Usp41, a Potential Candidate Gene in Development of Breast Cancer

rui ling (lingruiaoxue@126.com)
Xijing Hospital https://orcid.org/0000-0002-7994-0964
Meiling Huang
Xijing Hospital
Jian Zhang
Xijing Hospital
Jingjing Xiao
Xijing Hospital
Nanlin Li
Xijing Hospital

Research

Keywords: Ubiquitin-specific proteases 41, breast cancer, RACK1

Posted Date: March 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-272641/v1

Abstract

Background: Breast cancer is the most common cancer diagnosed among women and is the second leading cause of cancer death. It is of great significance to explore potential candidate targets.

Methods: Cell function assays, siRNA, western blot, mass spectrum, flow cytometry, and other molecular biology techniques were conducted to verify the function of USP41 to breast cancer cell line.

Results: The results indicate that USP41 (ubiquitin-specific proteases 41) expression is positively related to breast cancer progression. USP41 overexpression greatly enhanced breast cancer colony-forming ability, proliferation and migration. In contrast, USP41 knockdown significantly inhibited breast cancer colony-forming ability, proliferation and migration. Moreover, association of USP41 with RACK1 (Receptor for activated C kinase 1) was proved by mass spectrum, indicating its potential role in TGF-β signaling.

Conclusions: USP41 can be a potential therapeutic target against breast cancer via RACK1.

Background

Breast cancer is the most common cancer diagnosed among women and is the second leading cause of cancer death[1]. In 2019, approximately 268,600 new cases of invasive breast cancer and 48,100 cases of DCIS (ductal carcinoma in situ) will be diagnosed among US women, and 41,760 women will die from this disease[2].Similarly, in China, the incidence rate of breast cancer increased obviously both in urban and rural areas[3].However, the 5-year survival rate of breast cancer in China is 73%, significantly lower than that in developed countries[4].With the development of precision medicine and targeted therapeutics, breast cancer treatment has also begun to evolve toward a more individualized, precise and targeted approach[5]. But as a highly heterogeneous disease, breast cancer involved a complex etiology, including genetic alterations, reproductive factors and environmental factor[6]. Hence, it is of great significance to gain a better understanding of the mechanisms underlying breast cancer and to develop novel and effective therapeutic strategies[7].

Ubiquitination can regulate a variety of complex cellular processes and may lead to the activation or deactivation of tumorigenic pathways in cancer[8, 9]. Deubiquitinating enzymes (DUBs), reversing the ubiquitination process by removal of ubiquitin, have emerged as promising drug targets for cancer therapy. DUBs can be divided into six subclasses: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs or UBPs), ovarian tumour proteases (OTUs), Machado-Joseph disease proteases (MJDs), JAB1/MPN/Mov34 metalloenzymes (JAMMs) and monocyte chemotactic protein-induced deubiquitin family (MCPIPs). Among the six structurally different DUB families, the largest group comprises the ubiquitin-specific proteases (USPs) that belong to the cysteine protease family[10, 11]. Many studies indicate that USPs regulate tumor formation by modulating the proliferation and death of cancer cells. In 2019, Zhang KQ et al showed that USP22 play critical roles in the malignancy and progression of non-small cell lung cancer[12]. In 2020, USP 14 was proved to promote prostate cancer progression through deubiquitinating the transcriptional factor ATF2[13]. Lai CY et al found that low

expression of USP4 was associated with poor survival among lung cancer[14]. Yun SI et al considered USP21 as an attractive therapeutic target in metastatic colorectal cancer with high Fra-1 expression[15]. USP41 was first identified in human prostate, brain, lung, aorta, and kidney in 2004[16]. Until now, no data has been reported about the function of USP41 in cancer development, including breast cancer[17].

In this study, we reported for the first time that ubiquitin-specific protease 41 was overexpressed in breast cancer cells and tissues. We found that USP41 upregulation can enhance the growth, proliferation and invasion of breast cancer cells. The potential mechanism of USP41 in breast cancer was also explored and RACK1 (Receptor for activated C kinase 1)-related signaling maybe participated into this progression. Therapy targeting USP41 and RACK1 may provide a novel and promising approach to breast cancer treatment.

Methods

2.1 Clinical samples

Breast cancer specimens and paired adjacent normal breast tissue specimens were obtained from ten patients with primary breast cancer. All patients received no therapy before sample collection. The study protocol was approved by an independent ethics committee of Fourth Military Medical University. The study was undertaken in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. All patients were asked to provide written informed consent before enrollment. All tissues were collected immediately upon resection and transported in liquid nitrogen.

2.2 Cells, antibodies and reagents

Human breast cancer cell lines (MDA-MB-231, MCF-7) were obtained from ATCC. MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% FBS. MCF-7 cells were grown in DMEM medium supplemented with 10% and 0.01 mg/ml bovine insulin. All the cells were cultured in a humidifed atmosphere with 5% CO_2 at 37 °C.

2.3 TCGA database analyses

Clinical relevance of USP41 in breast cancer was analyzed based on the public database of TCGA. A total of 1104 cancer and 113 normal specimens were included to examine USP41 expression. A total of 541 USP41 high expression patients and 541 USP41 low expression patients were included to analyze overall survival (http://starbase.sysu.edu.cn/panGeneDiffExp.php#□Flold Changes=5.02□p Value=4.8*10E14).

2.4 Cell proliferation analysis

CCK-8 assay was performed to measure proliferation of breast cancer cells according to manufacturer's instructions. Briefly, 6000 cells/well were seeded in 96-well plates in medium containing 10% FBS and incubated under 37 °C□5% CO_2. After treatment, 10 ul CCK-8 reagent was added and incubated for 1 hour,

the absorbance under 450 nm was measured with a microplate reader. The same experiments were repeated after a defined incubation period.

2.5 Colony formation assay

The different cells were seeded in 6-cm dishes at a density of 300 cells/dish. Following incubation for 2 weeks in a humidified incubator at 37˚C in an atmosphere of 95% air and 5% CO_2, the cells were washed with phosphate-buffered saline (PBS), and colonies were fixed with methanol for 10 min and stained with 0.5% crystal violet for 15 min. The number of colonies was counted under a microscope (D750; Nikon, Tokyo, Japan). All experiments were performed in triplicate dishes in 3 independent experiments.

2.6 Transwell

Transwell migration assay was performed using transwell inserts. 8×10^4 cells in serum-free medium were seeded into the upper chamber of the insert and the bottom of the chamber contained the DMEM medium with 10% FBS. After 36 h incubation, the cells were fixed with methanol and stained with Giemsa. Then cells on the top surface of the membrane were wiped off, and cells on the lower surface were examined under an inverted light microscope. The number of migrated or invaded cells was quantified by counting the number of cells from 10 random fields at ×100 magnification.

