European Review for Medical and Pharmacological Sciences 2016; 20: 1115-1122

USP4 promotes invasion of breast cancer cells via Relaxin/TGF-β1/Smad2/MMP-9 signal

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Abstract. – OBJECTIVE: Ubiquitin-specific protease 4 (USP4) is a deubiquitinating enzyme with key roles in the regulation of TGF-β1 signaling, suggesting its importance in tumorigenesis. However, the underlying mechanisms causing this are not entirely clear. In the present study, we investigated the effect of USP4 on invasion and tumorigenesis of breast cancer cells, and explored its mechanism.

MATERIALS AND METHODS: Effects of USP4 overexpression or USP4 silencing by small interfering RNA (USP4 siRNA) on invasion of breast cancer MDA-MB-231 and T47D cells *in vitro* was detected. Using siRNAs and inhibitors to examine the USP4 signaling pathway.

RESULTS: The migration and invasion assays showed that USP4 promotes human breast cancer cell migration and invasion by USP4 overexpression, and knockdown of USP4 by siRNA inhibits human breast cancer cell migration and invasion. Treatment with RLX siRNAs, TGF-β1 siRNAs, Smad2 siRNAs or BB94 (MMPs inhibitor) to USP4-overexpressing breast cancer cells revealed that USP4- induced RLX via TGF-β1 pathway promotes the cell migration and invasion. Further studies demonstrated that USP4-mediated TGF-β1 activation not only enhances the phosphorylation of Smad2 through TGF-β, but also directly upregulate matrix metalloproteinase (MMP)-9-mediated cell migration and invasion of breast cancer cells.

CONCLUSIONS: Therapies targeting the USP4 inhibits invasion of breast cancer cells via Relaxin/TGF-β1/Smad2/MMP-9 signal. These results indicate that USP4 is an attractive target for breast cancer therapy.

Key Words:
Breast cancer, Ubiquitin-specific protease 4, Relaxin, TGF-β1.

Abbreviations

BC = breast cancer; TGF-b = transforming growth factor-beta; MMP-9 = matrix metalloproteinases-9; DMSO = dimethyl sulfoxide; BB94, batimastat; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; IMDM, Iscove's modified Dulbecco's medium.

Introduction

Breast cancer is the second-fourth leading cause of cancer-related deaths in women in the world, suggesting that early diagnosis and prevention of this disease is urgently needed[1]. Currently, breast cancer is treated with surgery, chemotherapy, and radiation therapy or combined modalities with remarkable success. In addition, patients with breast cancer or preneoplastic lesions are also treated with hormonal therapy either for treatment or prevention purposes. Although these treatment modalities are successful, a significant number of patients either do not respond to therapy, or the tumor may recur during therapy and develop metastasis, for which there is limited curative therapy[2,3]. This inadequate outcome strongly suggests that the evaluation of novel targeted therapeutic agents is urgently needed to improve the treatment outcome of patients diagnosed with this disease.

Ubiquitin-specific proteases (USPs) regulate post-translational modification of proteins by inhibiting ubiquitination of its targets, affecting multiple biological processes such as cell cycle, DNA repair, and cell signaling pathways[4]. USP4, a member of USP subfamily, is the first deubiquiting enzymes that have been identified in mammalian cells. TGF-β, a multifunctional cytokine, plays a tumor suppressive role in normal epithelia cells and precancerous tissues by inhibiting cell proliferation and inducing apoptosis, but accelerates the progression of established cancers by promoting cell proliferation, invasion, and metastasis[5-8]. TGF-β initiates signaling by binding to type

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I and type II receptor serine/threonine kinases on the cell surface, receptor-mediated Smads activation to regulate gene expression, which is a classical pathway for TGF-β signaling transduced from the cell membrane to the nucleus, and resulting in tumor suppression. Recently, it has been shown that USP4overexpression facilitates tumor cell invasion, migration, and aggressiveness by enhancing transforming growth factor-β (TGF-β) signaling[9-10]. However, the underlying mechanisms causing this are not entirely clear. Moreover, the contribution of USP4 to breast cancer progression has not been determined.

