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USF2 inhibits the transcriptional activity of Smurf1 and Smurf2 to promote breast cancer tumorigenesis

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ABSTRACT

Smurf1 (Smad ubiquitylation regulatory factor 1) and Smurf2 are negative regulators of the TGF-β (transforming growth factor-β) pathway. The protein stability and ubiquitin E3 activity regulation of Smurfs have been well studied. However, the mechanism of Smurfs expression at the transcriptional level remains uncharacterized. Here, we reported that USF2 (upstream stimulatory factor 2), a basic helix-loop-helix-leucine-zip transcription factor, is necessary for the transcriptional activity of Smurf1 and Smurf2. The 5′-flanking sequences of the Smurfs gene have more than one *E*-box motifs, and USF2 bounds the Smurfs promoter *in vitro* and *in vivo*. Over-expression USF2 inhibited the transcriptional activity of the Smurfs, and Smurfs mRNA was markedly decreased. Therefore, the activity of TGF-β was distinctly enhanced. Furthermore, in human breast cancers, USF2 was abnormally high expressed and correlated with cancer progression. USF2 was specifically inversely correlated with Smurfs in Luminal A subtype breast cancer patients. These findings suggest the mechanism regulation of Smurfs transcriptional activity, and shed new light on the cancer-promoting role of USF2.

1. Introduction

Protein ubiquitination is a dynamically covalent post-translational modification [1]. The ubiquitination of proteins is carried out by utilizing E1 to activate ubiquitin, then ubiquitin conjugates E2 and ligates with a variety of E3 ubiquitin enzymes, the last step is E3 transfers ubiquitin to target substrates for degradation or for other cellular fates [2,3]. E3s dictate the specificity recognition of the ubiquitination substrates. The number of E1s and E2s are few, but a great number of E3s are existed. Two types of E3s are commonly found: the HECT type and the RING type [4,5]. The HECT type E3 are characterized by a HECT (*h*omologous to *E*6-AP *C*-terminus) domain. The HECT domain usually locates in the C terminal region with approximately 350 amino acids. Ubiquitin conjugating enzymes E2 interacts with the HECT domain, *via* an evolutionally conserved cysteine residue to form a thioester complex with ubiquitin [6,7].

Smad ubiquitination regulatory factors (Smurfs), belonging to the HECT type ubiquitin ligases, regulate TGF-β receptors and various Smads *via* the ubiquitin-proteasome pathway [8]. It has been well known that Smurf1 targets the BMP signaling proteins Smad-1/5/8 for ubiquitin-dependent degradation [9], whereas Smurf2 degrades the phosphorylated Smad2/3 and TβRI during TGF-β signaling [10,11]. So far, several studies have demonstrated that Smurf1 and Smurf2 play key roles in physiological processes, for example, tumorigenesis, organ development and neurodegenerative progression [12,13]. In the post-translational level, the mechanism of how the protein stability and E3 activity of Smurfs are regulated have been well studied. Recent studies demonstrated the active regulation mechanisms of Smurf1 and Smurf2 by CKIP-1 and Smad7 [14,15], respectively, and the negative regulation mechanisms of Smurf1 by SCF^{FBXL15} and SCF^{FBXO3} [16,17]. MicroRNA-322 and microRNA-503 are identified as novel factors that regulate Smurf2 [18], and miR-15b and miR-497 are focused on Smurf1 as a target [19,20]. Some inflammatory cytokines and hormones, such as TNF, EGF, androgens, estrogen have been reported to enhance transcriptional activity of Smurf1 [21–24]. TGF-β could stimulate Smurf2 expression through PI3K/AKT pathway *via* TGF-β receptor, but not Smads [25]. However, up to now, little is understood about the activity mechanism of Smurfs at the transcriptional level. Moreover, the key transcriptional factor binding with the promoter regions of Smurfs remains unknown, especially for the passive regulate factor of Smurfs.

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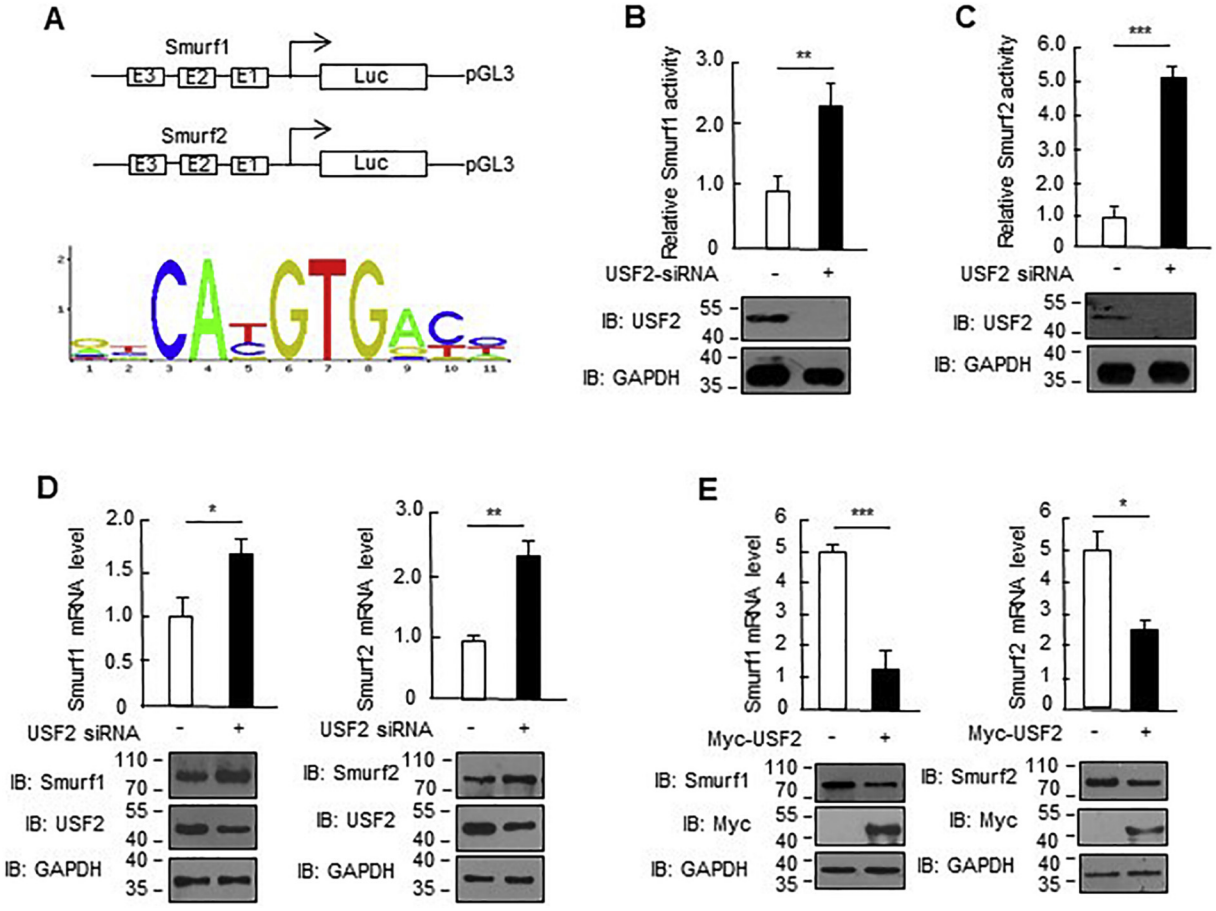

Fig. 1. USF2 inhibits the *Smurf1* and *smurf2* promoters in HEK293T cells.
A, schematic representation of the promoter constructs. The −1762 to-1900 bp promoter fragment of Smurf1 and the −279 to-590 bp promoter fragment of Smurf2 was inserted into the pGL3-enhance reporter. B and C, HEK293T cells transfected with siRNA of USF2 and pGL3 reporter gene with *Smurf1* or *Smurf2* promoter. Reporter activity was measured and represented as the mean ± s.d.n. n = 3 biology replicates, two-side Student's *t*-test. D and E, HEK293T cells were transfected with USF2 siRNA or control siRNA. HEK293T cells were transfected with Myc-tagged USF2 or control vector. Total RNA was subjected to QPCR analysis. n = 3 biology replicates, two-side Student's *t*-test. P < .05 was indicated with *, P < .01 was indicated with ** and P < .001 was indicated with ***. ns was abbreviated as "no significant".

