individual experiments were represented as mean ± SEM and analyzed with one-way ANOVA or Student's t-test using GraphPad Prism 9 software. A p-value <0.05 was considered as a statistical significance.

Results

GDF11 was upregulated in BC and correlated with poor prognosis. To characterize the role of GDF11, we first evaluated the expression pattern of GDF11 during BC progression. The results showed that GDF11 was highly expressed in human BC tissues and cell lines, and the highest expression was observed in T47D cells among the five BC cell lines (Figures 1A–1D). Therefore, T47D cells were chosen for the following research. Meanwhile, GEPIA database analysis indicated that GDF11 was significantly increased in BC samples compared with normal tissues (Figure 1E). Besides, the expression of GDF11 in patients at stage III–IV was found to be higher than those in stage I–II (Figure 1F). Our survival analysis indicated that BC patients with a high expression level of GDF11 had a shorter OS than those with low expressions of GDF11 (Figure 1G). Interestingly, Kaplan-Meier survival analysis also illustrated the same results (Figure 1H). Thus, these data suggested that GDF11 might play an essential role in BC progression and high expression of GDF11 was associated with the poor prognosis of BC patients.

GDF11 knockdown inhibited the progression of BC. An RNA interference technology was utilized to explore the role of GDF11 in the progression of BC. We first detected the transfection efficiency of shRNAs targeting GDF11 and found that shRNA-2 and shRNA-3 exhibited better interference efficiency compared with the NC shRNA group (Figures 2A, 2B). Therefore, GDF11 shRNA-2 and shRNA-3 were used as the GDF11 shRNAs for subsequent experiments. The results of MTT and colony formation assays indicated that GDF11 silencing remarkably inhibited the proliferation of T47D cells (Figures 2C, 2D). The apoptosis rate was notably elevated in the GDF11 silencing group compared with the NC shRNA group (Figure 2E). In addition, the knockdown of GDF11 reduced the number of migrated and invaded T47D cells (Figures 2F, 2G). These findings implied that GDF11 was involved in the progression of BC.

GDF11 knockdown deregulated SMURF1 to activate the p53 pathway. We speculated that there was a regula-