Relaxin (RLN) is a small peptide hormone expressed in several cancers of reproductive and endocrine organs. Increased expression of RLN in breast cancer correlates with aggressive cancer. Previous studies have found that RLN was the downstream of the TGF-β1/Smad2 signal, which could induce MMP-9 expression, suggesting that RLN plays a key role in breast cancer tumorigenesis[11,12].

In this study, we use USP4 gene-silenced and overexpressing breast cancer cells to determine if USP4 can promote the migration and invasion of breast cancer cells and clarify whether RLN/TGF-β/Smad2/MMP-9 are involved in the mechanisms that USP4 promotes the migration and invasion of breast cancer cells, providing a novel therapeutic target for the pathogenesis and gene therapy of breast cancer.

Materials and Methods

Cell Line and Culture

MDA-MB-231 and T47D human breast cancer cells were cultivated in HAM's F12 medium (Biochrom, Shanghai, China) substituted with 10% fetal calf serum (FCS), 2 mmol L-glutamine (Gibco, Invitrogen, Hangzhou, China), 6 ng/ ml insulin (Gibco; Invitrogen Corp, Hangzhou, China), 3.75 ng/ml hydrocortisone (Sigma, Oakville, Shanghai, China).

Agents

The primary antibodies against USP4, RLX2, TGF-β1, Phospho-Smad2 (Ser-465), MMP-9 and actin were all purchased from Cell Signaling Technology, Inc. (Shanghai, China). USP4 siRNA, RLX2 siRNA, TGF-β1 siRNA, Smad2 siRNA, BB94 (MMPs inhibitor) were from Cell Signaling Technology. Cells incubated with culture medium or culture medium with DMSO served as controls.

USP4 siRNA Transfection

MDA-MB-231 were seeded in six-well plates, grown to 50-80% confluence. Cells were transfected with USP4 siRNA (2 μg) in OptiMEM (Gibco, BRL, Grand Island, NY, USA) for 48 hs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

USP4 Plasmids Construction and Transfection

Human USP4 cDNA was cloned and amplified from leukocytes. The polymerase chain reaction (PCR) primers were: forward, 5′- GAA GGC CCT GGA TGT GAT GGT G-3′ and reverse, 5′- CAT TTC TTC CTG GGC TGC TTA TC-3′ with the restriction enzymes *Kpn*I and *Xho*I link. The cDNA was then subcloned into a linearized PMD-18T vector [Takara Biotechnology Co., Ltd., Dalian, China], digested and released with *Kpn*I and *Xho*I (Fermentas, Burlington, Ontario, Canada), and then cloned into pcDNA3.1 (+) vector (Invitrogen Life Technologies, Carlsbad, CA, USA). After amplification and DNA sequence confirmation, this vector was designated as pcDNA3.1-USP4 and used for the overexpression of USP4 in breast cancer cells. pcDNA3.1 and pCDNA3.1- USP4 plasmids were separately transfected into T47Dcells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and stable cell lines were selected with 600 μg/ml G418 (Invitrogen Life Technologies). Gene expression was confirmed by Western blot analysis. To determine the signaling pathways involved in the production of RLX, TGF-β1, TGF-βRI, Phospho-Smad2 (Ser-465), MMP-9. The stable pcDNA3.1 or pCDNA3.1- USP4 transfected T47D cells were transfected with RLX siRNA or TGF-β1 siRNA or Smad2 siRNA for 48 hs or treated withBB94 for 24 hs.

Western Blot Analysis

Cells were washed with ice-cold PBS and whole cell extracts were prepared using cell lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton-X, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 ug/mL aprotinin, and 10 ug/mL leupeptin] and cleared by centrifugation at 12,000 x g, 4°C. Total protein concentration was measured using the bicinchoninic acid assay kit (Sigma, St. Louis, MO, USA) with bovine serum albumin as a standard. Cell lysates containing 30 mg total protein were analyzed by immunoblotting. Chemoluminescent detection (Upstate, Lake

Placid, NY, USA) was done in accordance with the manufacturer's instructions.

