Deubiquitinating enzyme USP42 promotes breast cancer progression by inhibiting JNK/p38-mediated apoptosis

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Abstract

Ubiquitin-specific protease 42 (USP42) exerts crucial roles in various cancers, while the detailed functions and mechanisms of USP42 on breast cancer are still uncovered. In this study, we found that USP42 was upregulated in breast cancer tissues and cells compared with normal samples. Overexpression of USP42 significantly promoted breast cancer cells growth, invasion and migration while knockdown of USP42 exerted adverse effects. Additionally, silencing USP42 dramatically increased apoptosis rate of breast cancer cells and facilitated the activation of JNK in addition of p38. Moreover, JNK or p38-MAPK specific inhibitor partially eliminated effects of silencing USP42 on the progression of breast cancer cells. In conclusion, our present investigations revealed that USP42 facilitated breast cancer cells development by reducing the activation of JNK as well as p38 and suppressing cell apoptosis.

Introduction

Breast cancer is regarded as the leading malignancies associated with women and it contributes to > 458000 mortality rate every year all over the world (1, 2). The prognosis for patients with breast cancer is not satisfactory even though diagnosis and treatments have get improvements (3). Breast cancer belongs to a heterogeneous disease with complicated growth ways so that adjuvant treatments exhibited limited effects on patients (4, 5). Recently, gene therapy aimed at neoplasms has served as a promising research for treating various cancers (6). It is vital to expounded accurate mechanisms related with the progression of breast cancer for utilizing gene therapy against breast cancer.

Ubiquitin-specific protease 42 (USP42) is one of DUB (deubiquitinating enzyme) which expressed widely in a variety of human tissues (7). Ubiquitination as a type of reversible post-translational modification is associated with various biological processes including DNA damage, cell cycle in addition to apoptosis (8, 9). More and more evidence has proved that altering DUB function is involved in the development of multiple cancers (10). For example, high expression of USP37 is tightly related with poor prognosis of patients with breast cancer and increases sensitivity of breast cancer to cisplatin (11). DUB3 stabilizes NRF2 and up-regulates chemotherapy resistance of colorectal cancer (12). PD-1 monoclonal antibody combined with targeting USP7 provides a novel therapeutic therapy for lung cancer (13). Overexpression of USP22 accelerates cancer progress and results in poor prognosis of lung cancer (14), cervical cancer (15) and glioma (16). USP42 has been showed to be involved in acute myeloid leukemia (17) and gastric cancer (18). However, the detailed functions and mechanisms of USP42 in breast cancer is unclear now.

Previous study has revealed that JNK (c-Jun N-terminal kinase) in addition to p38 MAPK (p38 activated protein kinase) are tumor suppressors related to cell viability, cell cycle as well as apoptosis (19, 20). Cell apoptosis is the common effects of activating JNK and p38, which is induced by the change of Bcl-2 family proteins (21, 22). Additionally, JNK/p38 pathway has been found to exert essential role in breast cancer progress by triggering apoptosis (23, 24). In our present study, we verified that USP42 was up-regulated significantly in breast cancer tissues in addition to cells and facilitates breast cancer cells

growth, migration as well as invasion by inhibiting JNK/p38-mediated apoptosis, which implied that USP42 may be a potential gene target for treating breast cancer.

Materials And Methods

Cell culture

All cell lines (SKBP3, MCF-7, MDA-MB-231, Bcap-37 and MCF-10A) were brought from the Cell Resource Center of Beijing Xiehe (Bejing, China) and cultured with DMEM medium (Gibco, USA) at 37 ℃ incubator containing 95% O_2 in addition to 5% CO_2. The medium was added with 10% FBS (fetal bovine serum) (Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen, USA).

Cell transfection and treatment

Cells were seeded in 12-well plates and cultured to concentration of 60–70%. The cells were transfected specific vectors by utilizing Lipofectamine 2000 reagent (Invitrogen, USA) abiding the protocol of manufacture. Prior to introduce detailed analysis, cells were pretreated with JNK inhibitor (SP600125, MCE, USA) or p38-MAPK inhibitor (SB203580, MCE, USA) for 2 h according to the guidance of manufacture.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA of tissues or cells was obtained by using TRIzol reagent (Invitrogen, USA). Then reverse transcription kit (Bio-Rad, USA) was employed to transcript mRNA to cDNA. Finally, HT7500 system (Thermo Fisher Scientific, Inc.) was used to perform qPCR analysis with SYBR Green master mix (Promega, USA). All operations were according with manufacture's guidelines and GAPDH served as endogenous control. The primer sequence used in the study is: USP42-forward: 5'-ATGGAAAGCAGGGATGAC-3', USP42-reverse: 5'-ACGCAGATTGGAACAGAG-3'; GAPDH-forward: 5'-CACCCACTCCTCCACCTTTG-3', GAPDH-reverse: 5'-CCACCACCCTGTTGCTGTAG-3'.

Western blotting

Total proteins of tissues or cells were gained by utilizing cell lysates and mixed with loading buffer then treated with 95 ℃ for 10 min. Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to determine protein content. Then protein samples were isolated through 12% SDS-PAGE and transferred to 0.22 Polyvinylidene Fluoride membrane (Thermo Fisher Scientific, Inc.). Next, the membrane was blocked by 3% non-fat milk for 40–60 min and incubated with primary antibodies USP42 (HPA006752, MilliporeSigma, 1:1000), JNK (9252, Cell Signaling Technology, 1:1000), p-JNK (9251, Cell Signaling Technology, 1:1000), p38 (9212, Cell Signaling Technology, 1:1000), p-p38 (9211, Cell Signaling Technology, 1:1000), β-actin (sc-47778, Santa Cruz Biotechnology, 1:1000) overnight at 4℃. Subsequently, the membrane was incubated with secondary antibodies conjugated with horseradish

peroxidase for 1 hour at room temperature. Finally, the bands of proteins were visualized by utilizing ECL (enhanced chemiluminescence) kit (GlpBio, USA).