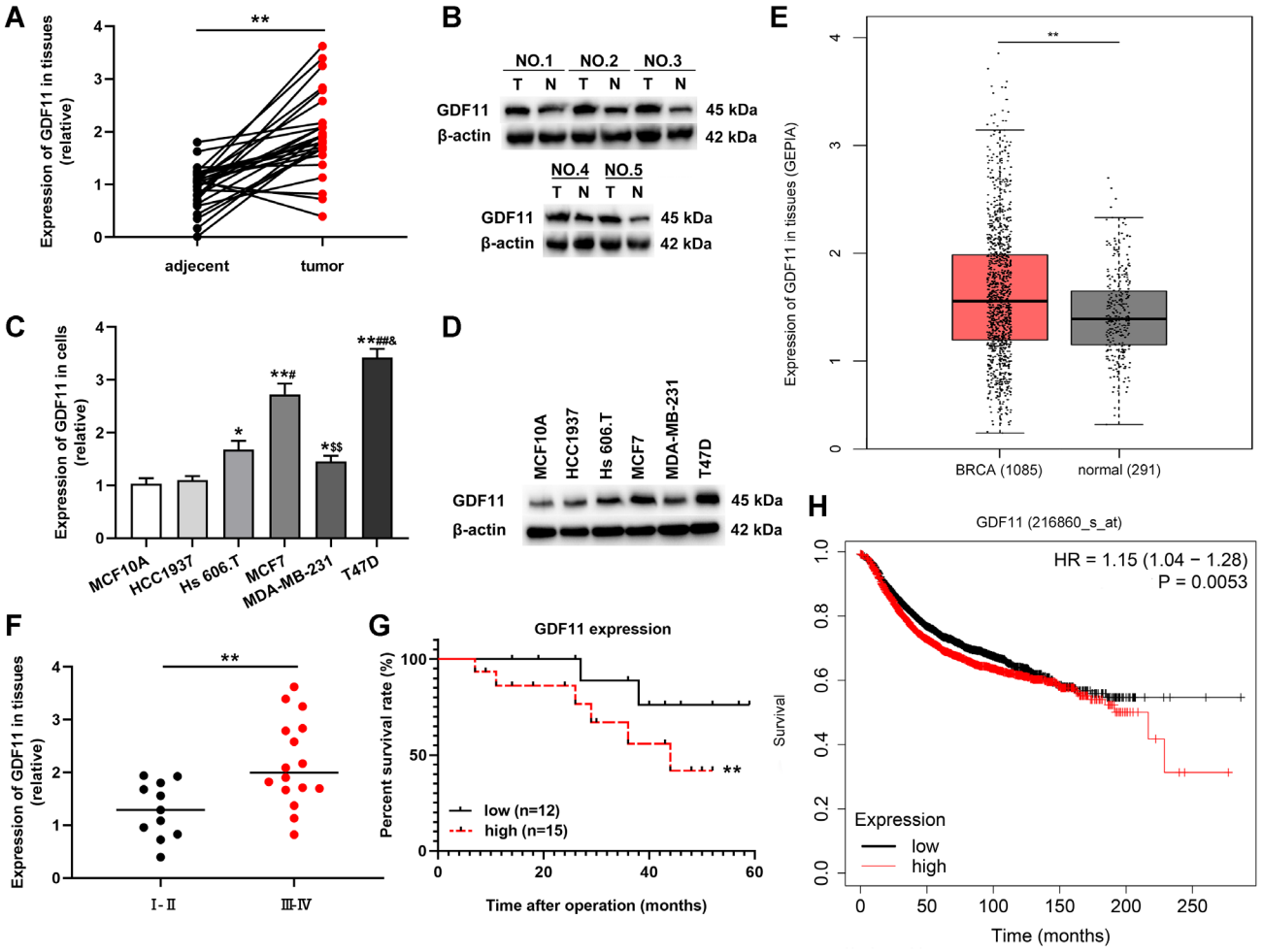

Figure 1. GDF11 was upregulated in BC and correlated with poor prognosis. A, B) qRT-PCR and western blot analyses of GDF11 expression levels in 27 couples of breast cancer (BC) tissues and adjacent normal tissues. **p<0.01 vs. adjacent tissues. C, D) qRT-PCR and western blot analysis of GDF11 expression levels in breast cancer cell lines (HCC1937, Hs 606.T, MCF7, MDA-MB-231, and T47D) and normal epithelial cells (MCF10A). *p<0.05, **p<0.01 vs. MCF10A; #p<0.05, ##p<0.01 vs. HS 606.T; $$p<0.01 vs. MCF7; &p<0.05 vs. MDA-MB-231. E) Expression analysis of GDF11 mRNA in BC tissues (n=1085) and normal tissues (n=291) based on the GEPIA database. **p<0.01 vs. normal tissues. Abbreviation: BRCA-breast invasive carcinoma. F) The expression of GDF11 in tissues of BC patients at different stages was evaluated using qRT-PCR. **p<0.01 vs. I-II stage. G) According to the quartile of GDF11 expression, 27 BC patients with follow-up information were divided into a low-expression group (n=12) and a high-expression group (n=15). The plotted survival curve of GDF11 in predicting the OS of BC cases. **p<0.01 vs. low expression of GDF11 cohort. H) Survival analysis of the level of GDF11 based on Kaplan-Meier Plotter. Each experiment was done at least in triplicates. Data are presented as mean ± SEM.

tory relationship between SMURF1 and GDF11. Thus, we first examined the transcription and expression of SMURF1. As depicted in Figures 3A–3C, SMURF1 was dramatically increased in BC tissues and cell lines compared with the control groups. Correlation analysis showed a positive correlation between SMURF1 and GDF11 expression (Figure 3D). Further investigations discovered that GDF11 knockdown declined the expression of SMURF1 in BC cells (Figures 3E, 3F). p53 pathway is one of the common typical pathways in BC. To identify whether GDF11/SMURF1 axis regulated the p53 pathway, we performed western blot to detect the expressions of key proteins in the p53 pathway. Among them, MDM2 is an essential target of the p53 gene, and p21 was a cell cyclin-dependent kinase inhibitor located downstream of the p53 gene. Our analysis revealed that compared with the MCF10A group, the expressions of MDM2, p53, and p21 were found to be markedly reduced in T47D cells. GDF11 knockdown enhanced MDM2, p53, and p21 expressions in BC cells, while SMURF1 overexpression and PFT-α (a p53 inhibitor) treatment reversed this effect (Figure 3G). These results confirmed that GDF11 knockdown regulated SMURF1 to activate the p53 signaling.