Invasion Assay

The upper chamber of each Transwell was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) 1:6 diluted with DMEM at 37°C for 3 h. Cells (2×10^4) were seeded in upper chambers in DMEM and incubated in 24-well-plates with 10% FBS supplemented DMEM. After 36 h of incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab. Cells that invaded through the Matrigel-precoated membrane filter were fixed, stained and counted using a microscope.

Wound Healing Assay

Cells (3×10^5 cells/well) were seeded into 24-well plates at 60-80% confluency, and the cell monolayer was wounded with a 200-µl pipette. After washing with PBS for three times, 500 µl IMDM medium containing 1% FBS was added to the 24-well plates. The remaining cells were cultured in an incubator at 37°C and 5% CO_2. Cell migration was monitored under a microscope at 100 × magnification at 48 h. The wound areas were measured by Image-Pro Plus 6.0 software. The changes in migration were determined by comparing the difference in wound-healing areas within 48 h ($n = 6$).

Statistical Analysis

Student's *t*-test was used to assess the significance of differences among the different groups. Differences were expressed as mean ±SD with *p*-values < 0.05 being considered as statistically significant.

Results

Effect of siRNA on USP4 Expression in MDA-MB-231 Cells

More USP4 protein expression was shown in the MDA-MB-231 cells. MDA-MB-231 cells were transfected with USP4 siRNA and control siRNA for 48 hs. Western blot analysis was used to detect the USP4 protein level after transfection. Cells transfected with USP4 siRNA displayed a significant reduction in the expression levels of USP4 protein (Figure 1A). Control siRNA did not exhibit any effect on protein levels of USP4 (Fig-