CCK-8 (Cell Counting Kit-8) assay

Cell growth was estimated through CCK-8 reagent (Promega, USA). Briefly, cells which have been transfected with specific vectors were seeded in 96-well plates (2×10^3/well) and cultured overnight. 10 µl CCK-8 plus 90 µl medium was added to every well. After 1 hour, cell viability was estimated spectrophotometrically at 450 nm.

Transwell assay

For invasion assay, cells which have been transfected with specific vectors were harvested and suspended in medium without FBS. Then cells were transferred to the hydrated matrigel chamber and the lower chambers were added into 500 µl DMEM containing 10% FBS. After overnight cultivation, the cells staying on the upper chamber was abolished while the cells on the bottom surface were regarded as invasive cells. The invasive cells were fixed and stained by using 0.1% crystal violet for 30 min at room temperature. For migration assay, the re-suspended cells were seeded into the upper chamber with non-coated membrane. The bottom parts were added 600 µl DMEM with 1% FBS and cultured for 24 hours at incubator. The remaining protocols were same as invasion assay.

Cell apoptosis analysis

The transfected cells were re-suspended in 500 µl binding buffer. 5 µl FITC-conjugated annexin V in addition to 5 µl PI (BD, USA) were added into suspension liquid. The mixed solution was incubated for 15 min at 37℃ keeping away from light and analyzed by utilizing FACSalibur flow cytometer (BD, USA).

Statistical analysis

All data were exhibited in the manner of mean values ± SD. A one-way analysis or Dunnett's t-test was introduced to perform statistical analysis. **$P < 0.05$ expressed significant differences in data.

Results

USP42 is significantly upregulated in breast cancer tissues and is associated with poor prognosis of patients

GEPIA database was employed to identify that the expression level of USP42 was increased dramatically in breast cancer tissues than that in normal tissues (Fig. 1A), indicating the important functions of USP42 in breast cancer tumorigenesis. The survival plot of USP14 illustrated that high level of USP14 brought low survival rate for patients with invasive breast carcinoma (Fig. 1B). To confirm the expression of USP42 in breast cancer, we conducted RT-qPCR and western blot analysis on breast cancer (n = 38) in addition to normal tissues samples (n = 38). The results proved that both mRNA and protein level of

USP42 were higher obviously in breast cancer tissues than in normal tissues ($P < 0.05$, Fig. 1C&D). Thus, we assumed that USP42 play crucial role in the development of breast cancer.

USP42 is significantly upregulated in breast cancer cells

To explore its role in breast cancer progression, we measured USP42 expression in breast cancer cells (SKBP3, MCF-7, MDA-MB-231, Bcap-37) and noncancerous cells (MCF-10A). We discovered that load of USP42 including mRNA and protein was increased in breast cancer cell lines compared with normal cells notably ($P < 0.05$, Fig. 2A&B), predicting its dramatic functions *in vitro*.

Overexpression of USP42 promotes the proliferation, invasion and migration of breast cancer cells

Comparing several kinds of breast cancer cell lines demonstrated that the protein level of USP42 in MCF-7 cells was lower than in other cell lines (MDA-MB-231, SKBP3, Bcap-37), so we chose MCF-7 to perform subsequent experiments. We transfected plasmid with or without USP42 into MCF-7 cells and estimated that transfection efficiency was successful by using RT-qPCR in addition to western blotting ($P < 0.05$, Fig. 3A&B). CCK-8 assay illustrated that overexpressing USP42 increased the OD_{450} of MCF-7 cells ($P < 0.05$, Fig. 3C), indicating that ectopic expression of USP42 facilitated MCF-7 cells proliferation. Furthermore, transwell assay showed that USP42 over-expression remarkably accelerated cells invasion and migration ($P < 0.05$, Fig. 3D&E). These findings manifested that USP42 promoted the progression of breast cancer cells.

USP42 knockdown inhibites the proliferation, invasion and migration of breast cancer cells

To further confirm the function of USP42 on breast cancer cell, we selected MDA-MB-231 cell lines with highest USP42 expression and introduced following experiments. We employed lentiviruses to construct cell lines stably silencing USP42 and determine that shRNAs-2# (small hairpin RNAs) targeting USP42 suppressed USP42 expression at mRNA and protein level in the most efficient way ($P < 0.05$, Fig. 4A). Next, CCK-8 assay illustrated that cell viability was reduced in MDA-MB-231 cells following with USP42 knockdown ($P < 0.05$, Fig. 4C) and transwell assay verified that knocking-down USP42 blocked cells to invade and migrate ($P < 0.05$, Fig. 4D&4E). These results proved that USP42 served as an oncogene to facilitate breast cancer cells progression.

USP42 knockdown promotes apoptosis in breast cancer cells

Subsequently, we detected whether USP42 was associated with apoptosis response in breast cancer cells. Annexin V/PI double staining assay was conducted to measure apoptotic cells after silencing USP42 and the results illustrated that knockdown of USP42 significantly increased the apoptosis rate of MDA-MB-231 cells (Fig. 5A). For the alteration of proteins related with apoptosis, consistently, silencing

USP42 notably up-regulated cleaved caspase-3 in addition to Bax whereas down-regulated Bcl-2 in MDA-MB-231 cells (Fig. 5B). These findings proved that USP42 inhibited breast cancer cells viability by suppressing cell apoptosis.

USP42 knockdown upregulates JNK and p38 activation

Given that activation of JNK in addition to p38 plays vital effects in cell apoptosis response, we investigated the protein level of JNK, p-JNK, p38 and p-p38 in MDA-MB-132 cells following with knocking-down USP42 by western blotting assay. We found that phosphorylation degree of JNK as well as p38 were obviously increased by silencing USP42 while pretreatment of SP600125 (JNK inhibitor) or SB203580 (p38-MAPK inhibitor) effectively reduced the activation of JNK and p38 (Fig. 6A&B). All data demonstrated USP14 inhibiting cell apoptosis by activating phosphorylation of JNK and p38 signaling pathway.