USF2, has a leucine-zip repeat and a helix-loop-helix (bHLH) motif, commonly recognizes *E*-box elements characterized by a central CANGTG sequence [26,27]. As a transcription factor, USF2 has been reported lots of target genes, including cell proliferation, glucose and lipid metabolism. It also has been proved that USF2 is participated in the pathogenesis of several metabolic disorders, including diabetic nephropathy and familial hypercholesterolemia [28–30]. Here, we show that USF2 binds to distinct *E*-box (CANGTG) sequences at the Smurf1 and Smurf2 promoter regions *in vitro* and *in vivo*, resulting in decreasing of Smurfs mRNA levels. As far as we are concerned, this is the first evidence to reveal a negative transcription factor for Smurfs. In addition, our data showed that USF2 was highly expressed in breast cancer and correlated with cancer progression. Interestingly, we also found that USF2 have a prominently negative correlation with Smurfs not in all type of breast cancer, but specifically in luminal A subtype tumors.

2. Materials and methods

2.1. Cell lines and antibodies

The cell lines HEK293T, MCF-7, MDB-MA-231 were obtained from American Type of Cell Collection or China Infrastructure of Cell line Resources and cultured according to the recommendations. Anti-USF2, Smurf1, Smurf2 antibodies were purchased from Abcam. Anti-Flag-tagged, Myc-tagged, phospho-Smad1/5 (Ser463/465), phospho-Smad2 (Ser245/250/255), phospho-Smad3 (Ser423/425), RhoA, ING2 were obtained from Cell Signaling Technology. Anti-GAPDH, secondary antibodies and USF2-siRNA were purchased from Santa Cruz Biotechnology.

2.2. Real-time PCR

Total RNA was isolated and converted to cDNA using the ReverTra Ace® (Toyobo, TRT-101). Quantitative PCR reactions were carried out using SYBR Green PCR master mix (Toyobo, QPK201). Primers used in these analyses were as follows:

Smurf1-F:5′-GAAACCCAATGGCAGAAA-3′,
Smurf1-R:5′-GCAGATGTTGAGGGATGAG-3′;
Smurf2-F:5′-GGCTCAATTCTTGGCTCTG-3′;
Smurf2-R:5′-ACCGGGTGTTTACCTTCC-3′;
GAPDH-F:5′-GGGAAGGTGAAGGTCGGAGT-3′;
GAPDH-R:5′-TTGAGGTCAATGAAGGGGTCA-3′;

2.3. Luciferase reporter assay

The −1100 to −1800 bp sequence in 5'UTR of Smurf1 promoter and −500 to −1000 bp of Smurf2 promoter sequence were cloned into the pGL3-Basic vector. Luciferase activity was measured by the Dual Luciferase Assay System (Promega).

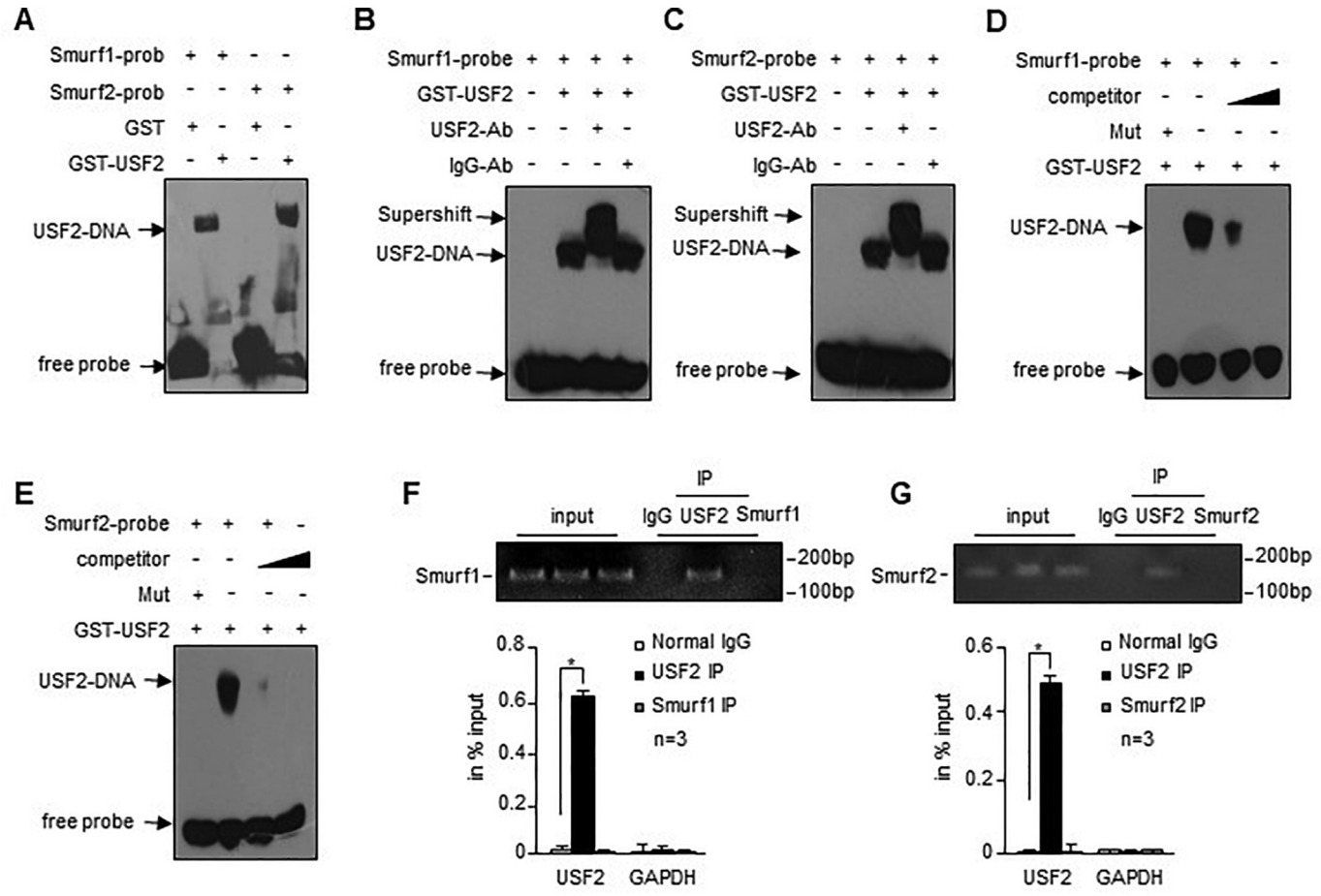

Fig. 2. USF2 binds to *Smurf1* and *smurf2* promoters *in vivo* and *in vitro*.
A-E, EMSA was performed with 100 ng of GST-USF2 and 5 μl of biotin-labelled *Smurf1* and *smurf2* promoter oligonucleotides (5 pmol) (A), the USF2 antibody (B and C) and an unlabeled competitor probe was added as indicated (D and E), the unlabeled competitor at a 10- or 50-fold excess was included in the incubation mixture prior to the EMSA as indicated. (F, G) ChIP assays in HEK293T cells. Data are represented as the means ± s.d. IP, immunoprecipitation.

2.4. Electrophoretic mobility shift assay

The double stranded oligo nucleotides used for EMSA were end-labelled with biotin. The labelled probes were incubated with USF2 (1–5 μg) for 30 min in binding buffer (10 mM Tris–HCl (pH 7.5), 5 mM KCl, 5 mM MgCl2, 10 mM ZnSO4, 50 mg/ml of poly[dI–dC], 5 mg/ml bovine serum albumin, 0.67 mM dithiothreitol, 0.67 mM phenylmethyl sulphonyl fluoride, 2.5% glycerol) in the presence or absence of unlabelled probes. The antibody and protein were pre-incubated for 20 min to detect the supershift. The EMSA assays details were performed as described previously [31,32].

The probe sequences are listed as in the following:
Smurfl-probe: 5′-CTCTGGGGCTCAAGTGATCCTCCCAC-3′
Smurf2-probe: 5′-TAATAAAATGCAGTTGGCCCTGCTTG-3′;
Smurfl -MP: 5′-CTCTGGGGCTGTTCACATCCTCCCAC-3′
Smurf2-MP: 5′-TAATAAAATGGTCAACGCCCTGCTTG-3.