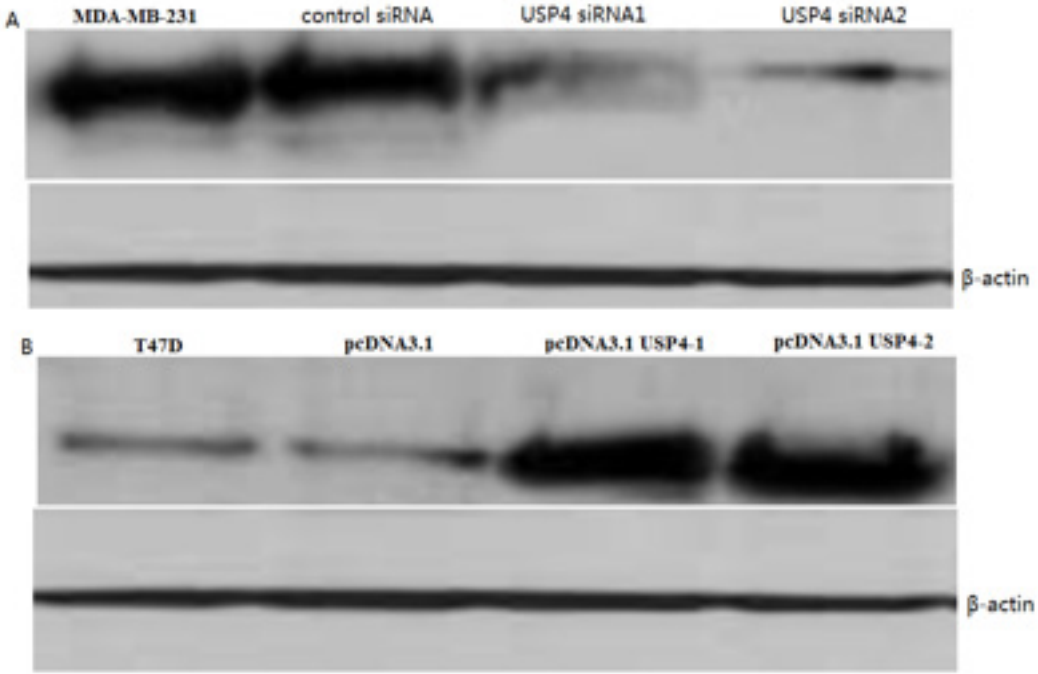

Figure 1. Effect of siRNA or USP4 plasmids transfection on USP4 expression in BC cells. ***A,*** MDA-MB-231 cells were transfected withUSP4 siRNA and control siRNA for 48 hs. Representative images showing expression of USP4 protein in control siRNA and USP4 siRNA-transfected cells as analyzed by Western blot; ***B,*** T47D cells were transfected with pCDNA3.1-USP4 plasmids and control pCDNA3.1 for 48 hs. Representative images showing expression of USP4 protein in control and USP4 transfected cells as analyzed by Western blot.

ure 1A). These data confirmed the suppression effect of siRNA and established the efficiency of siRNA transfection.

Effect of USP4 Plasmids on USP4 Expression in T47D Cells

Less USP4 protein expression was shown in the T47D cells. T47D cells were transfected with pCDNA3.1-USP4 plasmids and control pCDNA3.1 for 48 hs. Western blot analysis was used to detect the USP4 protein level after transfection. Cells transfected with pCDNA3.1-USP4 displayed a significant increase in the expression levels of USP4 protein (Figure 2A). Control pCDNA3.1 did not exhibit any effect on protein levels of USP4 (Figure 1B).

USP4 Promotes the Migration and Invasion of Breast Cancer Cells

To determine the effect of USP4 on the migration and invasion of breast cancer (BC) cells, wound-healing and invasion assays were performed using two independent USP4-knockdown clones (MDA-MB-231/USP4 siRNA1 and MDA-MB-231/USP4 siRNA2) and two USP4-overexpressing cell lines (T47D- USP4-1 and T47D-USP4-2). In the wound-healing assay, the results showed that the area changes for wound-healing in the MDA-MB-231/USP4 siRNA1 and MDA-MB-231/USP4 siRNA2 cells were reduced compared with the MDA-MB-231 or the control siRNA cells (p <0.05) (Figure 2A). In the T47D-USP4-1 and T47D-USP4-2cells, the area changes of wound-healing were significantly increased compared with the respective controls (p<0.05) (Figure 2C). In Matrigel invasion assays, the results showed that the cells in the lower chamber of Transwell were decreased in USP4-knockdown cells, compared with MDA-MB-231 or control siRNA (P < 0.05) (Figures 2B); in contrast, the cells in the lower chamber of Transwell were significantly increased in USP4-overexpressing cells compared with the control, respectively (p < 0.05) (Figures 2D).

USP4-Induced MMP-9 Promotes the Migration and Invasion of PC Cells

To study the mechanism of USP4-enhanced BC cell migration and invasion, MMP-9 levels in both USP4-knockdown and overexpressing BC cells were detected by Western blot assay. The results showed that the MMP-9 expression was inhibited in the USP4-knockdown cells, while the MMP-9 expression in USP4-overexpressing cells was increased significantly compared with the control groups (p < 0.05) (Figure 3A-3B). These results further confirm that USP4 promotes the MMP-9 expression in PC cells. Subsequently, to detect the effect of USP4-induced MMP-9 on cell migration and invasion, 20 μM BB94 were added to the culture media of T47D-USP4-1 and T47D-USP4-2 cells. The results showed that T47D-USP4-1 and T47D-USP4-2 cells were more migratory and invasive than T47D-USP4-1 and T47D-USP4-2 cells in the presence of the BB94 (Figure 3C-D).