Modulation of JNK/p38 activation is responsible for silencing USP42-activated cell progression

To further investigate whether USP42 regulated breast cancer cell development by mediating JNK/p38 activities, we employed SP600125 and SB203580 to simulate recovery experiments. As shown in Fig. 7, CCK-8 assay showed both SP600125 and SB203580 partially recover cell viability induced by silencing USP42 ($P < 0.05$, Fig. 7A). Transwell assay confirmed that SP600125 and SB203580 partly counteracted the inhibitory effects of USP42 on cell invasion in addition to migration ($P < 0.05$, Fig. 7B&C). Additionally, knockdown of USP42 dramatically increased apoptosis rate of MDA-MB-132 while pretreatment of SP600125 or SB203580 reduced apoptotic cells upregulated by silencing USP42 (Fig. 7D). Moreover, both SP600125 and SB203580 also partially eliminated the variation of apoptosis-related proteins induced by knocking-down USP42 (Fig. 7E). Taken together, all data expounded that activation of JNK and p38 was tightly associated with USP42-regulated the development of breast cancer cells.

Discussion

Recent studies have revealed that a part of members of DUB are involved in carcinogenesis such as USP1, USP7 and USP2 (25–27) while some DUB inhibited various cancer progression such as BAP1 and USP10 (28, 29). USP 42, a DUB related to p53 in addition to histone H2B has been discovered to be involved in multiple cancers (30, 31). Misaki Matsui, et al. revealed that USP42 played important role in DSB repair mediated by nuclear speckle by accelerating BRCA1 to load to DSB sites (32). Amélie Giguère et al. found that RUNX1 rearrangements combined USP42 played an essential part in acute myeloid leukemia, predicting a promising therapeutic target (33). Nevertheless, whether and how USP42 exerts crucial roles in breast cancer progress are not elucidated at all. In our present study, we uncovered for the first time that breast cancer tissues expressed higher load of USP42 at both mRNA and protein level compared to normal tissue samples. Moreover, expression level of USP42 was correlated with survival time of patients with breast cancer. Our primary findings provided compelling evidence for indispensable effects of USP42 on breast cancer.

Given these data, we assumed that USP42 served as an oncogene during the development of breast cancer. To confirm this hypothesis, we constructed plasmid with overexpressing USP42 and transfecting it into breast cancer cells. Comparing with control group, overexpressing USP42 dramatically promoted breast cancer cells growth, invasion and migration. Subsequently, we employed RNAi technology, the common strategy utilized in cancer research, to silence the expression of USP42 effectively in breast cancer cells. Knockdown of USP42 significantly suppressed breast cancer cells proliferation, invasion and migration. Taken together, USP42 was capable of facilitating the progression of breast cancer.

Apoptosis has been proved to be a well-organized and intricate process in various biological conditions and it exerts crucial effects in multiple diseases (34). One of the leading reasons for a variety of cancers occurrence is that too little apoptosis produces and tumor cells will not disappear (35, 36). In this study, we employed flow cytometry to confirm that silencing USP42 significantly increased apoptosis rate of breast cancer cell. In addition, Bcl-2, the member of Bcl-2 family, is the main regulator of apoptosis. Increased ration of Bax/Bcl-2 in addition to caspase-3 activation effectively triggered apoptosis and cell death (37). In this study, we investigated the load of apoptosis-related proteins including Bcl-2, Bax and cleaved-caspase-3, we found that silencing USP42 notably iecreased the expression of Bax in addition to cleaved-caspase-3 while decreased the expression of Bcl-2, indicating that USP42 promoted breast cancer cells progression by mediating cell apoptosis.

Previous studies have uncovered that MAPK signaling pathway is involved in various physiological progress such as cell proliferation and apoptosis (38). JNKs and p38 kinases are two indispensable subfamilies of MAPK signaling pathway and the activation JNKs as well as p38 kinases responds to multiple endogenous and exogenous stimuli (38, 39). Study has discovered that phosphorylation of JNK and p38 is associated with apoptosis tightly (37). In our present study, we revealed that knockdown of USP42 obviously up-regulated phosphorylation of JNK and p38 while JNK inhibitor or p38 inhibitor partly eliminated the above activation, which implied that silencing USP42 accelerated breast cancer cell apoptosis by activating JNK/p38 signaling pathway. Further investigation verified that pretreatment with JNK inhibitor of p38 inhibitor partially counteracted effects of silencing USP42 on breast cancer cells progression: increased cell viability, inhibited invasion in addition to migration and depressed apoptosis. However, we need more experiments to uncover that how USP42 affect the activation of JNK as well as p38. Additionally, we need to explore the direct target of USP42 on MAPK pathway and confirm the accurate deubiquitination sites.

In conclusion, our findings firstly expounded the functions of USP42 on breast cancer cells and revealed its mechanisms for the first time, which meant that USP42 may be regarded as a potential gene target for treating breast cancer.

Declarations

Ethics approval and consent to participate

This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University. All patients involved in this study have signed formal consent.

Disclosure statement

There are no conflict interest in this study.

Data availability statement

All data obtained in this study can be found in the published article.

Authors' contributions

Gongxian Wang and Chongwu He designed the study; Chongwu He, Jing Chen, and Wenrui He conducted experiments; Longbo He and Jianglong Li collected tissue samples; Chongwu He and Jing Chen analyzed these data; Gongxian Wang and Chongwu He wrote and revised the paper; all authors had read the final version of the article and agreed to submit it.