3. Chromatin Immunoprecipitation (ChIP)

The ChIP assays were performed as described previously [33]. Primer sequences used for ChIPs are listed as the following:

Smurf1 promoter: forward, 5′-GGATTACTTGGGAGCATTAG-3′ and reverse, 5′-GCAATAGCAGTAGAGCCACA-3′

Smurf2 promoter: forward, 5′-CCTTGGCTGCACCTGAAA-3′ and reverse, 5′-AATGCTCTGCTCCTGGTCTT-3′.

3.1. CCK8 assay

Cell growth was analyzed using the Cell Counting Kit-8 (Promega). The cells were plated on 96-well plates (2000 cells per well). The plates were incubated at 37 °C. Cell growth was analyzed using Cell Titer 96-Aqueous assay kit (Promega) according to the manufacturer's directions. Cell numbers were estimated every 24 h by adding CCK8 to the wells 1 h before measured by using an microplate reader (Perkin Elmer, Waltham, MA, USA) at 450 nm. Each cell line was set up in 4 replicate wells, and each data point represents a mean ± s.d.

4. Invasion assay

Cell invasion assay was performed in 24-well transwell plate with 8-mm polyethylene terephalate membrane filters (Falcon cell culture insert (Becton–Dickinson)) separating the lower and upper culture chambers. Cells were plated at a concentration of 1×10^6 cells per ml in serum-free DMEM medium. The bottom chamber contained DMEM with 10% FBS. Cells were allowed to migrate for 24 h in a humidified chamber. After the incubation period, filters were fixed with 4% formaldehyde for 15 min and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and photographed.

4.1. Wound healing assay

1.5×10^6 cells/well were seeded into a 6-well plate. A needle was used to create a wound of approximately 1500 μm. Wound cell

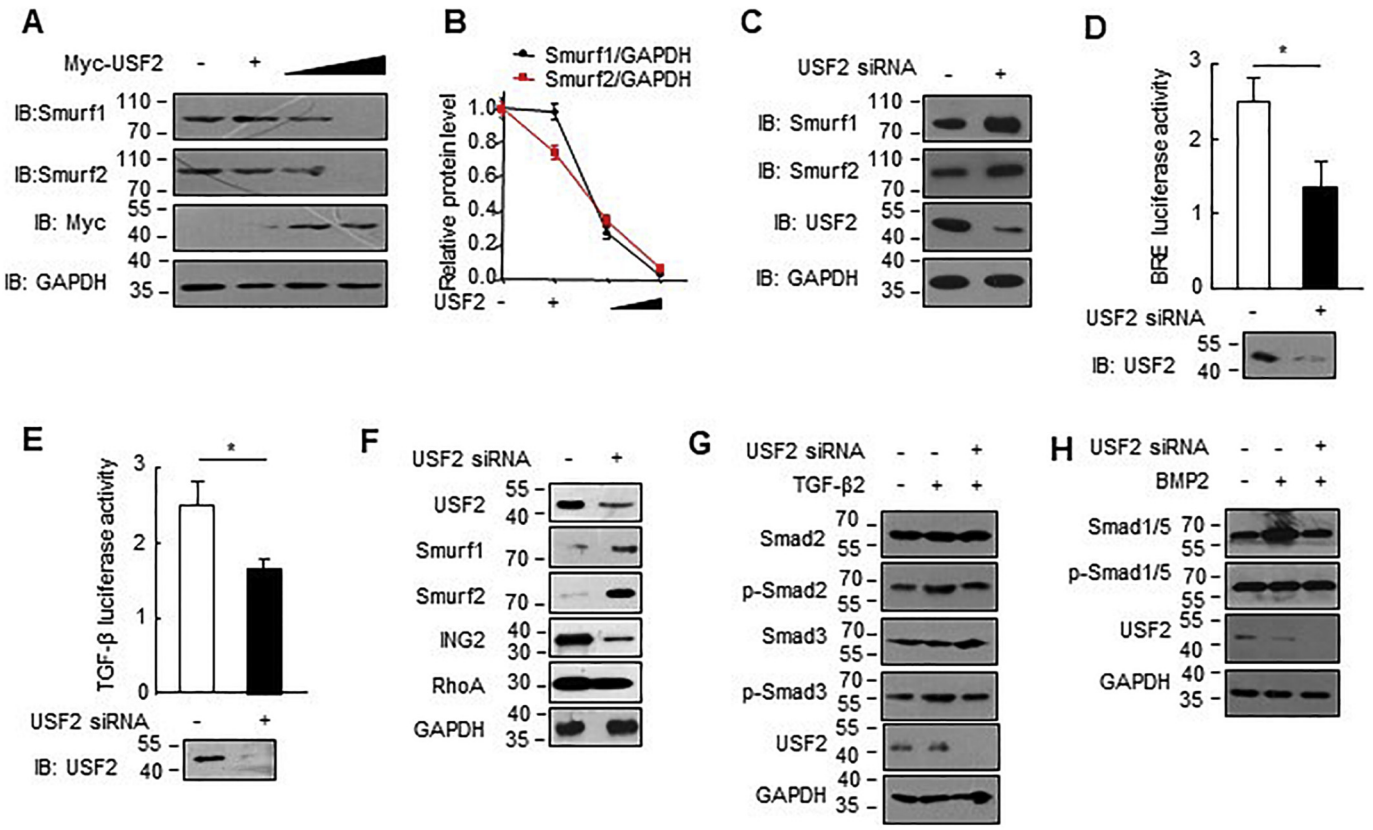

Fig. 3. USF2 negatively regulates Smurf1 and smurf2 stability to inhibit the activity of TGF-β and BMP signaling pathway.
A and B, increasing amounts of Myc-USF2 was transfected into HEK293T cells (0 μg, 0.2 μg, 0.4 μg, 0.6 μg). After 48 h, the expression of Smurfs was detected by western blot. C, knock-down of USF2 by siRNA transfection increased the expression of Smurf1/2 protein. D, HEK293T cells transfected with siRNA of USF2 and BMP luciferease reporter gene. E, HEK293T cells transfected with siRNA of USF2 and TGF-β luciferease reporter gene. Reporter activity was measured and represented as the mean ± s.d. F—H, HEK293T cells were transfected with siRNA of USF2. Then the cells were treated with TGF-β2 (10 ng/ml) for 1 h before harvested (G). Protein extracts were immunoblotted with Smurf1, Smurf2, p-Smad2, p-Smad3 antibodies. HEK293T cells were transfected with siRNA of USF2. Then the cells were treated with BMP2 (50 ng/ml) for 1 h before harvested (H). Protein extracts were immunoblotted with p-Smad1/5 antibodies.

migration was evaluated with motion analysis and Image J.

4.2. Immunohistochemical staining

Immunohistochemistry was performed as described previously [34]. The USF2 polyclonal antibody was incubated with 1:100 dilutions.

4.3. The Cancer Genome Atlas (TCGA)

The RNA-Seq data in breast cancer patients was obtained from www.synapse.org. The clinical information for breast cancer patients was downloaded from www.cbioportal.org. TCGA breast cancer patients were divided into five kind of unique molecular subtypes according to PAM50 condition.

4.4. GEO datasets

The microarray mRNA data in breast cancer tissues was obtained from GEO database (http://www.ncbi.nlm.nih.gov/geo/). USF2 expression was analyzed in GSE 20437 dataset using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/).

4.5. Statistical analysis

All data were representative of no less than three independent experiments. All the data were presented as the means ± SD. Data were analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The differences between groups were assessed by Student's *t*-test. The correlations were analyzed using Pearson correlation test. A value of $P < .05$ was considered to be statistically significant.

5. Results

5.1. USF2 inhibits Smurf1 and Smurf2 promoter activity

The mechanisms controlling Smurf1 and Smurf2 transcriptional regulation are not well understood. Here, we obtained USF2 as a potential interacting protein of human Smurf1 in the yeast two-hybrid screen. However, we failed to detect the binding between USF2 and Smurf1 in co-immunoprecipitation assay (Supplementary Fig. 1A). It has been well studied that USF2 interacts with high affinity to cognate *E*-box regulatory elements (CANGTG). The human Smurf1 promoter contains four E-box motifs in the upstream of transcription start sites (Supplementary Fig. 1B). Smurf2, which is the most similar member with Smurf1 in Nedd4 family, also processes four *E*-box motif within 2000 base pairs in the upstream of transcription initiation sites (Supplementary Fig. 1C).