GDF11 knockdown downregulated SMURF1 to inhibit BC progression through the activation of the p53 pathway. To validate the effects of the GDF11/SMURF1/p53 axis in BC progression, we conducted a series of functional rescued

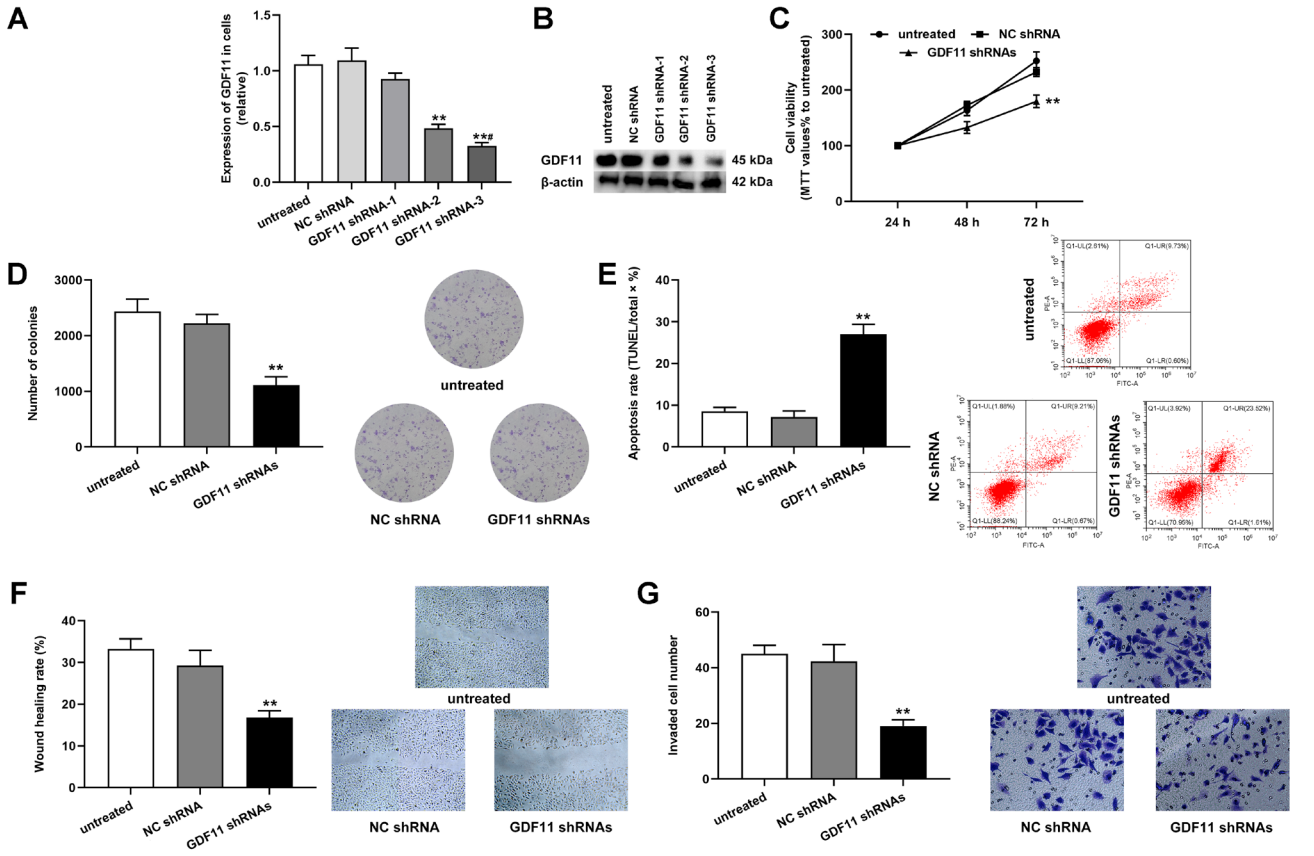

Figure 2. GDF11 knockdown inhibited the progression of BC. A, B) Determination of transfection efficiency of shRNAs targeting GDF11 by qRT-PCR and western blot assays. C, D) The effects of GDF11 on cell proliferation were detected using MTT assay and colony formation assay. E) The effects of GDF11 on cell apoptosis were measured using the TUNEL assay. F) After transfection for 24 h and 48 h, the effects of GDF11 on cell migration were examined using a wound-healing assay. G) The effects of GDF11 on cell invasion were evaluated using Transwell assay. Each experiment was done at least in triplicates. Data are presented as mean ± SEM. **p<0.01 vs. NC shRNA group; #p<0.05 vs. GDF11 shRNA-2 group.