2.7 Apoptosisassays

Additionally, cellular apoptosis was determined by flow cytometry (FAC). Cell lines cultured in DMEM supplemented with 10% FCS were seeded in 96-well plates (2 × 104 cells/well). After treatment with USP41 overexpression or knockdown, cellular apoptosis was determined by FAC after a stain with Annexin v-FITC or Propidium Iodide (PI) or both for 15 min in the dark at room temperature following the instruction of Annexin

v-FITC apoptosis detection kit (YEASEN, China).

2.8 Cell cycle analysis

Cell cycle distribution was analyzed using flow cytometric analysis. Briefly, after treatment with USP41 overexpression or knockdown, cells were harvested and fixed with precooled 75% (v/v) ethanol for 24h. Afterwards, ethanol was discarded by centrifugation. Fibroblasts were rehydrated with PBS at room temperature and resuspended in propidium iodide (PI) DNA staining buffer and incubated for 30 min at room temperature in the dark. Detection was performed on a flow cytometer (FACSAria; BD Biosciences).

2.9 RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the cells and tissues using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. cDNA was generated from 1 μg total RNA using SuperScript III (Invitrogen, USA) and polyN primers. qPCRwas performed using the ABI 7500 fast system (Thermofisher, USA) with

the primers as follow: USP41, 5'-TGGAGGGCAGTATGAGCTTTTT-3' (Forward Primer) and 5'-ATGACCGGAGTCTGCCATTC-3' (Reverse Primer); RACK1, 5'-CCACCACGAGGCGATTTGT-3' (Forward Primer) and 5'-CCCAGGGTATTCCATAGCTTGAT-3' (Reverse Primer). The relative levels of gene expression were represented as 2 - Δ Ct (Ct gene - Ct reference) . The experiments were repeated in triplicate.

2.10 USP41 overexpression and knockdown

USP41 overexpression lentivirus and small-interfering RNA (siRNA) were purchased from Beijing Syngentech Cooperation (Beijing, China) and HIPPOBIO (Wuhan, China), respectively. MCF7 and MDA-MB-231 cells were transfected with lentiviruses for 72 h. qPCR and western blot were used to verify the efficacy of USP41 knockdown. Then, USP41-overexpression and USP41-knockdown breast cancer cells were used for function detection.

2.11 Western blot

Cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM b-glycerophosphate, 2 mMsodium vanadate and protease inhibitor). Protein concentration was assayed using the micro-BCA protein assay (Pierce, Rockford, IL). 40 lg of protein per lane was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose (Amersham Pharmacia, Germany). Then, the membrane was blocked with 5% non-fat milk and incubated with antibodies against 4EBP1 (Cell Signaling, USA, 1:1000), P-4EBP1 (Cell Signaling, USA, 1:1000), Ps6 (Cell Signaling, USA, 1:1000),β-cantenin (Servicebio, China, 1:1000), Cleaved-caspase 3 (Cell Signaling, USA, 1:1000), CyclinD1 (Santa Cruz, USA, 1:1000), CyclinE (Santa Cruz, USA, 1:1000), FASN (Cell Signaling, USA, 1:1000), M-TOR (Cell Signaling, USA, 1:1000), PM-TOR(Cell Signaling, USA, 1:1000), p-cjun (Cell Signaling, USA, 1:1000), USP41 (Invitrogen, USA, 1:1000), P-P38 (Cell Signaling, USA, 1:1000), P53 (Santa Cruz, USA, 1:1000), PTEN (Cell Signaling, USA, 1:1000); E-cad (Cell Signaling, USA, 1:1000), HKII (Cell Signaling, USA, 1:1000), PSTAT3 (Cell Signaling, USA, 1:1000), N-Cad (Cell Signaling, USA, 1:1000), P21 (Cell Signaling, USA, 1:1000), PAKT (Cell Signaling, USA, 1:1000), P-bad (Cell Signaling, USA, 1:1000), PKM2 (Cell Signaling, USA, 1:1000), RACK1 (Proteintech, USA, 1:1000),β-actin (Servicebio, China, 1:1000), and GAPDH (Proteintech, USA, 1:1000). Incubation with the primary antibody was carried out overnight in a cold room. The membrane was then incubated with a secondary antibody conjugated to goat anti-mouse IgG (1:5 000, sigma) and developed using enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ).

2.12 CoIP-MS lysed by adding protease inhibitor to RIPA lysate

CoIP-MS was used to explore the interacting proteins with USP41. The process consists of 5 main steps: (1) incubation of cell lysates with Flag-tag antibodies (Sigma-Aldrich, USA), following cell transfection with Flag-USP41 and cell lysis with RIPA lysate, (2) binding of immune complexes to protein A/G agarose beads (Sigma-Aldrich, USA), (3) removal of non-interacting proteins, (4) elution to obtain protein interacting complexes, (5)mass spectrometry identification of protein interacting complexes. The enriched co-immunoprecipitation products were analyzed by mass spectrometry. Peptides with scores

b20 were removed, and higher scores meant a better degree of matching with the secondary atlas. Peptides were searched and compared qualitatively in UniPro. The UniquePep Count and the Cover Percent were also evaluated as auxiliary metrics for the final identification results.

2.13 Statistical analysis

The data are presented as the means ± SD. Student's t test was used for statistical analysis, unless otherwise indicated. GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA) and SPSS 23.0 Software (IBM Inc., Armonk, NY) were used for all statistical analyses. P-values <0.05 were considered to indicate statistically significant differences.

Results

3.1 Overexpression of USP41 in breast cancercell lines and tissues

To investigate the role of USP41 in breast cancer progression, we determined its expression in breast cancer. Our TCGA database analysis revealed that in metastatic breast cancer USP41 expression is upregulated (Figure 1A). The overexpression of USP41 is negatively correlated with breast cancer prognosis (Figure 1B). Quantitative real-time PCR (qRT-PCR) analysis revealed that the expression of USP41 was up-regulated in MCF 7 and MDA-MB-231 breast cancer cell lines (Figure 1A). The mRNA levels of USP41 were higher in tumor tissues than their adjacent non-tumorous tissues in 10 pairs of clinical breast cancer specimens (Figure 1C).

3.2 Establishment of stable USP41 expression in breast cancer cell lines

To further explore the function of USP41, we established USP41 overexpression cell lines. We also used siRNA to generate a stable cell line (MCF7 and MDA-MB-231 breast cancer cells) in which USP41 was knocked down. Transfection efficiency was confirmed by western blot analysis and RT-qPCR. Compared with the control, the MCF7 and MDA-MB-231 breast cancer cells that had been transfected with the USP41 overexpression plasmid exhibited obviously increased expression of USP41 at both the protein (Fig.2A, B) and mRNA (Fig.2C, D) level. On the contrary, the cells that had been transfected with the USP41 siRNA plasmid exhibited a significantly decreased expression of USP41 at both the protein (Fig. 2E, F) and mRNA level (Fig. 2G, H) compared with the cells transfected with the control siRNA.