To determine whether the USF2 regulates the activity of the human Smurf1 and Smurf2 promoter, luciferease reports with *E*-box motifs in Smurf1 and Smurf2 were constructed. A reporter, pGL3 enhancer-vector, containing the −1100 to -1800 bp wild-type Smurf1 promoter sequence, which overlap the E-box 1–3 sites in the reverse orientation was distinctly up-regulated with the knocking down of USF2 (Fig. 1A, B). Similarly, the activity of the Smurf2 promoter construct containing sequences −500 to −1000 bp, which overlap the E-box 1–3 sites, was

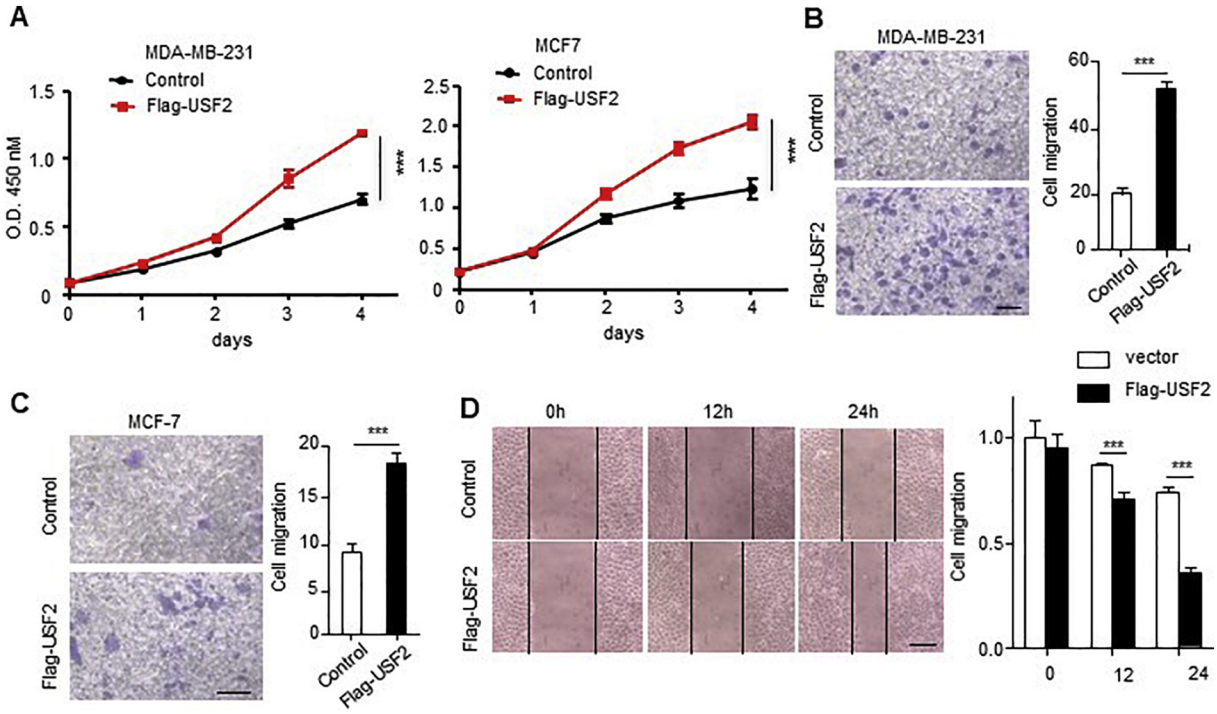

Fig. 4. Expression of USF2 enhanced breast cancer cell growth, invasion and migration.
A, Flag-tagged USF2 stable expression in MDA-MB-231 and MCF-7 cell lines were generated and cell viability was determined using CCK8 assays. B and C, Cells were subjected to invation analysis. Shown are representative images. Scale bar, 100 μm. D, Cells were subjected to wound healing analysis. Scale bar, 10 μm. n = 3 biology replicates, two-side Student's *t*-test.

also distinctly upregulated with the deletion of USF2 (Fig. 1A, C). Next, we detected whether USF2 changed the mRNA abundance of Smurf1 and Smurf2. QPCR assay showed that USF2 depletion significantly enhanced the mRNA level of both Smurf1 and Smurf2 (Fig. 1D). In contrast, overexpressing USF2 obviously decreased Smurfs mRNA level (Fig. 1E). In addition, the data showed that the protein stability of Smurfs were also changed with the transcriptional level. These results clearly illustrated USF2 inhibits the transcriptional activity of the Smurfs.

5.2. USF2 binds with Smurf1 and Smurf2 promoter in vivo and in vitro

Next we intended to detect whether the *E*-box of Smurfs could bind with USF2. Then an electrophoretic mobility shift assay (EMSA) was performed using purified GST-USF2 protein with double-stranded oligo nucleotides containing the E-box motifs as probes with biotin-labelled. The EMSA assay was analyzed with the purified GST-USF2 protein. *In vitro* data revealed that USF2 bounds to both Smurf1 and Smurf2 promoter (Fig. 2A). Incubation with an USF2 specific antibody resulted in a clear super-shift, which confirms the binding specificity, antibody for normal IgG was used as a negative control (Fig. 2B, C). To further affirm the specificity of the DNA-protein interactions, EMSAs competition experiments were performed. In competition these experiments, we used the unlabeled cold probes as competitors. The labelled DNA-protein complexes was competed by were dissociated upon addition of excess competitors in a dose depended manner (Fig. 2D, E). Furthermore, USF2 bound to the wild-type (WT) probe but not to any of the Mutant-type (MT) probes (Fig. 2D, E). Next, we performed chromatin immune-precipitations (ChIP) analysis to determine whether USF2 interacted with Smurf1 and Smurf2 promoter *in vivo*. As shown in Fig. 2F and G, the *E*-box containing sequence of the Smurf1 and Smurf2 were obtained from HEK293T cells, after immunoprecipitation of sheared genomic DNA with an anti-USF2 antibody, the quantitative PCR and DNA electrophoresis analysis were performed. Smurf1 and Smurf2 antibody were used as negative control. The results confirmed that USF2 binds with both the Smurf1 and Smurf2 E-box promoter region (Fig. 2F and G, bottom panels). Thus, USF2 appears to bind with Smurfs promoters both *in vitro* and *in vivo*.

5.3. USF2 reduced Smurfs expression to inhibit the BMP and TGF-β pathway responsiveness

Then Smurf1 and Smurf2 protein level were analyzed in the presence of ectopic USF2. USF2 significantly reduced the endogenous both Smurf1 and Smurf2 protein levels in a dose dependent manner (Fig. 3A). We thus analyzed the Smurf1 and Smurf2 protein levels by grayscale analysis of the ratio of Smurfs/GAPDH (Fig. 3B). In contrast, the stabilization of endogenous Smurf1 and Smurf2 protein level was enhanced with the deletion of USF2 using a specific siRNA (Fig. 3C). However, overexpressed Smurf1 and Smurf2 did not affect the protein level of USF2 (Supplementary Fig. 3A, B), suggesting that the stability of endogenous Smurfs protein were controlled by USF2.

Smurf1 acts as a negative regulator of BMP signaling pathway, as it promotes Smad1/5 for proteasome degradation. *Smurf1*−/− mice exhibited increased bone mineral density [9,35]. Smurf2 inhibits TGF-β signaling activity and targets Smad2/3 for degradation to impact a wide variety of cellular responses [10,11]. Next, the effects of USF2 on BMP signaling pathway was firstly delineated by luciferase reporter assay. Knockdown USF2 clearly inhibited the activity of BRE luciferase activity (Fig. 3D). Meanwhile, the activity of TGF-β responsive CAGA-luciferase was also potently reduced with the absence of USF2 (Fig. 3E). Next, we asked whether USF2 affects the protein stability of Smurfs downstream substrates. To this end, the levels of Smurf1 and Smurf2 were markedly upregulated with the deletion of USF2, and the substrate proteins Rho and ING2 were notably decreased (Fig. 3F). The treatment of TGF-β2 stimulate the phosphorylation of Smad2 and Smad3, depletion of USF2 decreased the effect (Fig. 3G). Similarity was found with the treatment of BMP2, the increased phosphorylation of Smad1/5 was reduced by the knockdown of USF2 (Fig. 3H).