experiments. The results indicated that SMURF1 overexpression and PFT-α treatment attenuated GDF11 knockdown-induced inhibition of proliferation, migration, and invasion in BC cells, and prevented GDF11 knockdown-mediated cell apoptosis (Figures 4A–4E). These data further clarified the tumor suppressive effects of GDF11 knockdown in BC progression through inhibition of SMURF1 expression to activate the p53 pathway.

GDF11 knockdown inhibited the activation of the SMURF1-mediated ERα signaling pathway in BC cells. ERα plays an important role in promoting BC development by forming a complex with ERE to initiate the transcription of target genes [19]. To investigate the link between GDF11 and ERα signaling pathway, we first detected the expressions of ERα and ERα-downstream target genes. As expected, GDF11 knockdown reduced the expressions of ERα, gene regulated in BC 1 protein (GREB1), PDZ domain containing 1 (PDZK1), and BC estrogen-inducing gene (PS2) in the presence of E2, whereas SMURF1 overexpression reversed these effects (Figures 5A, 5B). E2F1 and ERE are marker genes for ERα transcriptional activation. Luciferase reporter assays showed that GDF11 knockdown and SMURF1 silencing inhibited ERα-mediated luciferase activities of ERE and E2F1 (Figures 5C–5F). These data uncovered that GDF11 knockdown exerted its tumor inhibitory effects through the inactivation of the SMURF1-mediated ERα signaling pathway.

Discussion

BC is malignant cancer that threatens the health of women worldwide. It is urgent to understand new molecular mechanisms for therapeutic interventions in BC progression. Cumulative evidence has highlighted that GDF11 is an important modulator in the tumorigenesis of various human cancers. It was reported that GDF11 served as a tumor suppressor in hepatocellular carcinoma by inhibiting cell proliferation, cycle progression, spheroid formation, invasion, and epithelial to mesenchymal transition

