

USP18-deficiency in cervical carcinoma is crucial for the malignant behavior of tumor cells in an ERK signal-dependent manner

AONAN PAN¹, YUE LI², JIAN GUAN³, PENGXIA ZHANG⁴, CHUNBIN ZHANG⁴, YUPENG HAN⁵,
TAO ZHANG², YAO CHENG⁶, LUO SUN⁶, SHIZHEN LU⁴, JINRU WENG³, QIAOSHENG REN³,
SHENGJIE FAN⁷, WEIQUN WANG⁸ and JINGTAO WANG⁹

¹Department of Clinical Medicine, The Affiliated Second Hospital, Harbin Medical University, Harbin, Heilongjiang 150081;

²Departments of Immunology and Etiology, Basic Medical College, Jiamusi University, Jiamusi, Heilongjiang 154007;

³Department of Maxillofacial Surgery, Stomatological College, Jiamusi University, Jiamusi, Heilongjiang 154002;

⁴Department of Biochemistry and Cell and Molecular Biology, Basic Medical College, Jiamusi University, Jiamusi, Heilongjiang 154007; Departments of ⁵Gastroenterology and ⁶Clinical Laboratory, The First Affiliated Hospital, Jiamusi University, Jiamusi, Heilongjiang 154002; ⁷Department of Rehabilitation Medicine, Rehabilitation Medical College, Jiamusi University, Jiamusi, Heilongjiang 154007; Departments of ⁸Physiology and ⁹Human Anatomy, Basic Medical College, Jiamusi University, Jiamusi, Heilongjiang 154007, P.R. China

Received November 14, 2020; Accepted February 25, 2021

DOI: 10.3892/ol.2021.12682

Abstract. Ubiquitin-specific peptidase (USP)18 belongs to the USP family, and is involved in cleaving and removing ubiquitin or ubiquitin-like molecules from their target molecules. Recently, increasing evidence has suggested that USP18 is constitutively expressed in different types of human tumors, and ectopic expression or downregulation of USP18 expression may contribute to tumorigenesis. However, the role of USP18 in uterine cervical cancer (UCC) remains unclear. Thus, the present study aimed to investigate USP18 expression in a human tissue microarray constructed using UCC and non-cancer cervical tissues, and to determine the potential role and molecular mechanism by which USP18 is implicated in the tumor biology of human UCC HeLa cells. Microarray analysis demonstrated that USP18 protein expression was downregulated in tumor tissues compared with in normal tissues. In addition, *in vitro* analysis revealed that USP18-knockdown markedly promoted the

proliferation, colony formation, migration and aggressiveness of HeLa cells. Mechanistic analysis demonstrated that USP18-knockdown increased the levels of Bcl-2, STAT3 and phosphorylated-ERK in HeLa cells. Notably, USP18 silencing-induced malignant phenotypes were interrupted following exogenous administration of the ERK1/2 inhibitor PD98059. Overall, the results of the present study suggested that USP18 may be a potent inhibitor involved in UCC tumor-associated biological behaviors, which are associated with the ERK signaling pathway.

Introduction

Uterine cervical carcinoma (UCC) is one of the most common types of malignant cancer in women, representing the fourth most frequent malignancy worldwide (1). In 2018, ~570,000 new cases of UCC were identified, and up to 311,000 associated deaths were recorded worldwide (2). Epidemiological data have demonstrated that the incidence and mortality rates of UCC vary across different regions, with more cases in Sub-Saharan Africa and South-Eastern Asia, and less cases in North America, Australia, New Zealand and Western Asia (2). Although it has been reported that UCC incidence and mortality rates have decreased in several regions of the world over the last few decades (3), UCC remains a serious health issue in China, with an estimated 106,430 new cases and 47,739 mortalities in 2018 (3).