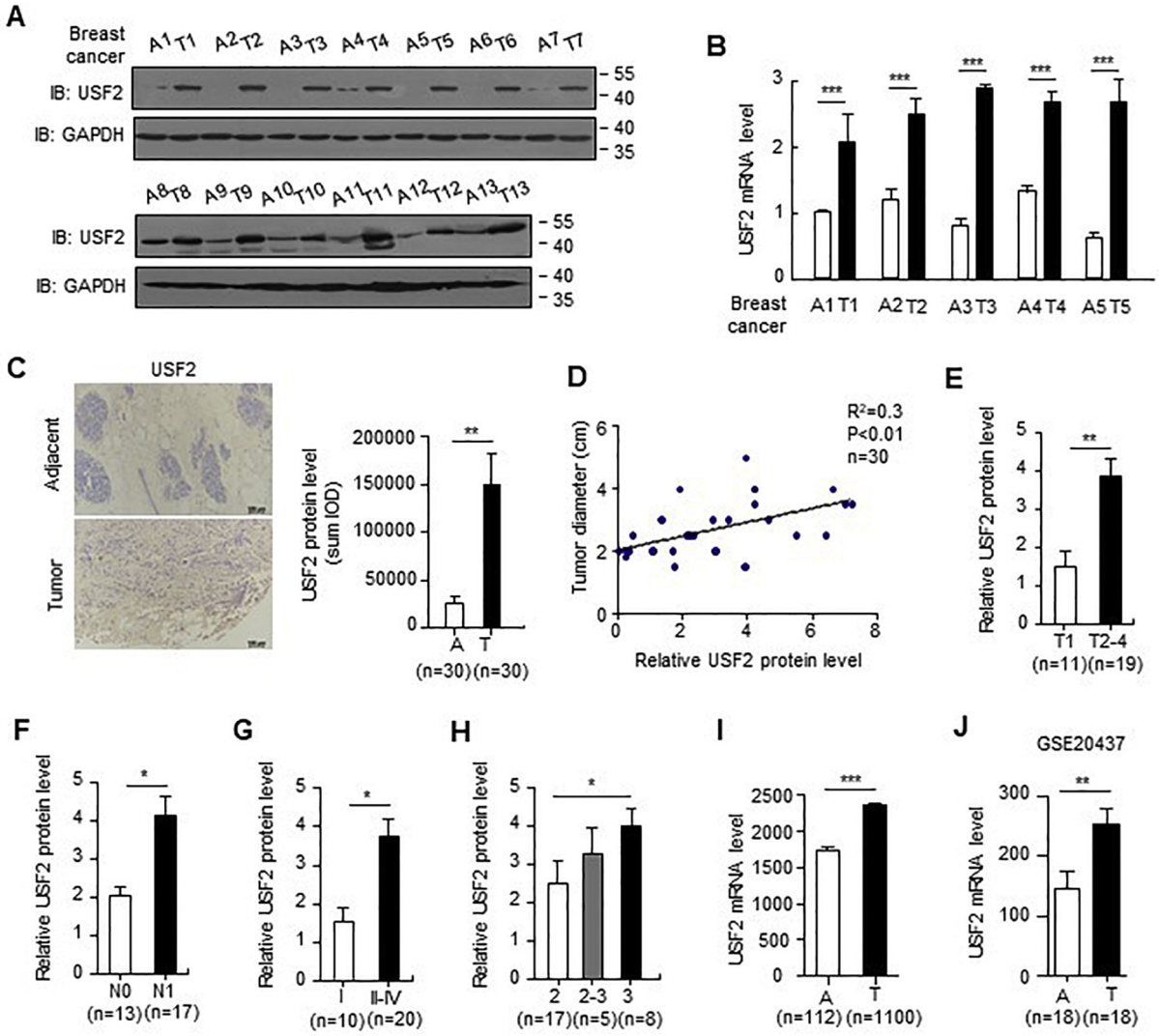

Fig. 5. USF2 was upregulated in breast cancer.
A and B, USF2 protein and mRNA level were examined by western blot and qRT–PCR in 13 paired human breast cancer patients. The representative immunoblotting was shown in (A, adjacent normal tissue; T, breast tumor tissue). C, Immunohistochemistry analysis of USF2 expression in breast tumor and adjacent normal tissue was shown. Scale bar, 100 μm. D, The correlation analysis between relative USF2 protein level and tumor diameter. E, USF2 expression in tumors with different T stages (T stage 1 *vs.* stages 2–4). F, USF2 expression in tumors with different N stages (N stage 0 *vs.* stage 1). G, USF2 expression in tumors with different AJCC stages (Stage I *vs.* II-IV). H, USF2 expression in tumors with different grades (grade2/grade2–3/grade 3). I, The USF2 mRNA level was upregulated in breast cancer tissues from TCGA_BRCA RNA seq v2 dataset. J, The USF2 mRNA level was upregulated in breast cancer from GSE20437 dataset. Group distributions were compared using the Student's *t*-test, P < .05 was indicated with *, P < .01 was indicated with ** and P < .001 was indicated with ***. ns was abbreviated as “no significant”.

5.4. USF2 promotes breast cancer cells proliferation, invasion and migration in vitro

Up to now, the role of USF2 function in breast cancer is unclear. To better understand the relationship of USF2 with breast cancer, we first applied the CCK8 assay *via* stable over-expression USF2 in MDA-MB-231 and MCF-7 cells (Supplementary Fig. 3A). The results showed that over-expression USF2 clearly promoted cell proliferation (Fig. 4A). Matrigel coated trans-well invasion assay revealed that over-expression USF2 significantly increased the invasion capacity of the MDA-MB-231 and MCF-7 cells. (Fig. 4B, C). Finally, results from wound healing assays also indicated that over-expression USF2 increased the cell migration in MCF-7 cells (Fig. 4D). Collectively, these results demonstrate that over-expression USF2 plays positive roles to promote breast cancer cells proliferation, invasion and migration.

5.5. USF2 is abnormally high expressed and correlated with malignant progression in breast cancer

In previous study, USF2 has been showed that processes tumor-suppressive activity in prostate carcinogenesis. In addition, the expression of USF2 gradually increased with increasing tumor grade and was correlated with poor prognosis in colon cancer. Up to now, the clinical association of USF2 in breast cancer development is still unclear. To explore the clinical significance of USF2 in breast cancer development, we collected 13 pairs of breast cancer tissues and matched adjacent tissues. Compared with the adjacent group, the expression of USF2 were much higher in tumor tissues (Fig. 5A). The mRNA level of USF2 was also upregulated in breast cancer samples (Fig. 5B). Then we applied the IHC staining with USF2 antibody to examine the USF2 expression in human clinical breast cancer samples. The results showed that higher USF2 expression in breast cancer than those from adjacent (Fig. 5C), suggesting USF2 may play positive roles in breast cancer. In addition, the protein level of USF2 was positively related with the