3.3 Effects of USP41 gene on breast cancer growth

Colony formation assays and CCK8 were used to exam the effects of USP41 expression on breast cancer cell proliferation. As shown in Figure 3, overexpression of USP41 promote the colony-forming ability of MCF 7 cells (Figure 3A) and MDA-MB-231 cells (Figure 3C), while its knockdown reduced the colony-forming ability of MCF 7 cells (Figure 3B) and MDA-MB-231 cells (Figure 3D). In addition , the overexpression of USP41 significantly enhanced the proliferation of MCF 7 cells (Figure 3E) and MDA-MB-231 cells (Figure 3G), whereas USP41 knockdown significantly inhibited their proliferation(Figure. 4F, H)

3.4 Effects of USP41 gene on breast cancer migration

Transwell assay indicated that overexpression of USP41 enhanced cell invasion in MCF 7 cells (Figure 4A), while its inhibition inhibited the migration compared to control cells (Figure 4B). Identically, USP41 overexpression promote cell migration in MDA-MB-231 cell line (Figure 4C), whereas USP41 knockdown significantly inhibited their invasion (Figure 4D).

3.5 Effects of USP41 gene on breast cancer cell cycle and apoptosis

We next confirm the role of USP41 in breast cancer cell cycle and apoptosis. As shown in Figure 5A, overexpression of USP41 did not affect MCF 7 cell apoptosis. However, the proportion of cell apoptosis was significantly increased in USP41-knockdown MCF 7 cells (Figure 5B). Consistent with the results above, USP41 overexpression significantly reduced cell apoptosis of MDA-MB-231 cell line (Figure 5E), while USP41 knockdown enhanced its apoptosis rate (Figure 5F). Cell cycle arrests of MCF 7 and MDA-MB-231 were also studied. For MCF 7, USP41 overexpression and knockdown could arrest cells in G0/G1 phase (Figure C,D). However, USP41 upregulation arrested MDA-MB-231 cells in G2/M phase (Figure G), whereas USP41 knockdown arrested cells in G0/G1 phase (Figure H).

3.6 Effects of USP41 on marker genes of common cellular signaling pathways

To elucidate the possible mechanism responsible for the effects of USP41 on breast cancer, we examined the level of several marker genes in breast cancer development, using MCF 7 cell line. As Figure 6A indicated, Ps6, β-cantenin, cyclinD1, cyclinE, FASN, M-TOR, N-cad, PAKT, P-cjun, and PKM2 were up-regulated, accompanied with USP41 overexpression. But the expression of cleaved caspase3 and PSTAT3 were decreased. When USP41 was knocked down, the level of Ps6, cyclinD1, cyclinE, FASN, PM-TOR and P53 were down-regulated. But p21 and PSTAT3 expression were increased (Figure 6B).

3.7 USP41 interacted with RACK 1 gene in breast cancer cell

Study of protein-protein interactions is an essential process to understand the biological functions of proteins and its underlying mechanism[18]. Here we performed Co-immunoprecipitation coupled with mass spectrometry (CoIP-MS) to discover novel proteins interacted with USP41. CoIP-MS analyses identified a list of genes that were interacted with USP41, including RACK 1 (Figure 6C). COIP results indicated that USP41 could combined with RACK 1 (Figure 6D). In breast cancer tissue samples, we also found the overexpression of RACK 1 in cancer tissue, compared with those in the adjacent normal tissue samples (Figure 6E). Next, we inhibited the expression of RACK1 by gene knockdown (Figure 7A,B). Colony-forming assay demonstrated that overexpression of USP41 promote the colony-forming ability of breast cancer cells (Figure 7C). Transwell assay indicated that knockdown of RACK1 inhibited the migration compared to control cells (Figure 7D).

Discussion

Ubiquitin-specific proteases (USPs) are emerging as potential therapeutic targets in many diseases. In breast cancer, several USPs were overexpressed, like USP9X, USP15, USP32, USP9Y, USP10, USP25, USP4 and USP11[19]. In 2016, Li Y et al proved that USP4 could inhibit breast cancer cell growth through the upregulation of PDCD4[20]. USP14 and USP21 were also involved in the progression of breast cancer[21, 22]. In 2018, USP2-69 was found over-expressed in breast invasive ductal carcinoma, and was closely related to proliferation promoting effects[23]. Qiu H et al demonstrated that USP8 might predict better clinical characteristics and might be a protective factor for patients with breast cancer[24]. In 2020, USP8 was proved as a positive regulator of Notch signaling, offering a therapeutic target for breast cancer[25]. Anupama Pal et al claimed that targeting USPs as breast cancer treatment is especially promising[26]. However, no results have been reported for USP41 in breast cancer, even in tumors. In this study, USP41 was found overexpressed in breast cancer and proved to be associated with the cancer progression for the first time.

Receptor for activated C kinase 1 (RACK1), a member of the tryptophan-aspartate-40 (WD-40) family of proteins, is an evolutionarily conserved a 36 kDa scaffold protein with seven WD-40 repeats, containing the subunit of G-proteins[27, 28]. The WD40 repeats of RACK 1 provides the complex protein–protein bio-interactions among signaling molecules such as integrins, phosphodiesterase 4D5, and Src tyrosine kinase, as well as protein kinase C (PKC)[29]. RACK1 seems to be essential for cellular functions because its amino acid sequence in human is 100% consistent with that in rats, chickens, mice, and cows[30]. As reported, RACK 1 can interact with numerous signaling proteins and is regarded as a scaffolding, anchor, or adaptor protein, participating into multiple intracellular signal transduction, immune defense, cell growth and migration pathways[31]. In 2018, RACK1 was verified as an oncogene in colon cancer[32]. In 2020, RACK1 overexpression was demonstrated to dampen the progression of cervical squamous cell carcinoma[33]. Wu H et al proved that RACK1 stimulates tumor invasion and lymph node metastasis of cervical cancer, providing promising means for cervical cancer treatment[34]. Dan HX et al found that RACK1 promotes cancer progression by increasing the M2/M1 macrophage ratio in oral squamous cell carcinoma[35]. For breast cancer, RACK1 was firstly identified as a superior independent biomarker for diagnosis and prognosis in 2010. Comparing with conventional diagnostic index (Ki67, ER, PR and HER-2) in breast carcinoma, RACK1 possessed its superiority in sensitivity and specificity as biomarker[36]. In this study, we also found the overexpression of RACK1 in breast cancer and its potential role in proliferation and migration. However, the mechanisms through which RACK1 regulates breast cancer progression are still in suspense.