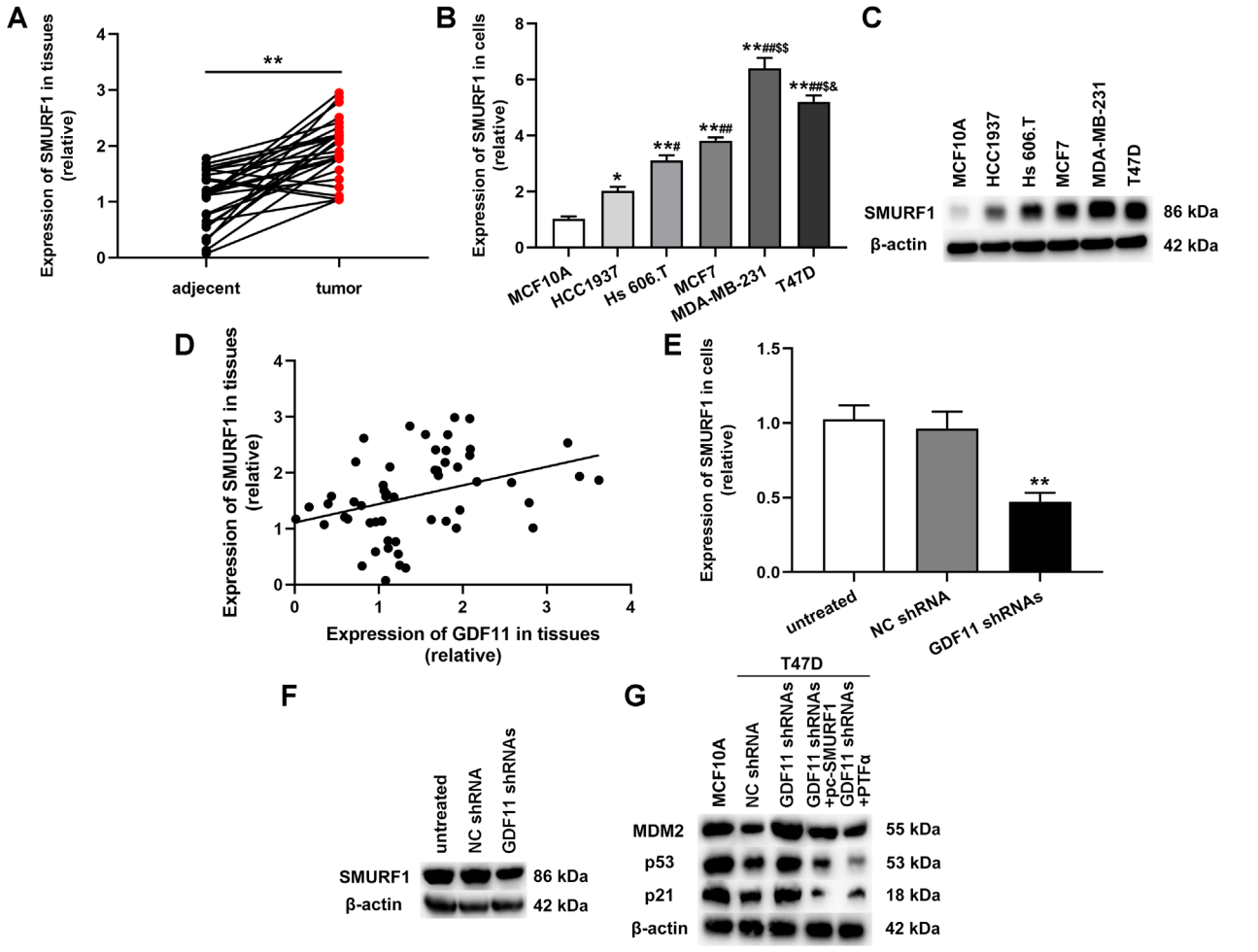

Figure 3. GDF11 knockdown deregulated SMURF1 to activate the p53 pathway. A) qRT-PCR analysis of SMURF1 expression levels in 27 couples of BC tissues and adjacent normal tissues. **p<0.01 vs. adjacent tissues. B, C) qRT-PCR and western blot analysis of SMURF1 expression levels in BC cell lines and normal epithelial cells. *p<0.05, **p<0.01 vs. MCF10A; #p<0.05, ##p<0.01 vs. HCC1937; $$p<0.01 vs. MCF7; &p<0.05 vs. MDA-MB-231. D) Correlation analysis of the expressions of SMURF1 and GDF11. E, F) The effects of GDF11 knockdown on the expression of SMURF1 were examined by qRT-PCR and western blot assays. **p<0.01 vs. NC shRNA group. G) The effects of GDF11 knockdown, SMURF1 overexpression, and PFT-α treatment on the expressions of MDM2, p53, p21 were assessed by western blot assay. Each experiment was done at least in triplicates. Data are presented as mean ± SEM.

(EMT) [9]. In addition, the high expression of GDF11 was associated with poor prognosis in colorectal cancer and uveal melanoma patients [10, 20]. GDF11 silencing inhibited the lymphangiogenesis of colorectal cancer [21]. The overexpression of GDF11 promoted EMT as is evident by reduced expression of E-cadherin and increased expressions of vimentin and metalloproteinase 9 in oral squamous cell carcinoma [22]. Interestingly, our data suggested that GDF11 was highly expressed in BC tissues and cell lines, and high expression of GDF11 was correlated with a poor OS of BC patients. GDF11 knockdown showed an anti-cancer role in BC cells by inhibiting the capabilities of proliferation, migration, and invasion, but promoting cell apoptosis. Similar to our results, Wallner et al. showed that GDF11 expression was significantly increased in BC cells and low-grade malignant breast adenocarcinoma (G1) tissues compared with normal breast epithelial cells and benign BC tissues, while GDF11 expression was decreased in high-grade malignant BC tissues [23]. This suggested that the expression of GDF11 was possibly related to the stage of BC progression. Another research indicated that GDF11 was abundantly expressed in triple-negative BC, but its biological activity was lost due to the massive accumulation of pro-GDF11 precursor [24]. Thus, it can be seen that the expression pattern of GDF11 was complicated. And we speculated that this difference in expression was caused by the regulation of GDF11 protein maturation through epigenetic modification. These data indicated that GDF11 was engaged in the progression of BC. However, the expression and functional characteristics of GDF11 still need to be further explored.