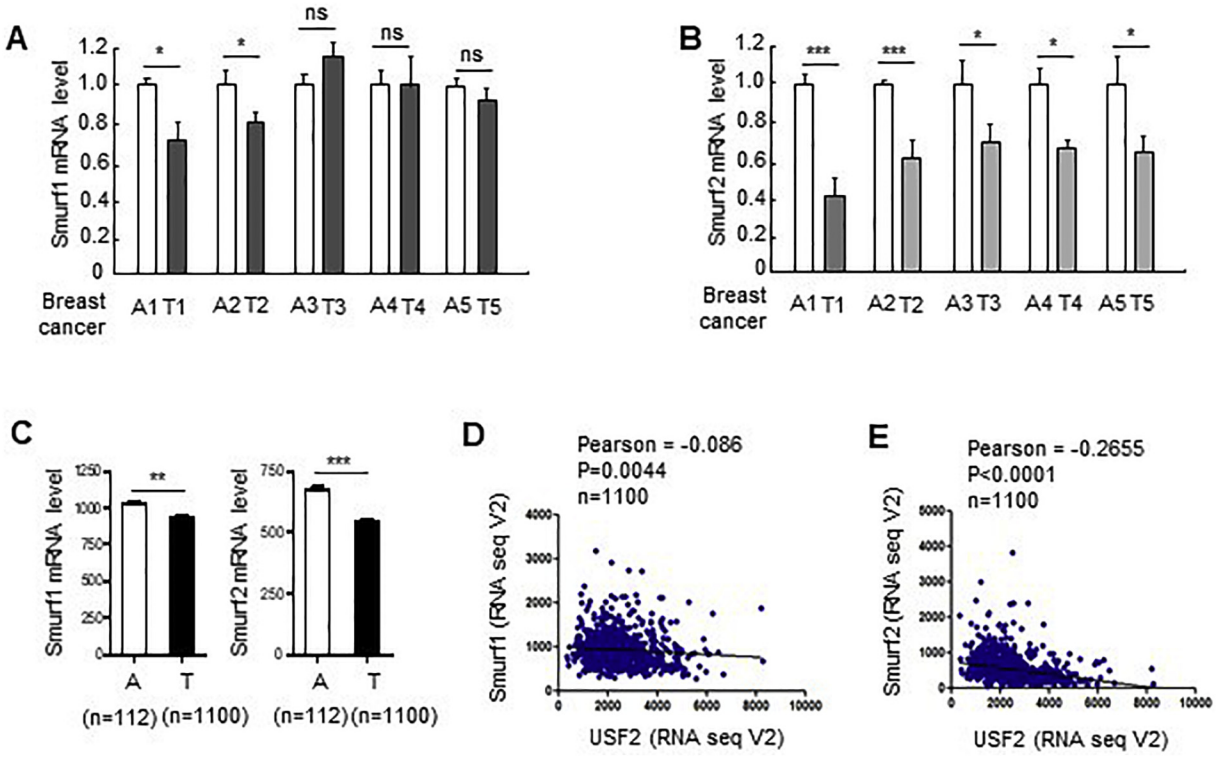

Fig. 6. Smurf1 and Smurf2 were downregulated and negatively correlated with USF2 in breast cancer.
A and B, Smurf1 and Smurf2 mRNA expression was examined by qRT–PCR in 5 paired human breast cancer tissues and adjacent normal tissue, respectively, two-side Student's *t*-test. C, The Smurf1 and Smurf2 mRNA level were downregulated in breast cancer tissues from TCGA_BRCA RNA seq v2 dataset (A, matched adjacent tissues, n = 110; T, tumor tissues, n = 1100). D and E, The Smurf1/Smurf2 mRNA level was negatively correlated with USF2 in breast cancer from TCGA_BRCA RNA seq v2 dataset, respectively. P < .05 was indicated with *, P < .01 was indicated with ** and P < .001 was indicated with ***. ns was abbreviated as "no significant".

tumor size (Fig. 5D). To gain the further role of USF2 in breast cancer progression, we analyzed the USF2 expression in different stage and grade of breast cancer patients. We found that USF2 expression was lower in low-T/N stage tumors and higher in high-T/N stage tumors (Fig. 5E, F). USF2 expression was also lower in early grade or stage tumors and higher in terminal tumors (Fig. 5G, H). To further validate the mRNA level of USF2 in breast cancer tissues, we examined the mRNA level of USF2 in public database (The Cancer Genome Atlas and Gene Expression Omnibus). Similarly, USF2 was abnormally high expression in breast cancer tissues (Fig. 5I, J). Together, the above results suggest that USF2 may play a positive role to promote the breast cancer progression.

5.6. The mRNA level of USF2 is inversely correlated with Smurfs in breast cancer

Our data showed that USF2 mRNA level was enhanced in breast cancer patients. Conversely, in Fig. 6A and B, the expression of Smurf1 and Smurf2 were decreased in tumor tissues compared with matched adjacent tissues. Meanwhile, TCGA_BRCA dataset showed that Smurf1 and Smurf2 were lower in breast cancer tissues than in adjacent normal tissues (Fig. 6C). To further investigate the regulatory effect of USF2 expression on Smurf1 and Smurf2 mRNA level in clinical samples, we systematically compared USF2 expression levels with Smurfs in breast tumors using the latest data set in TCGA_BRCA_IlluminaHiSeq_RNA-SeqV2, results showed that in the 1100 cases of human breast cancer samples, USF2 expression is negatively correlated with both Smurf1 and Smurf2 expression level (Fig. 6D, E). Thus, USF2 and Smurf1, Smurf2 expressions were inversely correlated in breast cancer samples.

5.7. USF2 is specifically inversely correlated with Smurfs in Luminal A breast cancer patients

It has been known that breast cancer patients were divided into five kind of unique molecular subtypes, and each subtype have distinct and predictive characteristics. To ascertain whether the relevance of USF2 and Smurf1 and Smurf2 expression is associated with expression of routinely used breast cancer biomarkers, we subtyped all the patients from the TCGA database into five groups according to PAM50 condition: Luminal A, Luminal B, HER2-enriched, normal-like and basal-like [36].

424 Luminal A type breast cancer patients and 112 patients with adjacent normal tissues were obtained from TCGA databases. Comparing these two groups, we found USF2 expression in the Luminal A group was significantly higher than in the normal control group. On the contrary, the expression of Smurf1 and Smurf2 in the Luminal A type tumor group was clearly lower than that in the normal control group (Fig. 7A). To further validate this hypothesis, we intended to analyze the correlation among USF2 and Smurfs, the data showed that USF2 and Smurf1, Smurf2 were inversely correlated in Luminal A type breast cancer samples (Fig. 7B). Meanwhile, the similar results of the expression of USF2 and smurfs were also found in Luminal B (Fig. 7C). Correlation analysis also indicated that USF2 was inversely correlated with Smurf2 in Luminal B type, but not Smurf1 (Fig. 7D). In HER2-enriched patients, it had no significant changes on the mRNA levels of USF2 and Smurf2 compared with adjacent normal tissues, however, Smurf1 was downregulated. The correlation analysis showed that USF2 and Smurf1 were inversely correlated and it has no correlation between USF2 and Smurf2 in these patients. We also found that USF2 expression was significantly higher in normal-like and basal-like patients (Fig. 7G, H). However, different from Luminal A and B, the mRNA level of