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Figures

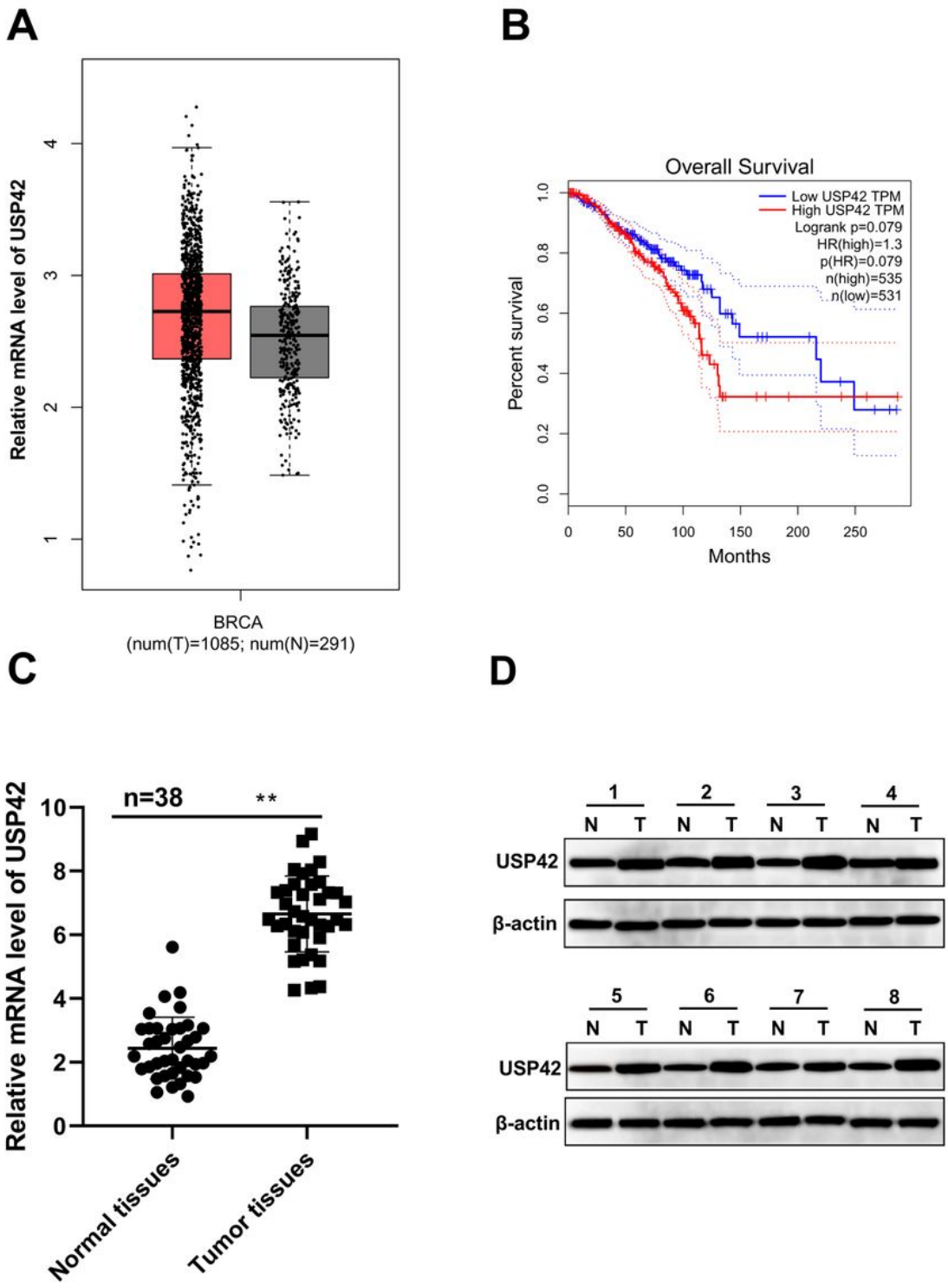

Figure 1

USP42 is significantly upregulated in breast cancer tissues and is associated with poor prognosis of patients. (A) The expression level of USP42 in breast cancer tissues and normal tissues from GEPIA database. (B) Survival analysis from GEPIA database. (C) The mRNA level of USP42 in breast cancer tissues and normal tissues measured through RT-qPCR (P<0.05). (D) The protein level of USP42 in breast cancer tissues and normal tissues measured through western blotting.