Ubiquitin-specific peptidase (USP)18, also known as ubiquitin-specific protease 43 (UBP43), is a member of the USP family and is involved in deubiquitinating activity, thereby resulting in stabilization of substrates (4). It is well known that USPs are the largest sub-family of deubiquitinase enzymes, and exert biological roles through their cysteine

Correspondence to: Professor Weiqun Wang, Department of Physiology, Basic Medical College, Jiamusi University, 258 Xuefu Street, Jiamusi, Heilongjiang 154007, P.R. China
E-mail: wangweiqun1974@163.com

Professor Jingtao Wang, Department of Human Anatomy, Basic Medical College, Jiamusi University, 258 Xuefu Street, Jiamusi, Heilongjiang 154007, P.R. China
E-mail: wjingtao@jmsu.edu.cn

Key words: uterine cervical carcinoma, ubiquitin-specific peptidase 18, malignant behavior, ERK

endopeptidase activity (5). The USP family consists of >100 members, which predominantly differ in amino acid sequence and protein size, but are characterized by several highly homologous sequences around the essential domains important for their catalytic activity (6,7). USP18 was originally identified from acute myelogenous leukemia 1- RUNX1 partner transcriptional co-repressor 1 knock-in mice and was characterized by Liu *et al.* (8). Previous studies have demonstrated that USP18 expression is present in multiple types of tissues, including liver, lung, spleen, thymus, bone marrow and adipose tissues (8,9), and is also expressed in different types of cells, such as macrophages, lymphocytes and hematopoietic cells (10,11). The function of USP18 has predominantly been associated with the regulation of cell proliferation, cell differentiation, stress, inflammatory reaction and immune response (9-11). Additionally, it has been suggested that USP18 serves a vital role in regulating T-cell activation and T helper 17 cell differentiation through its ability to remove the transforming growth factor β -activated kinase 1-TAK1-binding protein 1 complex (12).

Several studies have suggested that USP18 may be involved in tumor biology (10,13). USP18 is overexpressed in several types of human cancer including glioblastoma, hepatocellular carcinoma, bladder cancer and breast cancer, and its high expression is associated with a poor prognosis in patients with glioblastoma and bladder cancer (14-17). Furthermore, some studies have demonstrated that USP18 is important for the malignant behaviors of tumor cells, including cellular proliferation, migration, apoptosis and epithelial-to-mesenchymal transition (EMT) (14,17-19). Conversely, it has been demonstrated that USP18 may suppress tumorigenesis due to its involvement in the antitumor immune response (20).

Although the biological functions and clinical presentation of USP18 have been well characterized in several types of human cancer including glioblastoma, breast cancer and melanoma (14,17,19,20), its underlying molecular mechanisms in UCC remain unclear. Thus, the present study aimed to investigate USP18 expression in a cervix tissue microarray, and determine its potential role and molecular mechanism in UCC malignant phenotypes.

Materials and methods

Cell line, cell culture and cell transfection. Human UCC HeLa cells were purchased from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ in a humidified incubator.

To generate USP18-deficient HeLa cells, an RNA interference silencing strategy was used to design and construct a lentivirus vector carrying small interfering (si)RNA sequences targeting USP18 by GeneCopoeia, Inc. The lentiviruses containing USP18-siRNA vector and scrambled negative control vector (mock) were synthesized by GeneCopoeia, Inc (cat. nos. HSH117922-LVRU6GP-c and CSHCTR001-3-LVRU6GP, respectively). The sequences targeting USP18 were 5'-CCAACATTAATTCCATATGAA-3', and the scrambled sequences of 5'-ACGCGTATTCGTTTACTGT-3' were used as negative control. Following infection with the

vector-carrying lentiviruses, according to the manufacturer's protocol, cells were subsequently treated with 2 ng/ml puromycin (Gibco; Thermo Fisher Scientific, Inc.) and transfection efficiency was observed under a fluorescence microscope (magnification, x200). Western blot analysis was subsequently performed to detect USP18 protein expression.

Immunohistochemistry (IHC). A commercial cervix tissue microarray containing 15 squamous cell carcinoma tissues, five adenosquamous carcinoma tissues, 20 adenocarcinoma tissues, 20 cervical intraepithelial lesions tissues, 14 cervicitis tissues, four unpaired para-cancerous tissues and two cervical canal tissues (cat. no. F801301; Bioaitech Co., Ltd.) was used to detect USP18 expression. The characteristics of the tissues are listed in Table I.