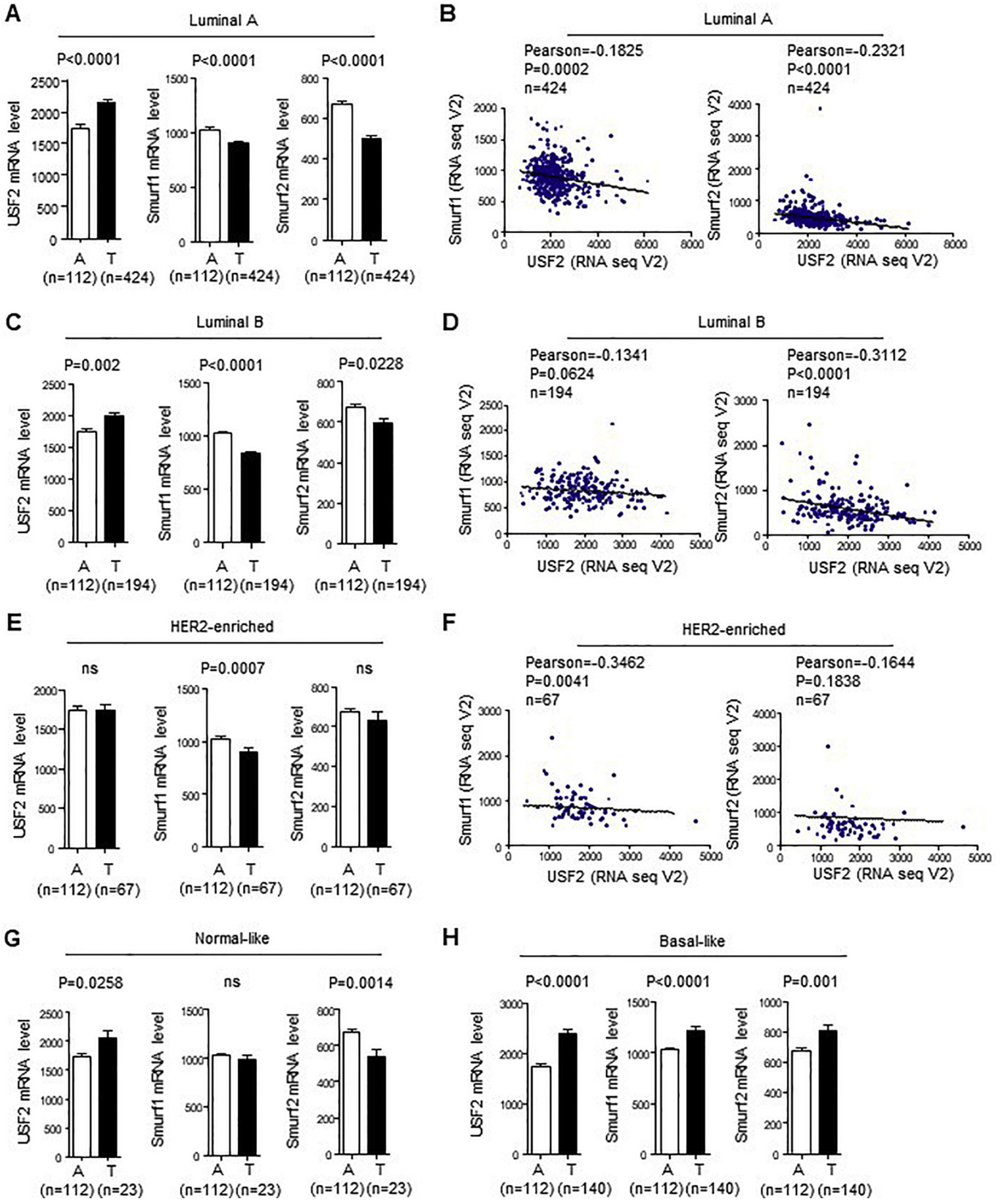

Fig. 7. The USF2 expression and correlation between USF2 and Smurf1/Smurf2 expression in breast cancer subtypes.
A, C, E, G, H, the expression of USF2, Smurf1 and Smurf2 in a variety of breast cancer subtypes (A: Luminal A, n = 424; C: Luminal B, n = 194; E: HER2+, n = 67; G: Normal-like, n = 23; H: Basal-like, n = 140) and adjacent normal tissues (n = 112) in TCGA data base. B, D, F, the correlation between USF2 and Smurf1/Smurf2 expression in a variety of breast cancer subtypes (B: Luminal A; D: Luminal B; F: HER2+).

Smurf1 have no statistical difference in normal-like patients. Both Smurf1 and Smurf2 were enhanced in basal-like group (Fig. 7G, H). There was no correlation between USF2 and Smurf1 in these two types of breast cancer patients. USF2 was negatively correlated with Smurf2 in HER2-enriched patients, however, it's very weak in basal-like patients (Supplementary Fig. 4A, B). According to above results, the relevance between USF2 with Smurf1 or Smurf2 may have a specificity correlation in Luminal A subtype tumors.

6. Discussion

USF2 has been reported to regulate several genes that involved in tumorigenesis, including cyclin B1 [37], BRCA2 [38], Adenomatous Polyposis Coli [39], and cathepsin D [40]. USF2 is also required for induction of HIF2α responsive genes (such as PAI-1) [41]. On the other hand, the transcriptional activity of Smurf1 and Smurf2 remains largely unknown. Although TNF, androgens, estrogen, EGF have been reported to increase Smurf1 mRNA levels, the underlying mechanisms are unclear. Here, we demonstrated that Smurf1 and Smurf2 are downstream target genes of USF2. The promoter regions of both Smurf1 and Smurf2 process more than one *E*-box motif, and a luciferase-promoter assay showed the transcription activity of Smurfs were negatively responsive to USF2. The functionally important E-box among the four need further investigation. Gel mobility super-shift assays and chromatin immunoprecipitation showed that USF2 specifically binds with the promoter region of Smurfs *in vivo* and *in vitro*. Up until now, the transcriptional factor to stimulate or inhibit the expression of Smurfs is not revealed. Our present work shows for the first time that USF2 interacts with the E-box motifs on the Smurfs promoter, indicating that USF2 is a transcriptional activity repressor of both Smurf1 and Smurf2.

Consistent with these mRNA results, USF2 decreased Smurfs protein levels in a dose-dependent manner. Smurf1 was identified due to its abilities to modulate bone morphogenetic protein (BMP) signaling by inducing ubiquitin modification on Smad1/5/8. Smurf2 has been recognized as a negative regulator of TGF-β signaling activity by targeting TGF-β type I receptor and Smads for degradation. Here, we found USF2 modulates TGF-β and BMP signaling by downregulating Smurf1 and Smurf2. It is now recognized that TGF-β signaling promotes invasion and metastasis of breast cancer at the late phase of tumor progression [42]. Overexpression of USF1, another member of the E-box binding transcription factors, could prompt the EMT process through the accumulation of TGF-β1 [43]. USF1 and USF2 have different, though perhaps overlapping, sets of down-stream targets, but many of the genes regulated by USF2 are implicated in the control of cellular proliferation and cancer. In most cases both transcription factors act in form of the USF1/USF2-heterodimer, however, a few cases are known where USF homodimers have been found. USF1 and USF2 may process different function in controlling transcription. Here, the role of USF1 on how USF2 inhibit Smurfs transcription need to be further investigated, and this may help to fulfil their diverse tasks. Although USF2 has tumor-suppressor function in prostate carcinogenesis [44], the role of USF2 in breast tumorigenesis is not clear. In this study, our data showed over-expression USF2 promotes breast cancer cells proliferation, invasion and migration. In previous study, USF2 is one of most likely transcription factor candidates for maintaining open chromatin within the regulatory elements in MCF-7 but not in MCF-10A [45]. In clinical level, our data showed that USF2 expression was significantly upregulated in breast cancer tissues compared with adjacent tissues and associated with the cancer progression. The level of USF2 may change in different microenvironment of tissues. In addition, we also found in the mRNA level of USF2 was lower in breast cancer than in normal (The human protein altas, data was not shown). However, the mRNA levels of USF2 in TCGA and GEO database were enhanced in tumor tissues than in adjacent. This could be simply because USF2 was reported higher in normal epithelial cells than in cancer cells. Thus we compared the expression of USF2 in adjacent with tumor, not normal tissues. Furthermore, we detected the no mutation of USF2 gene was found in breast cancer patients (The human gene mutation database, HGMD, data was not shown).

Moreover, according to the TCGA Breast cancer RNA-seq with PAM50, the mRNA expression of USF2 is inversely correlated with Smurfs in breast cancer patients, especially in luminal A subtype. The downregulation of Smurf2 has been confirmed that could lead to tumor invasion, EMT and metastasis, and Smurf2 has been suggested as a tumor suppressive factor. Aged *Smurf2*−/− mice displays a wide spectrum of tumor phenotypes [46,47]. Although Smurf1 protein has been proved abnormally high in tumors and promote tumor genesis in pancreatic cancer and colon cancer, the reason may attributes low level of Smurf2 could enhance the protein stability of Smurf1 [48]. Accordingly, further study needs to focus on the mechanism of the specificity correlation between USF2 with Smurfs in luminal A subtype tumors. Further study on the mechanism of Smurfs and USF2 link would be innovative and shed new light on breast cancer tumorigenesis.

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Conflict of interest statement

No conflicts of interests were disclosed.

Author contributions

P.X. conceived the project and performed the project planning. Y.W. and Y.C. performed the experimental work of transcriptional factor analysis, including ChIP, EMSA and Report gene; Z.P. performed the CCK8, invasion, wound healing, and analyzed the clinical IHC and TCGA data base. M.D. participated in cell culture and data analysis. Z.P. analyzed the data. P.X. and Z.P. designed the experiments, analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

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References

[1] K.N. Swatek, D. Komander, Cell Res. 26 (2016) 399–422.
[2] C.M. Pickart, Ann. Rev. Biochem. 70 (2001) 503–533.
[3] B.A. Schulman, J.W. Harper, Nat. Rev. Mol. Cell Biol. 10 (2009) 319–331.
[4] R.J. Deshaies, C.A. Joazeiro, Ann. Rev. Biochem. 78 (2009) 399–434.
[5] D. Rotin, S. Kumar, Nat. Rev. Mol. Cell Biol. 10 (2009) 398–409.
[6] J.M. Huibregtse, M. Scheffner, S. Beaudenon, P.M. Howley, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 2563–2567.
[7] M. Scheffner, U. Nuber, J.M. Huibregtse, Nature 373 (1995) 81–83.
[8] H. Zhu, P. Kavsak, S. Abdollah, J.L. Wrana, G.H. Thomsen, Nature 400 (1999) 687–693.
[9] L. Xing, M. Zhang, D. Chen, J. Cell. Biochem. 110 (2010) 554–563.
[10] X. Lin, M. Liang, X.H. Feng, J. Biol. Chem. 275 (2000) 36818–36822.
[11] Y. Zhang, C. Chang, D.J. Gehling, A. Hemmati-Brivanlou, R. Derynck, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 974–979.
[12] Y. Cao, L. Zhang, Cell. Mol. Life Sci. 70 (2013) 2305–2317.
[13] D. David, S.A. Nair, M.R. Pillai, Biochim. Biophys. Acta 1835 (2013) 119–128.
[14] K. Lu, X. Yin, T. Weng, S. Xi, L. Li, G. Xing, X. Cheng, X. Yang, L. Zhang, F. He, Nat. Cell Biol. 10 (2008) 994–1002.
[15] A.A. Ogunjimi, D.J. Briant, N. Pece-Barbara, C. Le Roy, G.M. Di Guglielmo, P. Kavsak, R.K. Rasmussen, B.T. Seet, F. Sicheri, J.L. Wrana, Mol. Cell 19 (2005) 297–308.
[16] Y. Cui, S. He, C. Xing, K. Lu, J. Wang, G. Xing, A. Meng, S. Jia, F. He, L. Zhang, EMBO J. 30 (2011) 2675–2689.
[17] D. Li, P. Xie, F. Zhao, J. Shu, L. Li, Y. Zhan, L. Zhang, Biochem. Biophys. Res. Commun. 458 (2015) 941–945.
[18] S. Cao, L. Xiao, J.N. Rao, T. Zou, L. Liu, D. Zhang, D.J. Turner, M. Gorospe, J.Y. Wang, Mol. Biol. Cell 25 (2014) 1234–1243.
[19] W. Wang, F. Ren, Q. Wu, D. Jiang, H. Li, Z. Peng, J. Wang, H. Shi, Biochem.