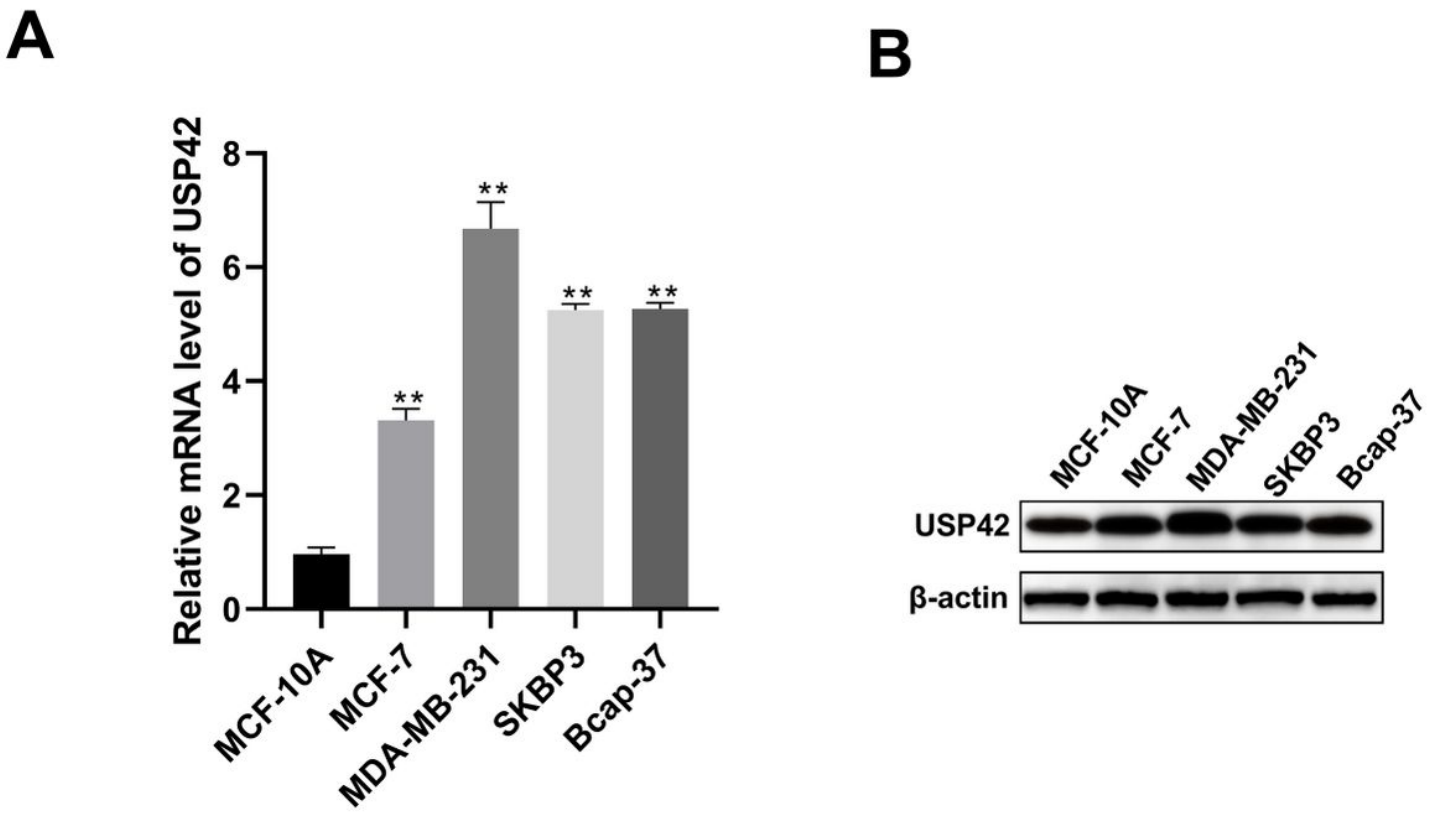

Figure 2

USP42 is significantly upregulated in breast cancer cells. (A) The mRNA level of USP42 in breast cancer cells and normal cells measured through RT-qPCR ($P<0.05$). (B) The protein level of USP42 in breast cancer cells and normal cells measured through western blotting.

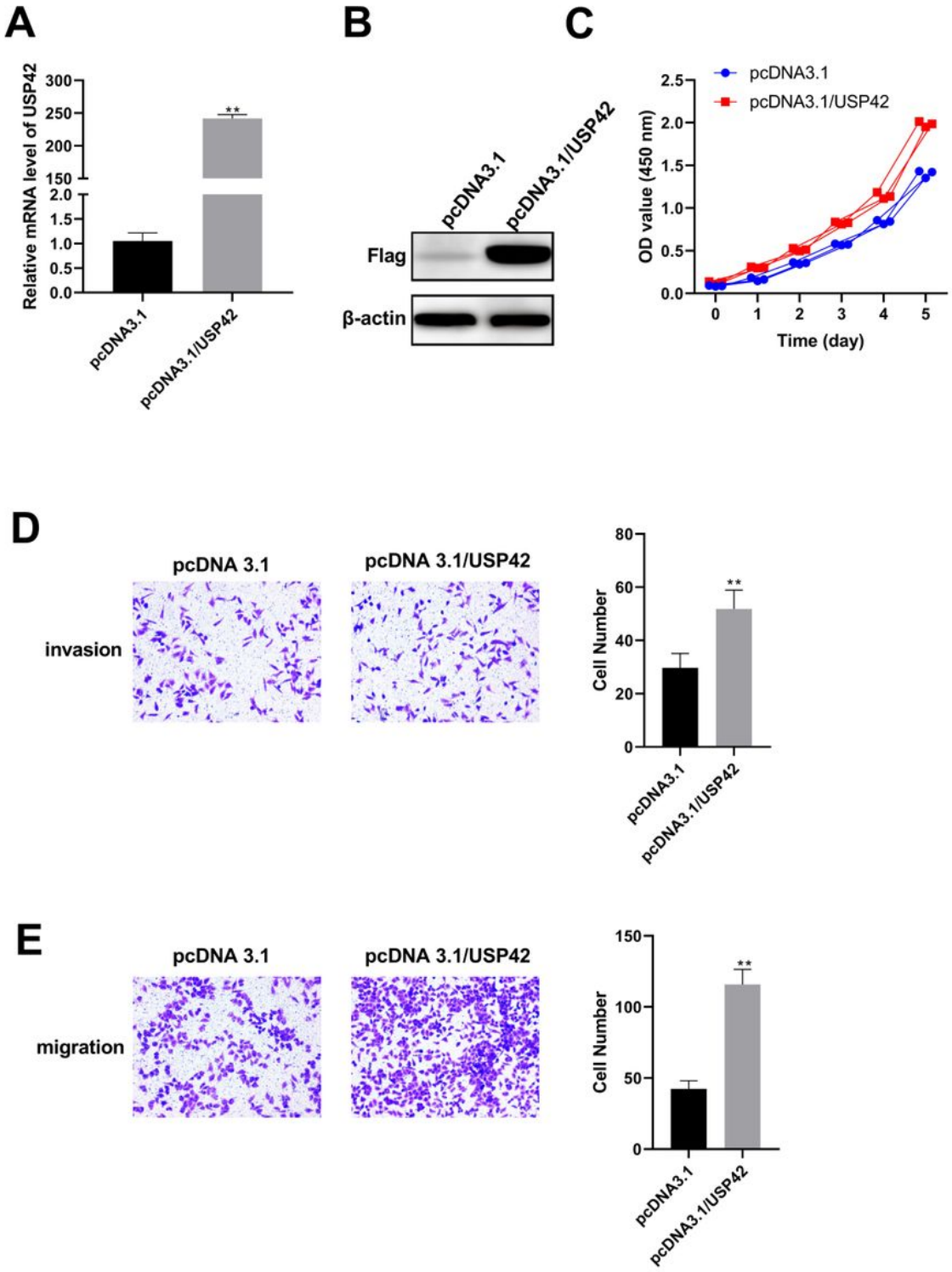

Figure 3

Overexpression of USP42 promotes the proliferation, invasion and migration of breast cancer cells. (A) The mRNA level of USP42 measured by using RT-qPCR ($P<0.05$). (B) The protein level of USP42 measured by using western blotting. (C) The cell viability detected by using CCK-8 assay ($P<0.05$). (D) The invasion ability of breast cancer detected by transwell assay ($P<0.05$). (E) The migration ability of breast cancer detected by transwell assay ($P<0.05$).