USP4 Induced MMP-9 Through Activation of TGF-β1/Smad2 Promotes Migration and Invasion of PC Cells

We found that the effect of USP4 upregulating BC cell migration and invasion is correlated to USP4-induced MMP-9, but the mechanisms remained largely unknown. In the present study, we found that that the TGF-β1 and phosphorylation of Smad2 was significantly elevated in the USP4-overexpressing cell lines (T47D-USP4-1 and T47D-USP4-2) compared with the respective control cells (Figure 4A). In contrast, knockdown of USP4 in MDA-MB-231 cells inhibited TGF-β1 and phosphorylation of Smad2 activation (Figure 4B). These results suggested that enhances the TGF-β1/phosphorylation of Smad2 activation. To further clarify that the migration and invasion were promoted by the USP4/TGF-β1/Smad2/MMP-9 signaling pathway in BC cells, TGF-β1 or Smad2 was blocked by TGF-β1siRNA or Smad2 siRNA transfection. The results showed that the MMP-9 was significant downregulated when the activation of TGF-β1 /Smad2 was inhibited by TGF-β1 siRNA or Smad2 siRNA transfection (Figure 4A). The migration and invasion of both USP4-overexpressing cells) and the control cells were almost abolished by TGF-β1 siRNA or Smad2 siRNA transfection (p < 0.05) (Figure 4C-D).

USP4 Through Relaxin-Mediated TGF-β1/Smad2/MMP-9 Promotes Migration and Invasion of PC Cells

As shown in Figure 4A and 4B, USP4 expression promoted relaxin2 expression in the T47D cells, and knockdown of USP4 inhibited relaxin2 expression in the MDA-MB-231 cells. To further verify whether USP4 promoted the migration and invasion of BC cells through relaxin2/TGF-β1/Smad2/MMP-9 signaling pathway, the relaxin2 was blocked by relaxin siRNA transfection. The results showed that the TGF-β1, Smad2, MMP-9