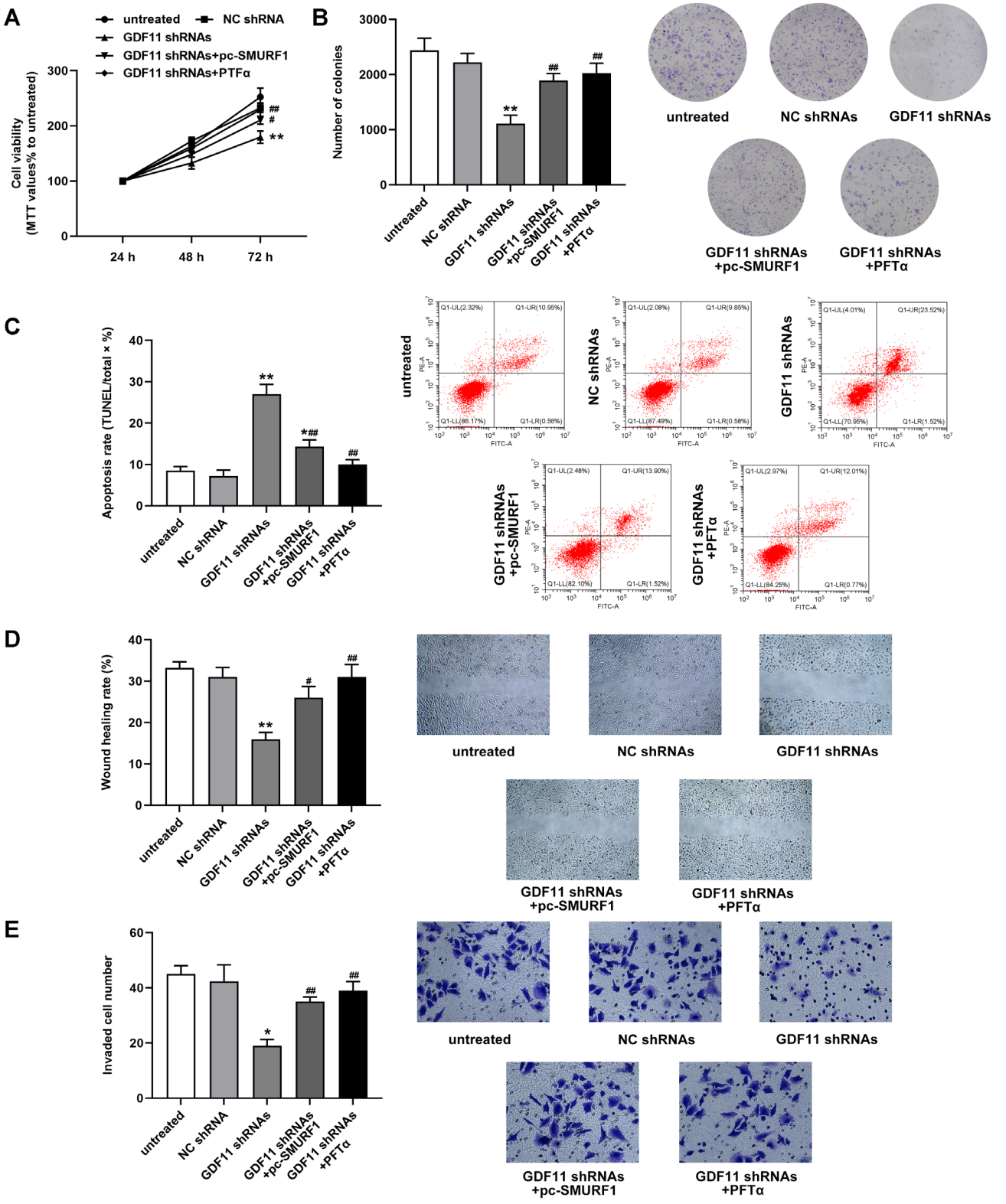

Figure 4. GDF11 knockdown downregulated SMURF1 to inhibit BC progression through the activation of the p53 pathway. The effects of SMURF1 overexpression and PFT-α treatment on A) cell viability, B) clone formation capability, C) cell apoptosis, D) cell migration, and E) cell invasion was measured by a series of functional assays. T47D cells were pre-treated with 10 mmol/l PFT-α (a p53 inhibitor) for 2 h. Each experiment was done at least in triplicates. Data are presented as mean ± SEM. *p<0.05, **p<0.01 vs. NC shRNA group; #p<0.05, ##p<0.01 vs. GDF11 shRNAs group.