USPs-regulated signaling pathways implicated in breast cancer is more like to be transforming growth factor beta (TGFβ) signaling, which has a well-documented role in mediating epithelial-to-mesenchymal transition (EMT), tumor progression and metastasis[19]. In 2012, USP11 was found to augment TGFβ signalling by deubiquitylating ALK5[37]. Eichhorn PA et al demonstrated that USP15 stabilized TGF-β receptor I and promoted oncogenesis through the activation of TGF-β signaling[38]. In 2019, Zhang J et al proved that USP4 inhibition prevented the activity of the TGF-β/Smad pathway signaling[39]. Galant C et al found that overexpression of several USPs amplifies fibrotic responses induced by TGF-β[40]. In this study, USP41 was found to be associated with RACK1 and RACK1 has been identified as concomitant

regulator of TGF-β1[41]. Zhou P et al indicated that RACK1 can inhibit collagen synthesis in KFs via inhibition the TGF-β1/Smad signaling pathway[42]. RACK1 silencing was proved to attenuate renal fibrosis by inhibiting TGF-β signaling[43]. Above all, USP41 maybe exert oncogenetic function via TGF-β signaling by combination with RACK1. However, the mechanisms through which RACK1 regulates breast cancer are wide ranging and diverse. Further study was still needed to explore its role in USP41 mediated breast cancer progression.

Conclusions

In conclusion, USP41 overexpression promotes the proliferation and invasion of breast cancer by combination with RACK1, which may served as emerging therapeutic potential target for breast cancer treatment.

Abbreviations

USP: ubiquitin-specific proteases

DUBs: Deubiquitinating enzymes

RACK1: Receptor for activated C kinase 1

Declarations

- **Ethics approval and consent to participate:** The study protocol was approved by an independent ethics committee of Fourth Military Medical University. The study was undertaken in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. All patients were asked to provide written informed consent before enrollment.
- **Consent for publication:** Not applicable
- **Availability of data and materials:** All data generated or analysed during this study are included in this published article
- **Competing interests:** The authors declare that they have no competing interests
- **Funding:** This work was supported by The National Natural Science Foundation of China [No. 81572917, 81472598]; Project of Xijing Hospital [No.XJZT18MJ30].
- **Authors' contributions:** Rui Ling and Nanlin Li participate into the design of the work, Meiling Huang and Jian Zhang participated into the acquisition, analysis and interpretation of data, Meiling Huang and Jing jing Xiao have drafted the work, Rui Ling and Nanlin Li substantively revised the manuscript. All authors read and approved the final manuscript
- **Acknowledgments:** Not applicable

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Figures

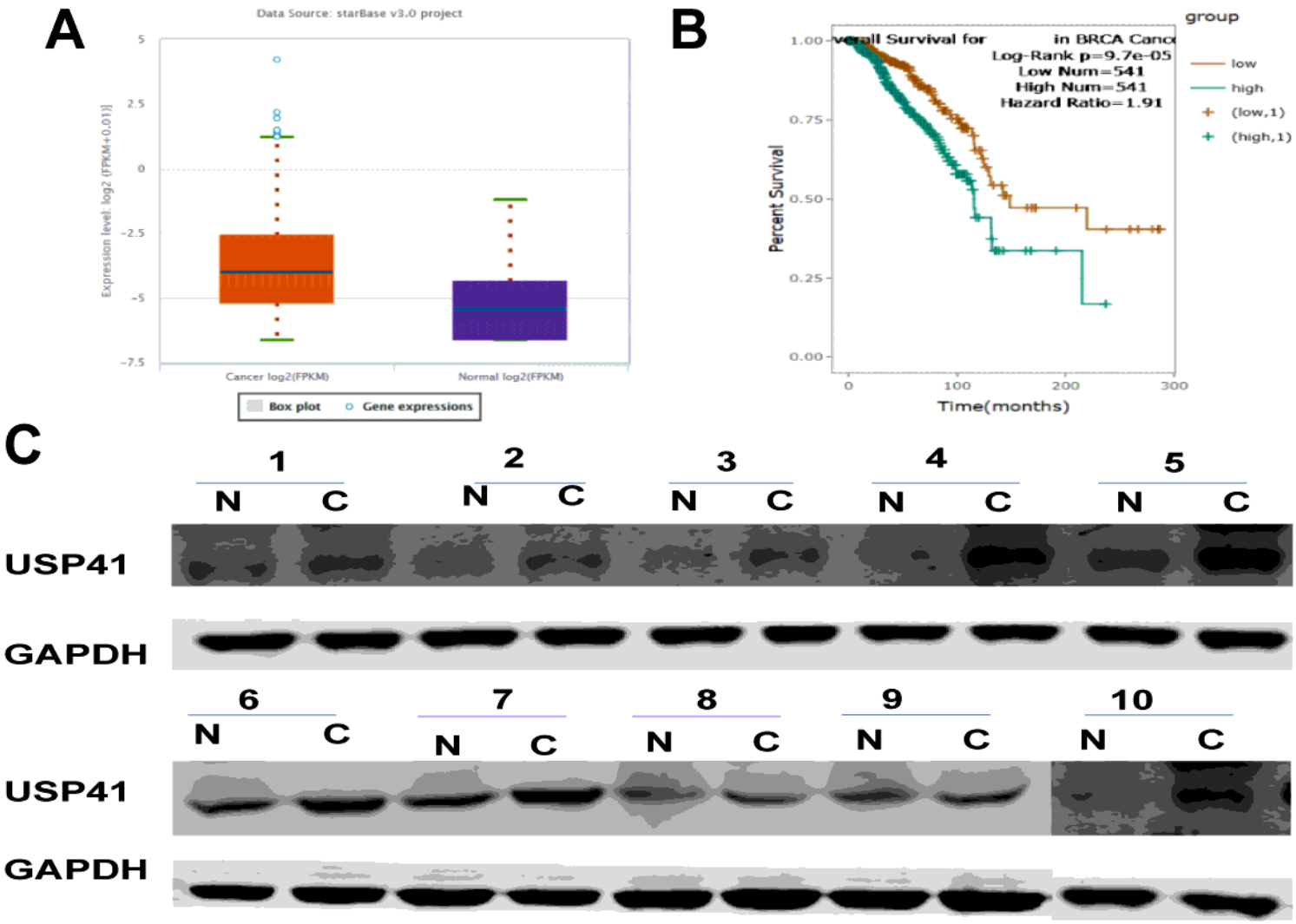

Figure 1

Overexpression of USP41 gene in breast cancer. (B) Overexpression of USP41 in breast cancer by TCGA database analysis. (B) Association of USP41 with breast cancer prognosis by TCGA database analysis. (C) The protein levels of USP41 in clinical breast cancer specimens (n=10) (P<0.05, Wilcoxon's signed-rank test). Notes: N = non-tumorous tissues; C = breast cancer specimens