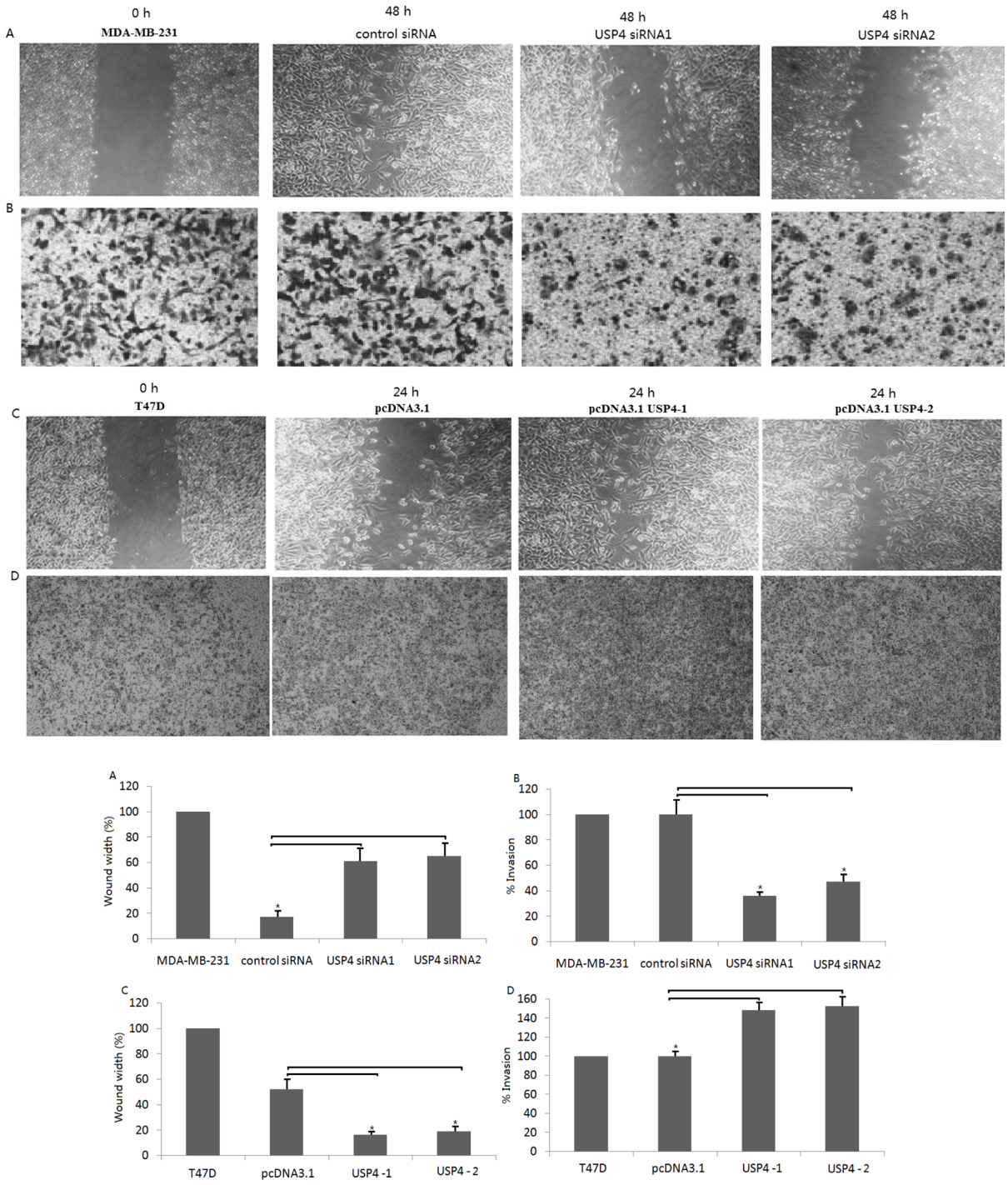

Figure 2. Effect of USP4 on the migration and invasion of breast cancer (BC) cells. ***A-B,*** Knockdown of USP4 inhibits MDA-MB-231 cells migration and invasion in wound healing assays and Transwell migration assay. ***C-D,*** USP4 overexpression promoted T47D cell migration and invasion in wound healing assays and Transwell migration assay ($^{*}p<0.05$).

expression were inhibited by relaxin siRNA transfection in USP4-overexpressing cells compared with control cells (Figure 4A). Furthermore, the migration and invasion of USP4-overexpressing cells were suppressed (Figure 4C-D). The results further indicate that USP4 promote the migration and invasion of BC cells through the relaxin/TGF-β1/Smad2/MMP-9 signaling pathway.

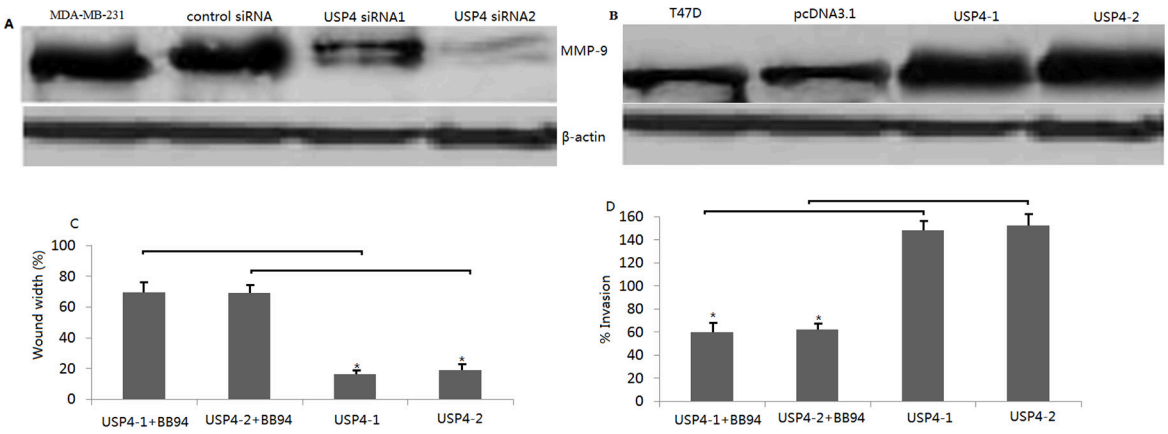

Figure 3. USP4-induced MMP-9 promotes the migration and invasion of BC cells. ***A,*** MMP-9 protein was detected in MDA-MB-231 cells transfected with USP-4 siRNA; ***B,*** MMP-9 protein was detected in T47D cells transfected with USP-4; C, D, The effect of BB94 (20 μM) on the migration and invasion of T47D-USP4-1 and T47D- USP4-2 cells detected by Transwell migration and matrigel invasion assays, respectively. The data are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$, compared with respective controls.