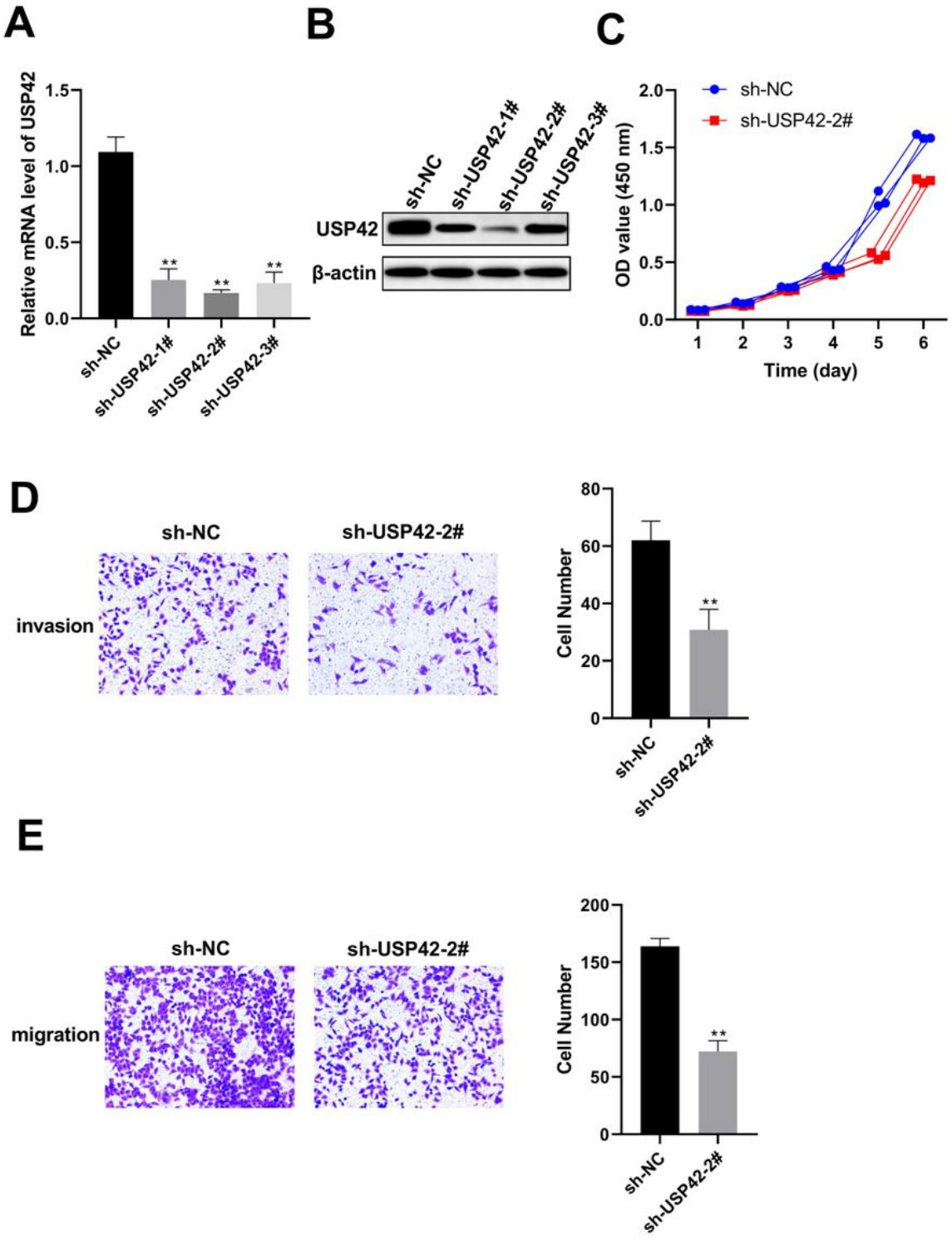

Figure 4

USP42 knockdown inhibites the proliferation, invasion and migration of breast cancer cells. (A) The mRNA level of USP42 measured through RT-qPCR (P<0.05). (B) The protein level of USP42 measured through western blotting (P<0.05). (C) The cell viability detected by using CCK-8 assay (P<0.05). (D) The invasion ability of breast cancer detected by transwell assay (P<0.05). (E) The migration ability of breast cancer detected by transwell assay (P<0.05).

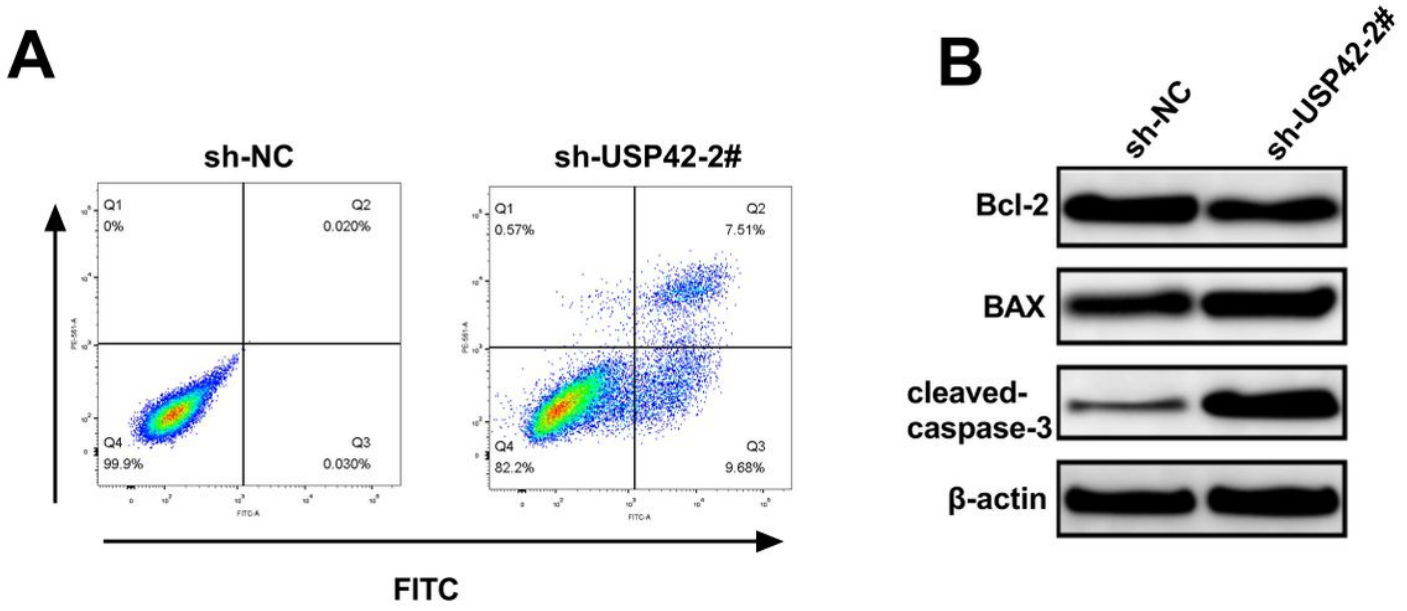

Figure 5

USP42 knockdown promotes apoptosis in breast cancer cells. (A) The apoptosis rate of MDA-MB-231 cells measured by using annexin V/PI double staining assay. (B) The proteins related to apoptosis detected by using western blotting.