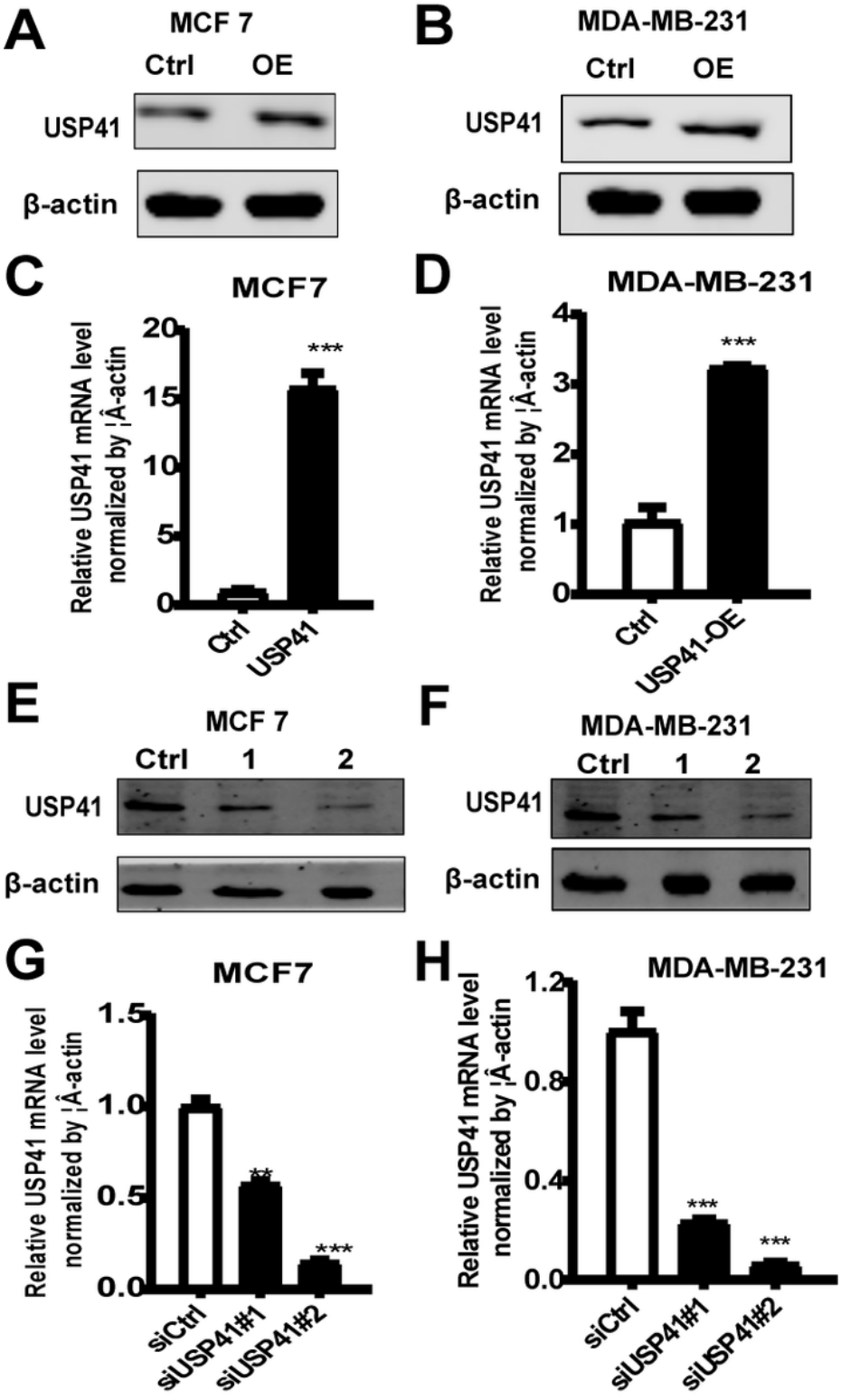

Figure 2

Establishment of USP41 gene overexpression and knockdown. (A) Protein levels of USP41 overexpression in MCF-7 breast cancer cells. (B) Protein levels of USP41 overexpression in MDA-MB-231 breast cancer cells. (C) Up-regulation of USP41 mRNA level in MCF 7 cell line were assessed by RT-qPCR. (D) Up-regulation of USP41 mRNA level in MDA-MB-231 cell line. (E) Protein levels of USP41 knockdown in MCF-7 breast cancer cells. (B) Protein levels of USP41 knockdown in MDA-MB-231 breast cancer cells.

(C) Knockdown of USP41 mRNA level in MCF 7 cell line were assessed by RT-qPCR. (D) Knockdown of USP41 mRNA level in MDA-MB-231 cell line. Notes: Ctrl = control; OE = overexpression; 1=siUSP41#1; 2=siUSP41#2; ** = P < 0.05, *** = P < 0.01.