The microarray was processed routinely via deparaffinization, rehydration, endogenous peroxidase quenching and antigen retrieval, and subsequently blocked with 20% normal goat serum (Wuhan Boster Biological Technology, Ltd.) for 30 min at 37°C to remove the background from staining. The microarray was incubated with a mouse antibody against human USP18 (1:50; cat. no. sc-374064; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following the primary antibody incubation, the microarray was incubated for 35 min at 37°C using the SABC kit (cat. no. SA1021; Wuhan Boster Biological Technology, Ltd.). DAB and hematoxylin were used for visualization and nuclear counterstaining at room temperature for 30 and 5 sec, respectively. The USP18 protein expression profiles were estimated based on the staining intensity and the percentage of positive cells using a fluorescence microscope (magnification, x200). The staining intensity was ranked as follows: 1, weak; 2, moderate; 3, intensive; and 4, super intensive. The percentage of positive cells was scored as follows: 1, <25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The total score for each sample was the sum of the two parameters, as previously described (21).

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8; Wuhan Boster Biological Technology, Ltd.) assay was performed to assess the proliferation of HeLa cells following USP18-knockdown. Briefly, mock- and USP18-siRNA-transfected HeLa cells were seeded into 96-well plates at a density of 1.5×10^3 cells/well in a final volume of 100 μ l RPMI-1640 medium supplemented with or without 80 μ M of the ERK1/2 blocker PD98059 (Sigma-Aldrich; Merck KGaA). Following incubation at 37°C for 24, 48 or 72 h, 10 μ l CCK-8 reagent was added to each well and incubated at 37°C for 1 h. Cell proliferation was subsequently analyzed at a wavelength of 450 nm, using a microplate reader (Omega Bio-Tek, Inc.). The inhibitory role of PD98059 on the proliferation of HeLa cells was estimated as follows: Inhibition rate (%)=(treatment with 0 μ M PD98059-treatment with 80 μ M PD98059)/treatment with 0 μ M PD98059 x100.

Clonogenic ability assay. Mock- and USP18-siRNA-transfected HeLa cells were seeded into 24-well plates at a density of 5×10^2 cells/well in 500 μ l RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were cultured at 37°C and the medium was replaced every 2-3 days. Following incubation for 10 days, the medium was removed and cells were stained with 0.01% crystal violet for 15 min at room temperature.

A minimum of 3 mm diameter or more was considered as a colony. Images of cell colonies were captured using an imaging system (Tanon Science & Technology Co., Ltd.) and counted under a fluorescence microscope (magnification, x50).

Cell migration assay. The migratory ability of HeLa cells was assessed using 24-well Transwell chambers with polycarbonate filter of 8- μ m pore size. A total of 2×10^4 mock- and USP18-siRNA-transfected HeLa cells were plated in the upper chambers of Transwell plates in 100 μ l serum-free RPMI-1640 medium. A total of 600 μ l RPMI-1640 medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) supplemented with or without 80 μ M of the ERK1/2 blocker PD98059 was plated in the lower chambers. Following incubation for 24 h at 37°C, cells in the upper chambers were removed using a cotton swab, while the migratory cells were fixed and stained using a solution of crystal violet in ethanol for 15 min at room temperature. Stained cells were counted using a fluorescence microscope (magnification, x200). The inhibitory role of PD98059 on the migration of HeLa cells was estimated as follows: Inhibition rate (%)=(treatment with 0 μ M PD98059-treatment with 80 μ M PD98059)/treatment with 0 μ M PD98059 x100.

Wound healing assay. Mock- and USP18-siRNA-transfected HeLa cells were seeded into 6-well plates at a density of 2×10^6 cells/well in 1 ml RPMI-1640 medium supplemented with 10% fetal bovine serum. After incubation until confluent, the culture medium was replaced with serum-free medium, and a cell-free wound zone was created by scraping the monolayer with a sterile pipette tip. The images of the wounds were captured and the numbers of migrating cells were counted at 0 and 24 h after wounding using a fluorescence microscope by eye (magnification, x100).