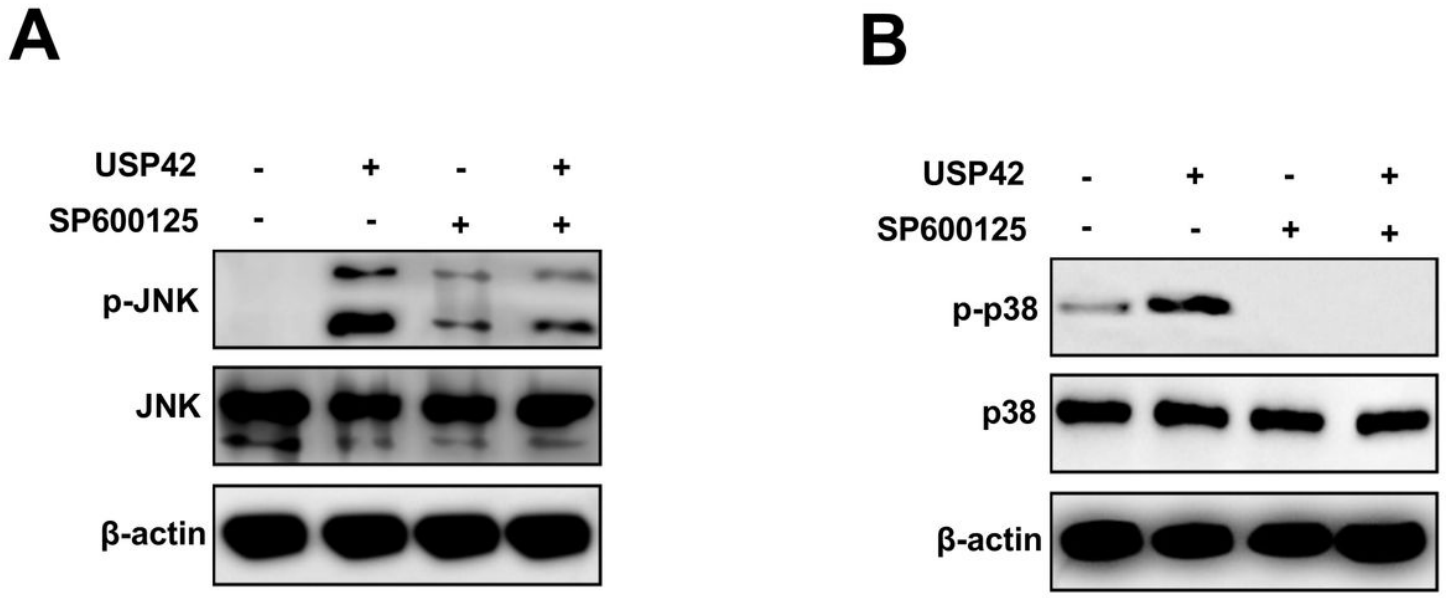

Figure 6

USP42 knockdown upregulates JNK and p38 activation. (A) The protein level of JNK and p-JNK detected by using western blotting. (B) The protein level of p38 and p-p38 detected by using western blotting.