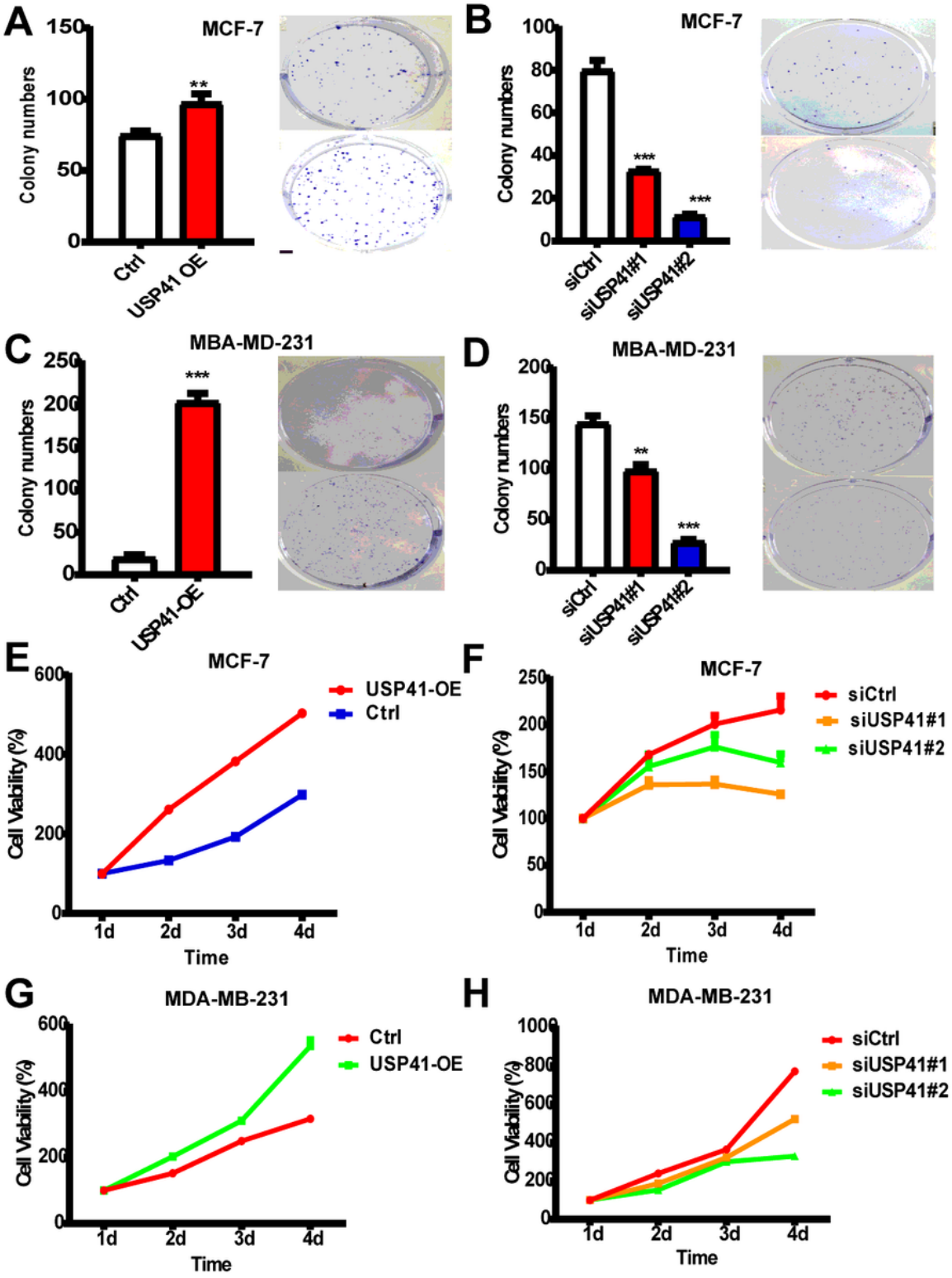

Figure 3

Effects of USP41 gene on breast cancer growth. (A) Cell colony formation after USP41 overexpression in MCF 7 cells was measured by colony formation assays. (B) Cell colony formation after USP41

knockdown in MCF 7 cells. (C) The result of colony formation assays that were conducted after the overexpression of USP41 in MDA-MB-231 cell. (D) The result of colony formation assays that were conducted after USP41 knockdown in MDA-MB-231 cell. (E) Cell proliferation after USP41 overexpression in MCF 7 cells was measured by colony formation assays. (F) Cell proliferation after USP41 knockdown in MCF 7 cells. (G) The result of CCK8 assay that were conducted after the overexpression of USP41 in MDA-MB-231 cell. (H) The result of CCK8 assay that were conducted after USP41 knockdown in MDA-MB-231 cell. ** = P < 0.05, *** = P < 0.01.

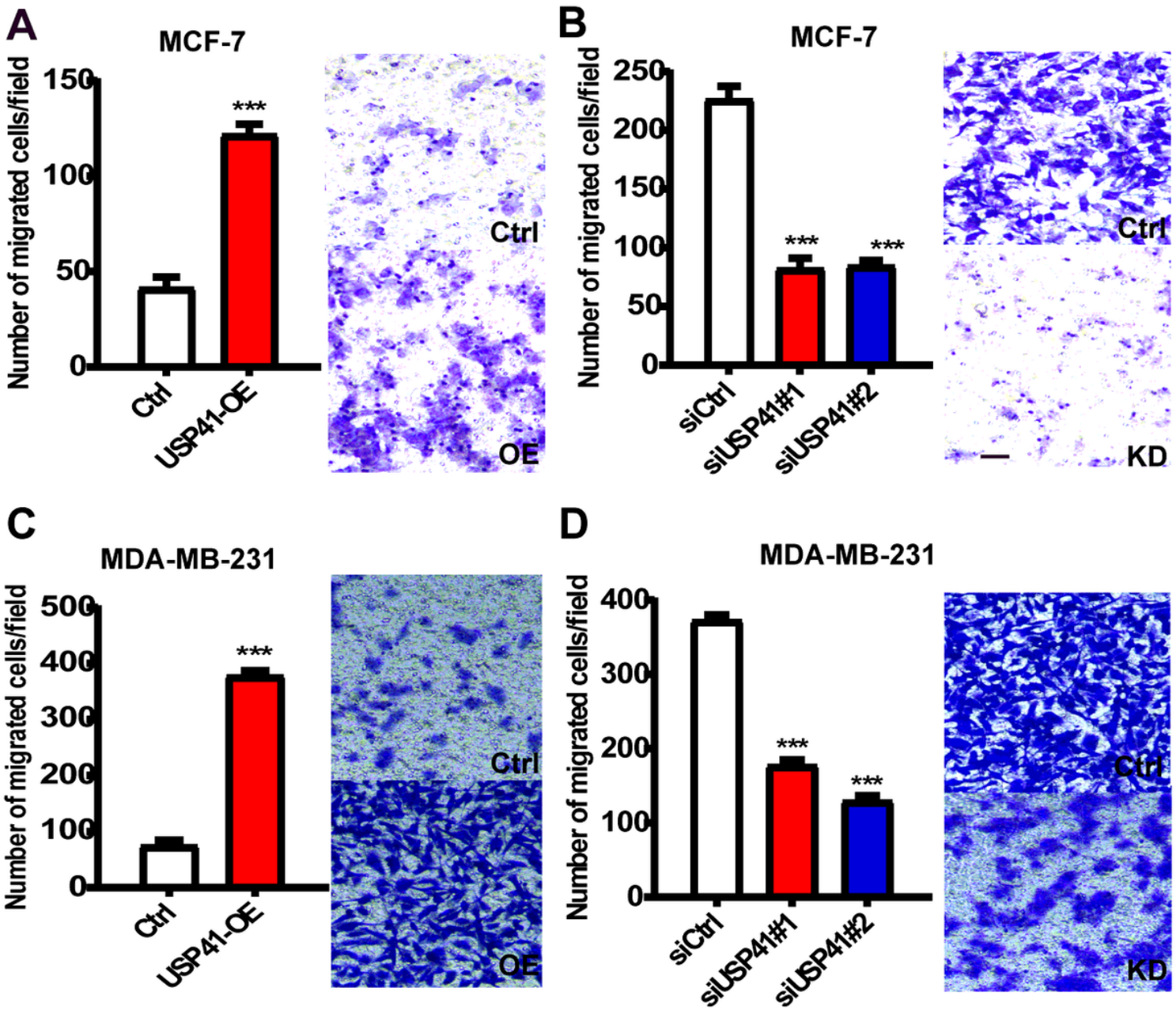

Figure 4

Effects of USP41 gene on breast cancer migration. (A) Cell invasion analysis after USP41 overexpression in MCF 7 cells. (B) Transwell invasion of MCF 7 cells after USP41 knockdown. (C) The result of cell migration assay that were conducted after the overexpression of USP41 in MDA-MB-231 cell. (D) The

result of transwell assay that were conducted after USP41 knockdown in MDA-MB-231 cell. Transwell staining results are shown as a reference (scale bar = 50 μm). Notes: Ctrl = control; OE = overexpression; KD = knockdown; ** = P < 0.05, *** = P < 0.01.