Western blotting. Untransfected parental, mock- and USP18-siRNA-transfected HeLa cells were harvested and lysed using a commercial RIPA buffer kit (cat. no. P0013C; Beyotime Institute of Biotechnology) supplemented with phenylmethylsulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology) and 40 μ g protein/lane was subjected to 12% SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk in PBS for 60 min at 37°C. The membranes were incubated with primary antibodies against USP18 (1:1,000; cat. no. DF7968; Affinity Biosciences), Bcl-2 (1:1,000; cat. no. AF6139; Affinity Biosciences), STAT3 (1:1,000; cat. no. CY5165; Shanghai Abways Biotechnology Co., Ltd.), ERK (1:1,000; cat. no. CY5487; Shanghai Abways Biotechnology Co., Ltd.), phosphorylated (p)-ERK (1:1,000; cat. no. CY5277; Shanghai Abways Biotechnology Co., Ltd.) and β -actin (1:5,000; cat. no. AB0011; Shanghai Abways Biotechnology Co., Ltd.) overnight at 4°C. Following the primary antibody incubation, membranes were incubated with HRP-coupled goat anti-rabbit and anti-mouse secondary antibodies (1:10,000; cat. nos. BA1054 and BA1050, respectively; Wuhan Boster Biological Technology, Ltd.). Protein blots were visualized using ECL reagent (Thermo Fisher Scientific, Inc.). The protein expression levels of USP18, Bcl-2 and STAT3 were normalized to β -actin, whereas p-ERK expression was

Table I. Patient characteristics of tissues in cervix tissue microarray.

Characteristics	Cancer tissues, n=40	Non-cancer tissues, n=40
Age, years		
Range	26-63	32-66
Median	48.68	48.20
Stage, n		
I	29	
II	9	
III	2	
Grade, n		
1	10	
2	15	
3	10	
Lymph node metastasis, n		
Yes	4	
No	34	

normalized to total ERK expression using ImageJ software (version 1.45 s; National Institutes of Health).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean \pm SD. Comparisons between two groups were analyzed using unpaired Student's t-test (if the variance was homogeneous) or Cochran and Cox separate variance estimation t-test (if the variance was not homogeneous). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

USP18 expression is downregulated in UCC tissues. To evaluate the clinical significance of USP18, USP18 expression was determined via IHC analysis in a commercial cervix tissue microarray (Fig. 1). The final score demonstrated that USP18 was expressed at significantly lower levels in UCC tissues compared with in normal tissues (Table II). However, there was no significant difference in USP18 expression between different stages, grades, age and lymph node metastasis (Table II).

USP18 is expressed in HeLa cells. To assess the potential involvement of USP18 in the malignant phenotypes of HeLa cells, USP18 protein expression was detected. Western blot analysis confirmed that USP18 protein was expressed in untransfected parental HeLa cells (Fig. 2A). Following transfection with lentiviruses, fluorescent imaging demonstrated that siRNA- and mock-transfected cells expressed high-intensity screening fluorescence, suggesting that the cells were effectively infected by lentiviruses (Fig. 2B). Subsequent western blotting showed that USP18 protein expression was significantly decreased in siRNA-transfected HeLa