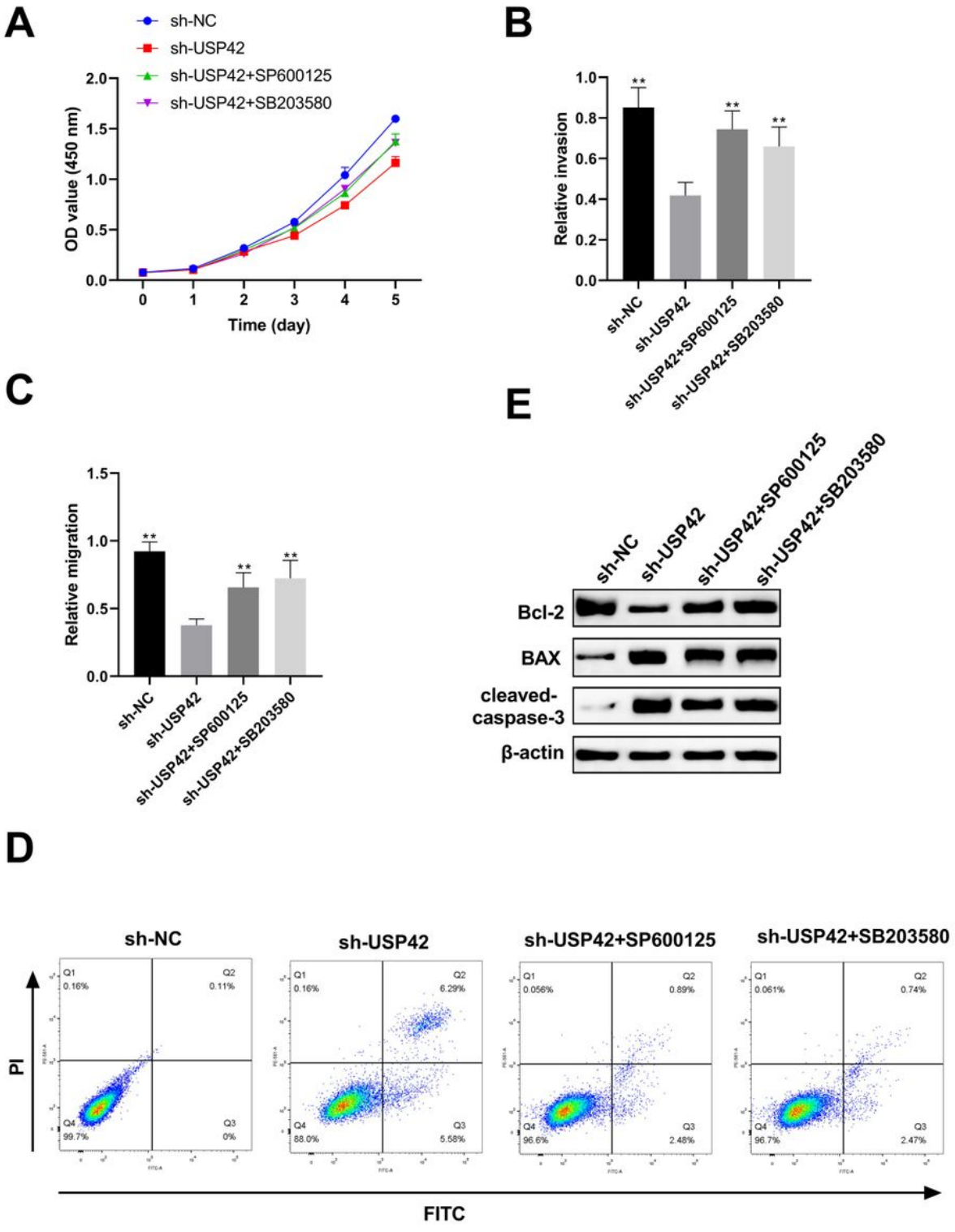

Figure 7

Modulation of JNK/p38 activation is responsible for silencing USP42-activated cell progression. (A) The cell viability detected by using CCK-8 assay (P<0.05). (B) The relative invasion ability of breast cancer detected by transwell assay (P<0.05). (C) The relative migration ability of breast cancer detected by transwell assay (P<0.05). (D) The apoptosis rate of MDA-MB-231 cells measured by using annexin V/PI double staining assay. (E) The proteins related to apoptosis detected by using western blotting.

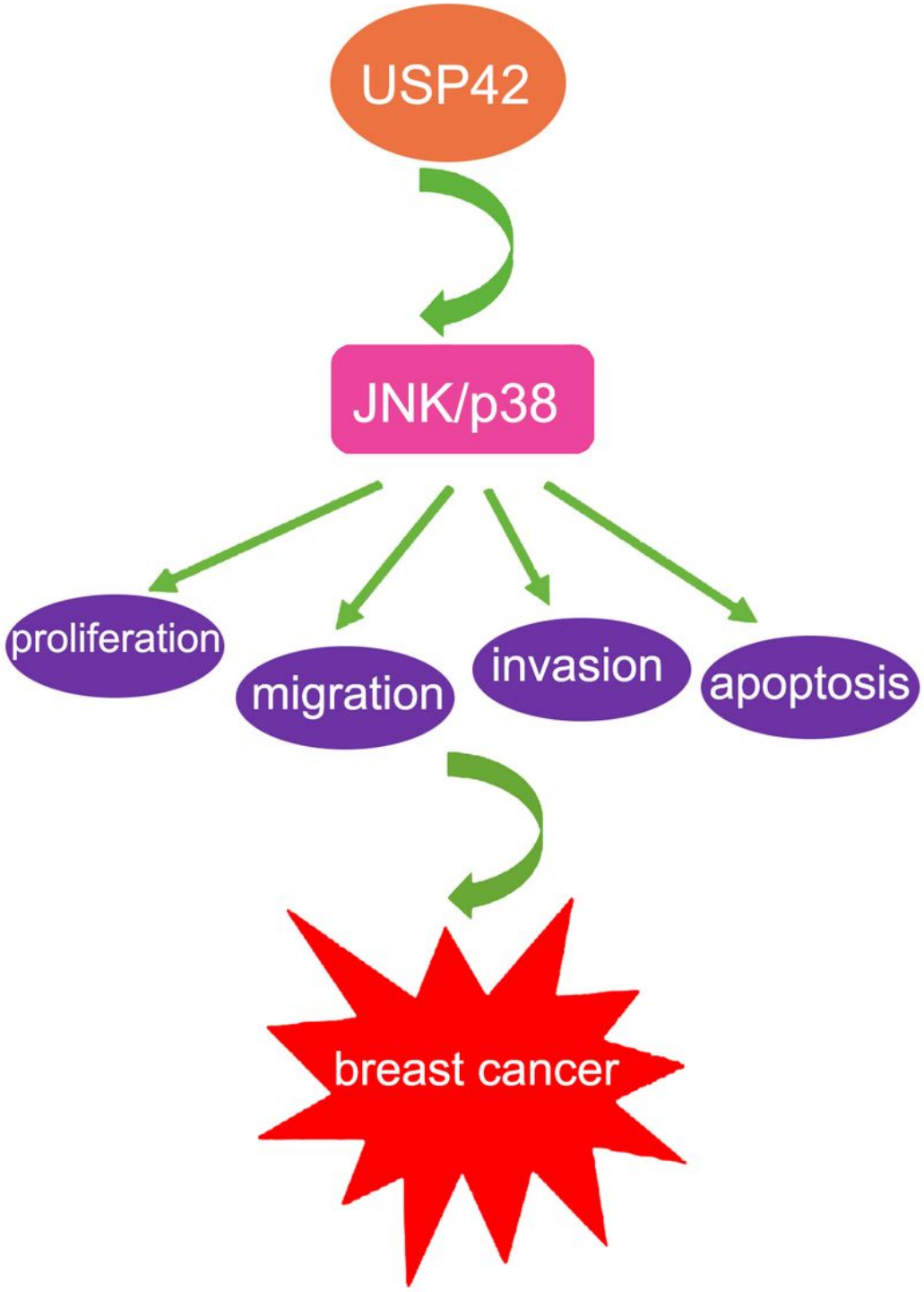

Figure 8

Graphical abstract of USP42 regulating breast cancer cell progression via JNK/p38 signaling pathway.