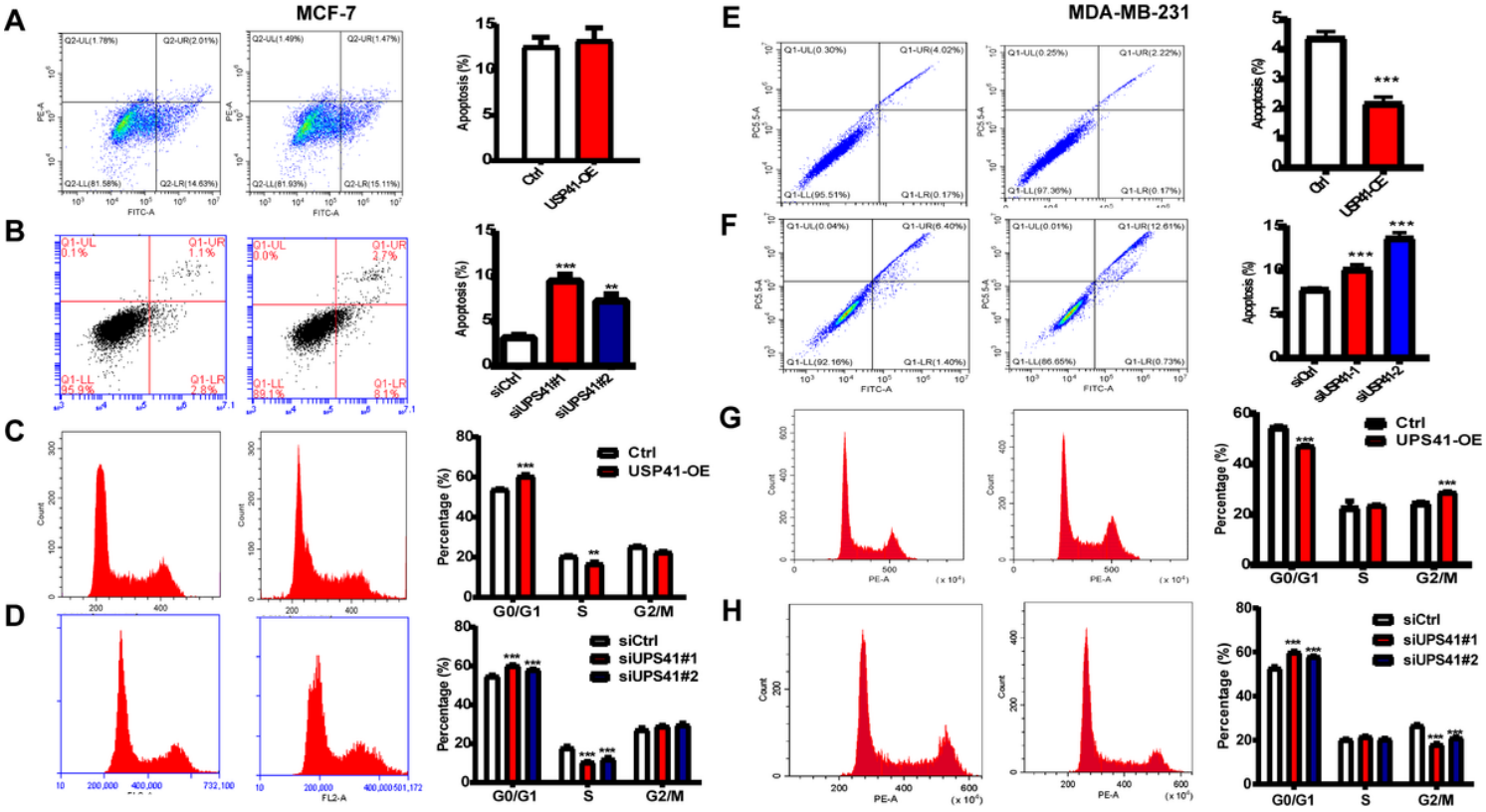

Figure 5

Effects of USP41 gene on breast cancer cell cycle and apoptosis. (A) Cell apoptotic analysis after USP41 overexpression in MCF 7 cells. (B) Cell apoptotic analysis after USP41 overexpression in MCF 7 cells. (C) Cell cycle assay that were conducted after USP41 knockdown in MCF 7 cell. (D) The result of cell cycle assay that were conducted after the of USP41 knockdown in MCF 7 cell. (E) Cell apoptotic analysis after USP41 overexpression in MDA-MB-231 cells. (F) Cell apoptotic analysis after USP41 overexpression in MDA-MB-231 cells. (G) Cell cycle assay that were conducted after USP41 knockdown in MDA-MB-231 cell. (H) The result of cell cycle assay that were conducted after the of USP41 knockdown in MDA-MB-231 cell. Notes: Ctrl = control; OE = overexpression; ** = P < 0.05, *** = P < 0.01.