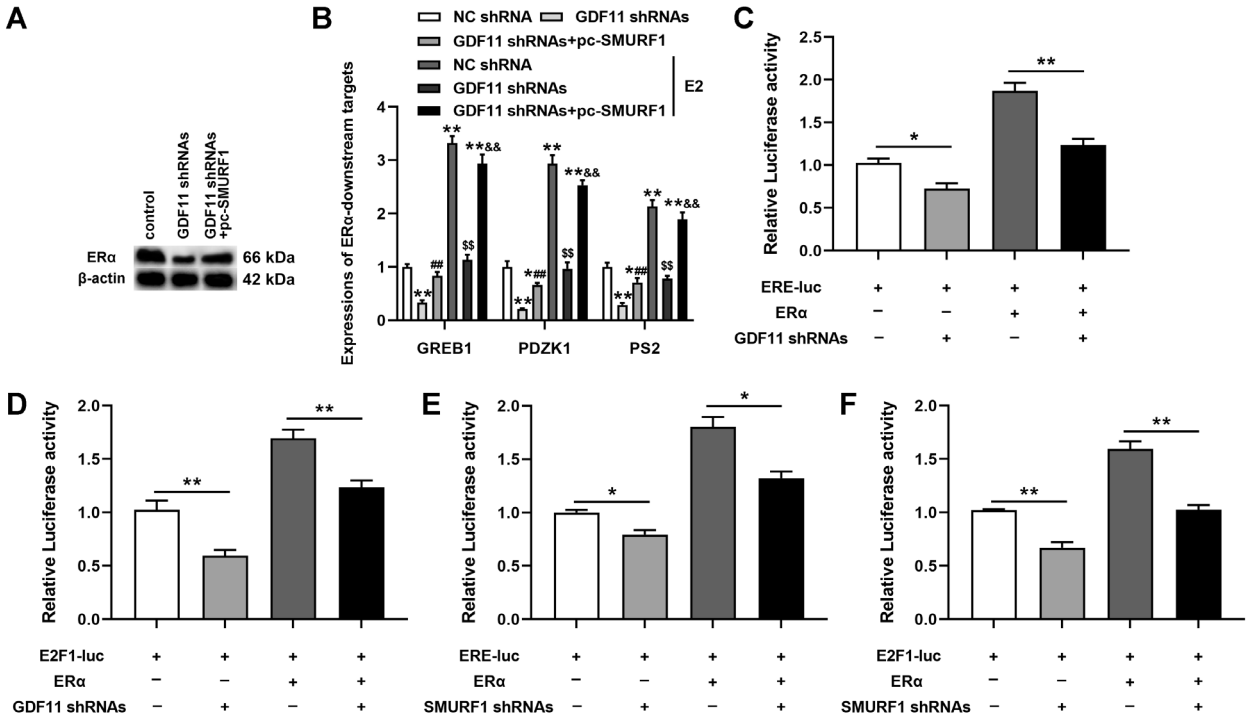

Figure 5. GDF11 knockdown inhibited the activation of the SMURF1-mediated ERα signaling pathway in BC cells. A) The effects of GDF11 knockdown and SMURF1 overexpression on ERα protein level. B) The effects of GDF11 knockdown and SMURF1 overexpression on the expressions of ERα target genes. After transfection of T47D cells with GDF11 shRNAs or NC shRNAs or pc-SMURF1 for 48 h, the cells were cultured in a serum-free medium and treated with 10 nmol/l estradiol for 6 h. The expressions of endogenous ERα target genes PS2, GREB1, and PDZK1 were determined by qRT-PCR. *p<0.05, **p<0.01 vs. NC shRNA group; #p<0.05, ##p<0.01 vs. GDF11 shRNAs group; $$p<0.01 vs. E2+NC shRNA group; &&p<0.01 vs. E2+GDF11 shRNAs group. C-F) The luciferase activities of ERE-luc and E2F1-luc mediated by GDF11 knockdown, SMURF1 overexpression, and ERα were examined by luciferase reporter assays. Each experiment was done at least in triplicates. Data are presented as mean ± SEM. *p<0.05 vs. ERE-luc group; **p<0.01 vs. ERE-luc+ERα group or E2F1-luc group, E2F1-luc+ERα group.