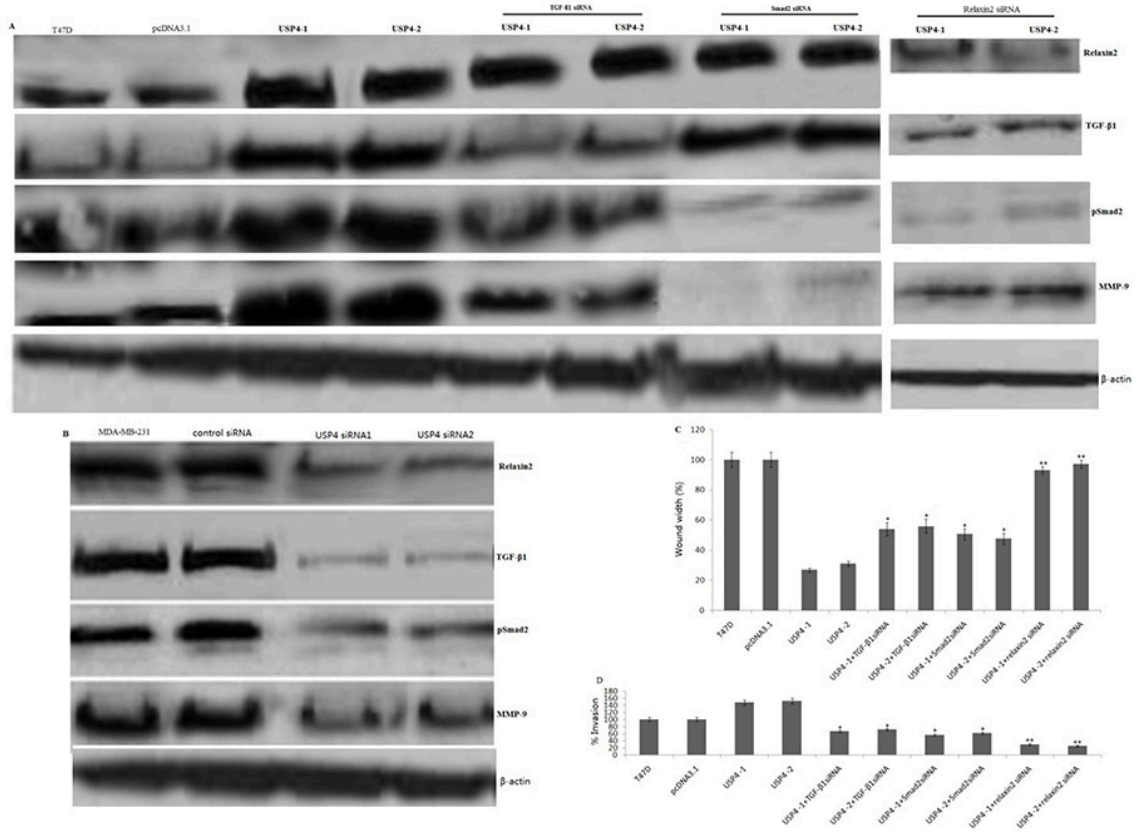

Figure 4. USP4 through TGF-β1-mediated phosphorylation of Smad2 promotes the MMP-9-mediated cell migration and invasion of BC. ***A-B,*** Cell lysates of USP4-knockdown MDA-MB-231 cells, and USP4-overexpressing T47D cells or/and TGF-β1 siRNA or Smad2 siRNs or relaxin2 siRNA transfection were analyzed by Western blotting for the relaxin2, TGF-β1, phosphorylation of Smad2, and MMP-9; actin was used as a loading control. C, D. The effect of TGF-β1 siRNA or Smad2 siRNs or relaxin2 siRNA transfection on the migration and invasion of T47D-USP4-1 and T47D-USP4-2 cells detected by Transwell migration and matrigel invasion assays, respectively. The data are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$, compared with respective contr ols. $^{*}p < 0.05$, $^{**}p<0.01$ compared with respective controls.