Biophys. Res. Commun. 449 (2014) 432–437.
[20] S. Vimalraj, N.C. Partridge, N. Selvamurugan, J. Cell. Physiol. 229 (2014) 1236–1244.
[21] H. Kaneki, R. Guo, D. Chen, Z. Yao, E.M. Schwarz, Y.E. Zhang, B.F. Boyce, L. Xing, J. Biol. Chem. 281 (2006) 4326–4333.
[22] A. Kwon, H.L. Lee, K.M. Woo, H.M. Ryoo, J.H. Baek, Mol. Cells 36 (2013) 548–555.
[23] X. Gang, G. Wang, H. Huang, Prostate 75 (2015) 561–572.
[24] H. Yang, N. Yu, J. Xu, X. Ding, W. Deng, G. Wu, X. Li, Y. Hou, Z. Liu, Y. Zhao, M. Xue, S. Yu, B. Wang, X. Li, G. Niu, H. Wang, J. Zhu, T. Zhuang, J. Exp. Clin. Cancer Res. 37 (2018) 24.
[25] N. Ohashi, T. Yamamoto, C. Uchida, A. Togawa, H. Fukasawa, Y. Fujigaki, S. Suzuki, K. Kitagawa, T. Hattori, T. Oda, H. Hayashi, A. Hishida, M. Kitagawa, FEBS Lett. 579 (2005) 2557–2563.
[26] P.D. Gregor, M. Sawadogo, R.G. Roeder, Genes Dev. 4 (1990) 1730–1740.
[27] M. Sirito, S. Walker, Q. Lin, M.T. Kozlowski, W.H. Klein, M. Sawadogo, Gene Expr. 2 (1992) 231–240.
[28] V.S. Vallet, A.A. Henrion, D. Bucchini, M. Casado, M. Raymondjean, A. Kahn, S. Vaulont, J. Biol. Chem. 272 (1997) 21944–21949.
[29] V.S. Vallet, M. Casado, A.A. Henrion, D. Bucchini, M. Raymondjean, A. Kahn, S. Vaulont, J. Biol. Chem. 273 (1998) 20175–20179.
[30] S. Corre, M.D. Galibert, Pigment Cell Res. 18 (2005) 337–348.
[31] C. Tian, G. Xing, P. Xie, K. Lu, J. Nie, J. Wang, L. Li, M. Gao, L. Zhang, F. He, Nat. Cell Biol. 11 (2009) 580–591.
[32] L. Yuan, C. Tian, H. Wang, S. Song, D. Li, G. Xing, Y. Yin, F. He, L. Zhang, EMBO Rep. 13 (2012) 363–370.
[33] P. Xie, M. Zhang, S. He, K. Lu, Y. Chen, G. Xing, Y. Lu, P. Liu, Y. Li, S. Wang, N. Chai, J. Wu, H. Deng, H.R. Wang, Y. Cao, F. Zhao, Y. Cui, J. Wang, F. He, L. Zhang, Nat. Commun. 5 (2014) 3733.
[34] M. Yamashita, S.X. Ying, G.M. Zhang, C. Li, S.Y. Cheng, C.X. Deng, Y.E. Zhang, Cell 121 (2005) 101–113.
[35] J.P. Cogswell, M.M. Godlevski, M. Bonham, J. Bisi, L. Babiss, Mol. Cell. Biol. 15 (1995) 2782–2790.
[36] S. Nik-Zainal, H. Davies, J. Staaf, M. Ramakrishna, D. Glodzik, X. Zou, I. Martincorena, L.B. Alexandrov, S. Martin, D.C. Wedge, P. Van Loo, Y.S. Ju, M. Smid, A.B. Brinkman, S. Morganella, M.R. Aure, O.C. Lingjærde, A. Langerød, M. Ringnér, S.M. Ahn, S. Boyault, J.E. Brock, A. Broeks, A. Butler, C. Desmedt, L. Dirix, S. Dronov, A. Fatima, J.A. Foekens, M. Gerstung, G.K. Hooijer, Jang SJ21, Jones DR1, H.Y. Kim, T.A. King, S. Krishnamurthy, H.J. Lee, J.Y. Lee, Y. Li, S. McLaren, A. Menzies, Mustonen V1, S. O'Meara, I. Pauporté, X. Pivot, C.A. Purdie, K. Raine, K. Ramakrishnan, F.G. Rodríguez-González, G. Romieu, A.M. Sieuwerts, P.T. Simpson, R. Shepherd, L. Stebbings, O.A. Stefansson, J. Teague, S. Tommasi, I. Treilleux, G.G. Van den Eynden, P. Vermeulen, A. Vincent-Salomon, Yates L1, C. Caldas, L. Van'T Veer, A. Tutt, S. Knappskog, B.K. Tan, J. Jonkers, Å. Borg, N.T. Ueno, C. Sotiriou, A. Viari, P.A. Futreal, P.J. Campbell, P.N. Span, S. Van Laere, S.R. Lakhani, J.E. Eyfjord, A.M. Thompson, E. Birney, H.G. Stunnenberg, M.J. van de Vijver, J.W. Martens, A.L. Børresen-Dale, A.L. Richardson, G. Kong, G. Thomas, M.R. Stratton, Nature 534 (2016) 47–54.
[37] K. Wu, S.W. Jiang, M. Thangaraju, G. Wu, F.J. Couch, J. Biol. Chem. 275 (2000) 35548–35556.
[38] A.S. Jaiswal, S. Narayan, J. Cell. Biochem. 81 (2001) 262–277.
[39] W. Xing, T.K. Archer, Mol. Endocrinol. 12 (1998) 1310–1321.
[40] L. Qi, C.E. Higgins, S.P. Higgins, B.K. Law, T.M. Simone, P.J. Higgins, J. Cell. Biochem. 115 (2014) 1840–1847.
[41] J. Massague, Nat. Rev. Mol. Cell Biol. 13 (2012) 616–630.
[42] H. Moses, M.H. Barcellos-Hoff, Cold Spring Harb. Perspect. Biol. 3 (2011) a003277.
[43] N. Chen, M.N. Szentirmay, S.A. Pawar, M. Sirito, J. Wang, Z. Wang, Q. Zhai, H.X. Yang, D.M. Peehl, J.L. Ware, M. Sawadogo, Oncogene 25 (2006) 579–587.
[44] Y. Liu, N.M. Walavalkar, PLoS Genet. 13 (2017) e1006761.
[45] P.M. Ismail, T. Lu, M. Sawadogo, Oncogene 18 (1999) 5582–5591.
[46] M. Blank, Y. Tang, M. Yamashita, S.S. Burkett, S.Y. Cheng, Y.E. Zhang, Nat. Med. 18 (2012) 227–234.
[47] C. Ramkumar, Y. Kong, H. Cui, S. Hao, S.N. Jones, R.M. Gerstein, H. Zhang, Cancer Res. 72 (2012) 2714–2719.
[48] E. Fukunaga, Y. Inoue, S. Komiya, K. Horiguchi, K. Goto, M. Saitoh, K. Miyazawa, D. Koinuma, A. Hanyu, T. Imamura, J. Biol. Chem. 283 (2008) 35660–35667.