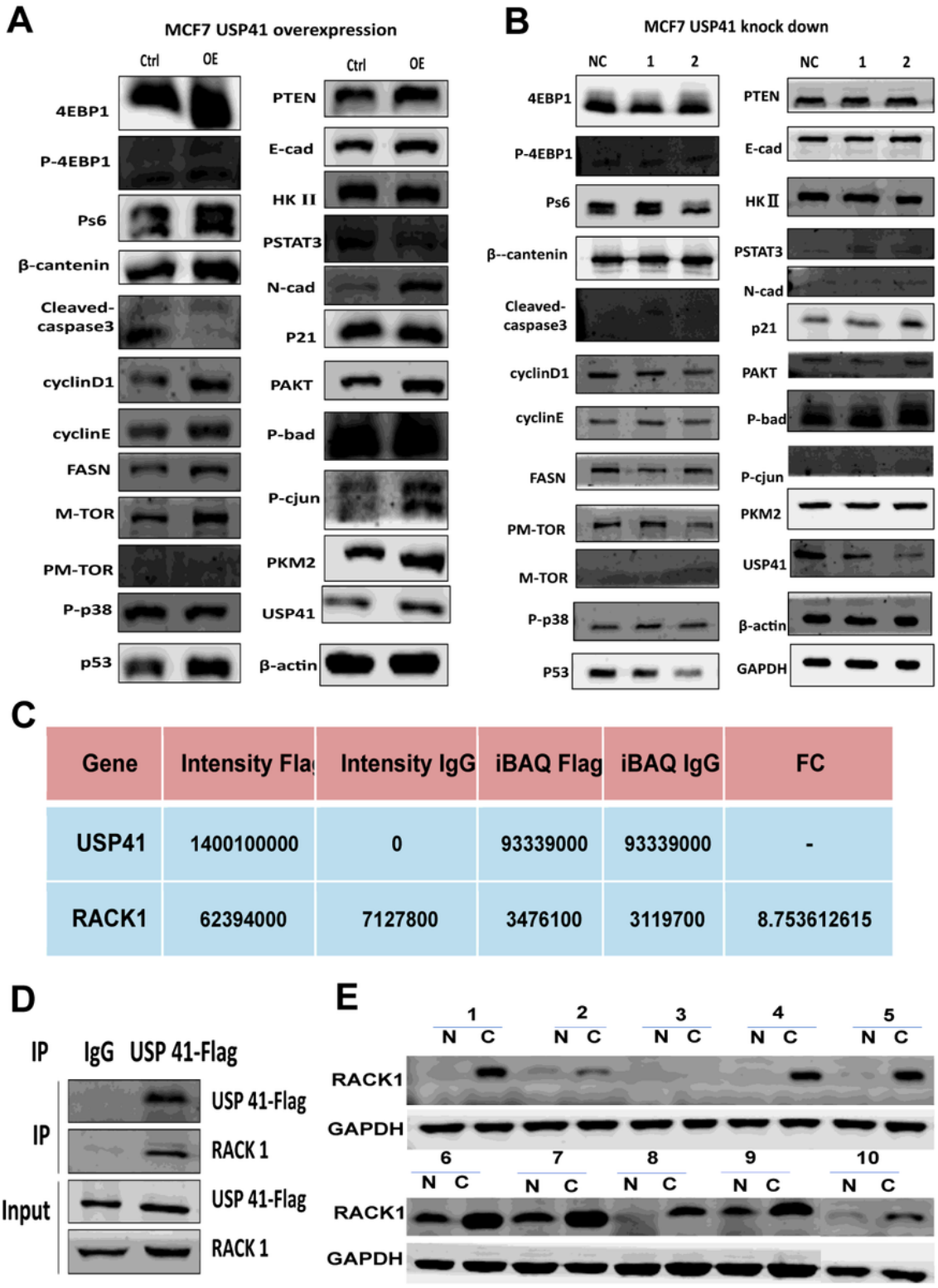

Figure 6

Effects of USP41 on marker genes in cellular signaling. (A) Protein levels of marker genes when USP41 overexpressed in MCF 7 breast cancer cells. (B) Expression of marker genes when USP41 knock down in MCF 7 breast cancer cells. (C) Combination of USP41 and RACK1 by mass spectrum. (D) Combination of USP41 and RACK 1 by CoIP analysis. (E) Overexpression of RACK 1 in cancer tissue that in normal tissue

(n=10). Notes: Ctrl = control; OE = overexpression; NC = control; N = non-tumorous tissues; C = breast cancer specimens; 1=siUSP41#1; 2=siUSP41#2

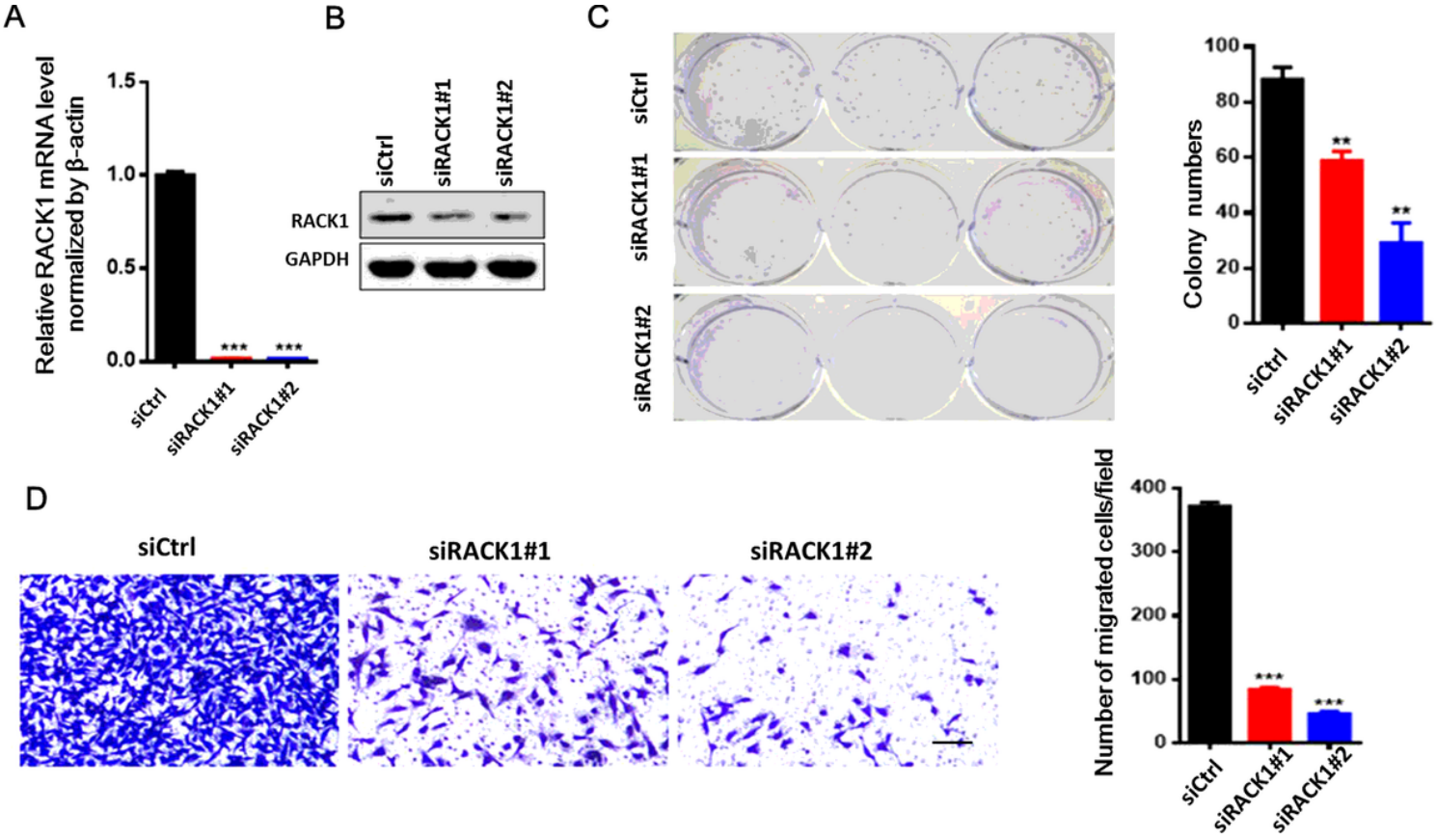

Figure 7

RACK 1 knock down inhibited the proliferation and metastasis of breast cancer cell. (A) The mRNA level of RACK1 after gene knockdown. (B) The protein level of RACK1 after gene knockdown. (C) Effects of RACK1 knockdown on breast cancer cell colony formation. (D) Effects of RACK1 knockdown on breast cancer cell migration. Transwell staining results are shown as a reference (scale bar = 50 μm). ** = $P < 0.05$, *** = $P < 0.01$.