Discussion

USP4 regulates the growth, invasion, and metastasis of colorectal cancer. Knockdown of USP4 diminished colorectal cancer cell growth, colony formation, migration, and invasion *in vitro* and metastasis *in vivo*[13]. However, whether USP4 could promote the migration and invasion of breast cancer (BC) cells remains largely unclear.

In this study, to investigate the effect of USP4 on the migration and invasion of BC cells, we used USP4-knockdown clones and USP4-overexpressing cell lines and then performed wound-healing, Transwell migration, and matrigel invasion assays. The results demonstrated that USP4 promoted the migration and invasion of BC cells, and knockdown of USP4 inhibited the migration and invasion of BC cells.

RLX accelerates tumor progression through enhancing cell proliferation, migration, and invasion in cancer cells[14-16]. Our recent study reveals that USP4 could induce RLX expression in BC cells[17], which suggests that a novel mechanism for USP4 may promote the migration and invasion of BC cells via RLX signaling. In the present study, RLX protein expression was detected by Western blot, and we found that USP4 induced RXL and USP4 silencing inhibited RXL expression. To investigate the effect of RLX on BC cell migration and invasion, the pcDNA3.1 or pCDNA3.1- USP4 transfected T47D cells were transfected with RLX siRNAs. The results revealed that USP4-induced RLX promoted the migration and invasion of BC cells.

As described above, USP4 upregulated the BC cell migration and invasion, and is closely correlated to USP4-induced RLX, but the molecular mechanism remains largely unknown. TGF-*β* (transforming growth factor-*β*) is a prototypical member of a multifunctional cytokine family that regulates a wide variety of cellular functions. TGF-β has known to promote tumor progression through increasing tumor cell invasion and metastasis[18-20]. Previous studies[11,12] found that TGF-β was regulated by RLX. This leads to the hypothesis that USP4-induced RLX upregulated the migration and invasion of BC cells via the activation of TGF-*β*. In this study, we found that USP4 enhances the activation of TGF-*β* and cell migration and invasion are almost completely inhibited in USP4-overexpressing BC cells when TGF-*β* is blocked by TGF-*β* siRNA transfection, suggesting that USP4-induced the activation of TGF-*β* promotes the cell migration and invasion of BC cells.

Intracellular Smad2 proteins play a pivotal role in mediating antimitogenic effects of TGF-β1, but their function in TGF-β1 induced invasion and metastasis is unclear. Furthermore, there has been debate whether or not Smad2 are required for the TGF-β1-induced metastasis.

To investigate whether a molecular mechanism downstream of TGF-β1 is involved in the migration and invasion of BC cells, the phosphorylation of Smad2 were detected. In the present study, the results of Western blot revealed that the Smad2 level was consistent with the migration and invasion activities of both USP4 gene silenced and USP4-overexpressing BC cells.

A recent study has found that Smad2 could promote breast cancer cell migration and invasion by upregulation of MMP-9. In our study, we found that USP4 upregulated the phosphorylation of Smad2 and the expression of MMP-9 by Western blotting analysis. Especially, when pcDNA3.1 or pCDNA3.1- USP4 transfected T47D cells were treated with RLX siRNA or TGF-β1 siRNA or Smad siRNA, we found that USP4-induced MMP-9 activation, cell migration and invasion was deleted. These results suggest that USP4 promotes the migration and invasion of BC cells through the RLX/ TGF-β/Smad2/MMP-9 signaling pathway.

Conclusions

Our study reveals that USP4 promotes the migration and invasion of BC cells via RLX-mediated TGF-β/Smad2/MMP-9 pathway, providing the theory basis for the targeted therapy of BC. Our present results demonstrate that USP4 is an attractive target for breast cancer therapy.

This study was supported by grants from the National nature scientific research fund (No: 81572616).

Conflicts of interest

The authors declare no conflicts of interest.

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