In consideration of the regulatory mechanism of GDF11, various studies indicated that GDF11 could regulate SMURF1 to govern the progression of several diseases. For example, GDF11 promoted SMURF1-mediated sarcoplasmic reticulum Ca ATPase (SERCA) degradation to accelerate cardiomyocyte pathology through the activation of activin type II receptor signaling [25]. Furthermore, the administration of exogenous GDF11 was involved in the SMURF1-activated ubiquitin proteasome system in the liver, thus affecting hepatic iron homeostasis [26]. Notably, SMURF1 has been found to be an oncogenic factor in multiple different cancers. In prostate cancer cells, the elevated SMURF1-induced PTEN facilitated tumorigenesis through activation of the PI3K/AKT/mTOR signaling pathway [15]. Silenced SMURF1 contributed to the suppression of cell migration and invasion in glioma [27]. The activation of SMURF1 promoted RhoA ubiquitination to enhance BC development [28]. Consistent with these studies, our study proved that SMURF1 overexpression alleviated GDF11 knockdown-induced reduction in BC cell proliferation, invasion and migration, and the enhancement in cell apoptosis. Thus, it was further confirmed that GDF11 knockdown downregulated SMURF1 to attenuate the tumorigenic effects of SMURF1.

Growing studies showed that the p53 signaling pathway took an important part in regulating BC progression. The p53 signaling pathway is activated by Salt Inducible Kinase 1 (SIK1) to promote oxidative phosphorylation, and thus suppresses aerobic glycolysis and cell proliferation in BC [29]. Inhibiting p53 signaling contributed to BC development as indicated by increased cell proliferation, mobility, and invasiveness [30]. The activation of the p53 pathway triggered by lncRNA maternally expressed 3 (MEG3) induced endoplasmic reticulum (ER) stress and apoptosis of BC cells [31]. Notably, a microarray analysis of hypoxia identified that SMURF1 was a novel p53-targeting gene [32]. Previous research indicated that the protein levels of p53 depended on the interaction between SMURF1 and MDM2 [33]. The other study showed that targeting SMURF1 indirectly elicited p53-induced cell apoptosis in colorectal cancer cells with miR-596 overexpression [34], which further clarified the regulatory relationship between p53 and SMURF1. Interestingly, SMURF1 overexpression and the presence of PTF-α diminished the GDF11-mediated enhancement in MDM2, p53, and p21 expressions in our study. Our data verified that GDF11 knockdown inhibited the development of BC through the downregulation of SMURF1 to trigger the p53 pathway.

In addition, the ERα signaling pathway has been described as a modulator to control BC development [35]. The inhibition of ERα signaling via Poly ADP-ribose polymerase 7 (PARP7) exerted anti-cancer effects in E2-responsive BC cells [36]. The ERα signaling activated by BC Amplified Sequence 2 (BCAS2) accelerated the growth of BC cells [37]. ERα signaling activation was associated with Tripartite Motif Containing 11 (TRIM11) which provided favorable conditions to support the development of BC [38]. Therefore, the ERα signaling pathway is considered a potential target for BC treatment. Moreover, both ERα and SMURF1 inhibited TGFβ signaling leading to the instability of Smads [39]. The depletion of SMURF1 suppressed the proliferation of BC cells by dampening the activity of the ERα signaling [17]. This evidence implied that SMURF1 participated in the modulation of the ERα signaling. Our results revealed that SMURF1 overexpression prevented the reduction in ERα target genes' expressions induced by GDF11 knockdown, and the silencing of SMURF1 blocked the transcriptional activation of ERα signaling. Taken together, these data suggested that GDF11 knockdown inactivated ERα signaling by suppressing the expression of SMURF1 to limit the malignant progression of BC.

In this study, we elucidated that GDF11 was highly expressed in BC cell lines and tissues, and upregulation of GDF11 expression was associated with the poor OS of BC patients. GDF11 knockdown induced the suppression of SMURF1 to inhibit the development of BC through activated p53 and inactivated ERα signaling pathway. Our study indicates that GDF11 is an effective molecular target, providing a novel perspective for the treatment of BC.

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