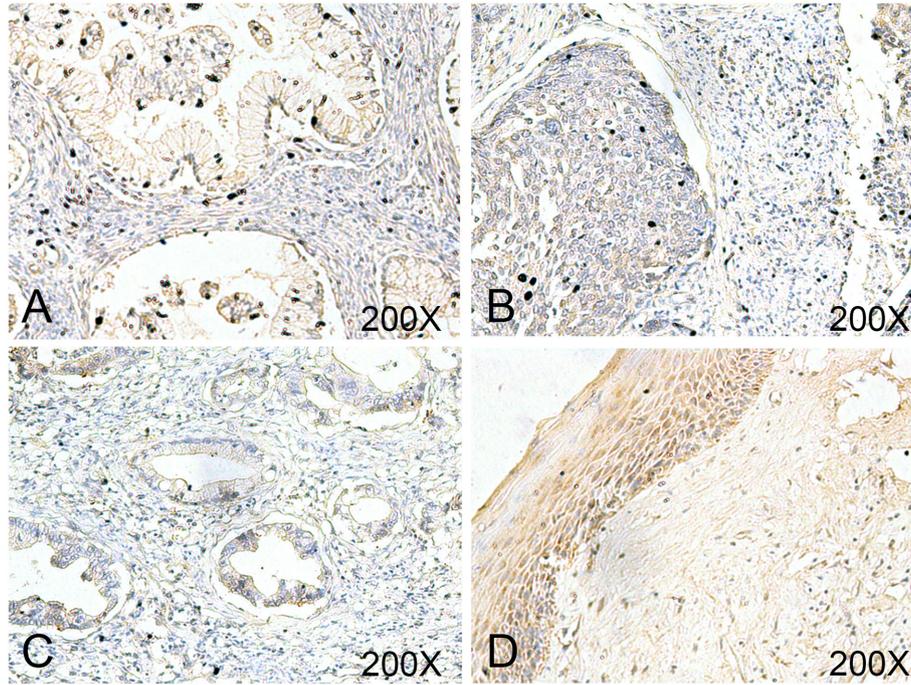


Figure 1. Downregulated USP18 expression in tumor tissues of a cervix tissue microarray. UCC tissues with grade (A) 1 and (B) 2 exhibited moderate staining, and grade (C) 3 exhibited weak staining of USP18. (D) Non-cancer tissues exhibited super intensive staining of USP18. Magnification, x200. USP18, ubiquitin-specific peptidase 18.

Table II. Ubiquitin-specific peptidase 18 expression in cervix tissue microarray (mean \pm S).

Characteristics	USP18 level	t	P-value
Tissues			
Non-cancer	5.25 \pm 1.532	2.1750	0.0327
Cancer	4.50 \pm 1.553		
Stage			
I	4.62 \pm 1.568	0.7945	0.4318
II-III	4.18 \pm 1.537		
Grade			
1	5.00 \pm 1.155	1.0299	0.3105
2-3	4.40 \pm 1.683		
Age, years			
\leq 50	4.35 \pm 1.522	0.8511	0.4001
\geq 50	4.79 \pm 1.626		
Lymph node metastasis			
No	4.62 \pm 1.615	1.0452	0.3029
Yes	3.75 \pm 0.957		

cells compared with untransfected parental and mock-transfected HeLa cells (Fig. 2A and C), which indicated that USP18-deficient HeLa cells were successfully established.

Downregulation of USP18 promotes the proliferation, colony formation, migration and aggressiveness of HeLa cells. The CCK-8 assay was performed to assess the effect of USP18

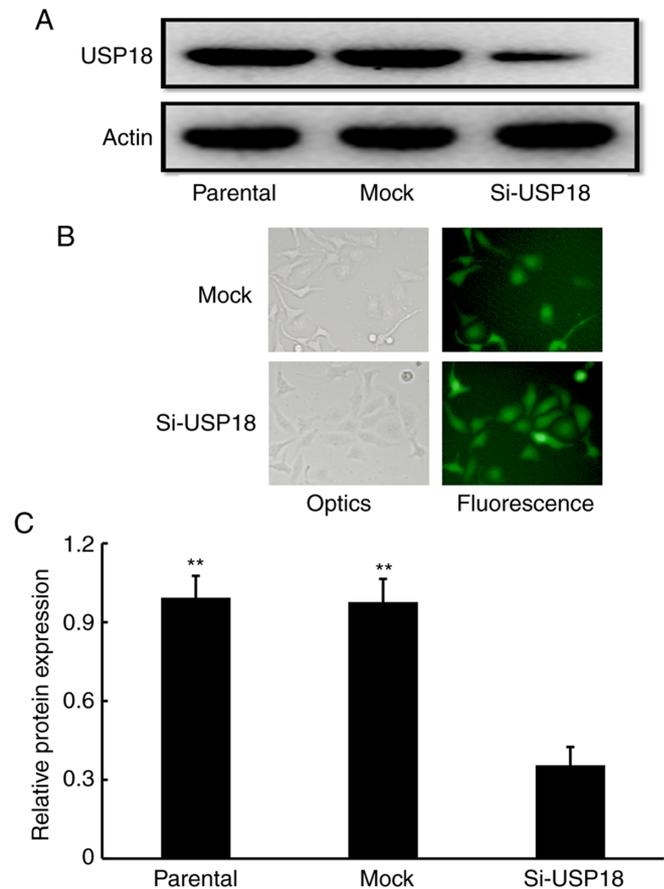


Figure 2. USP18 expression in HeLa cells. (A) Representative images of USP18 protein expression from western blot assay. (B) Fluorescence images of mock- and USP18-siRNA-transfected HeLa cells. Magnification, x200. (C) Statistical analysis of USP18 protein expression. **P<0.01 vs. si-USP18. USP18, ubiquitin-specific peptidase 18; siRNA, small interfering RNA.

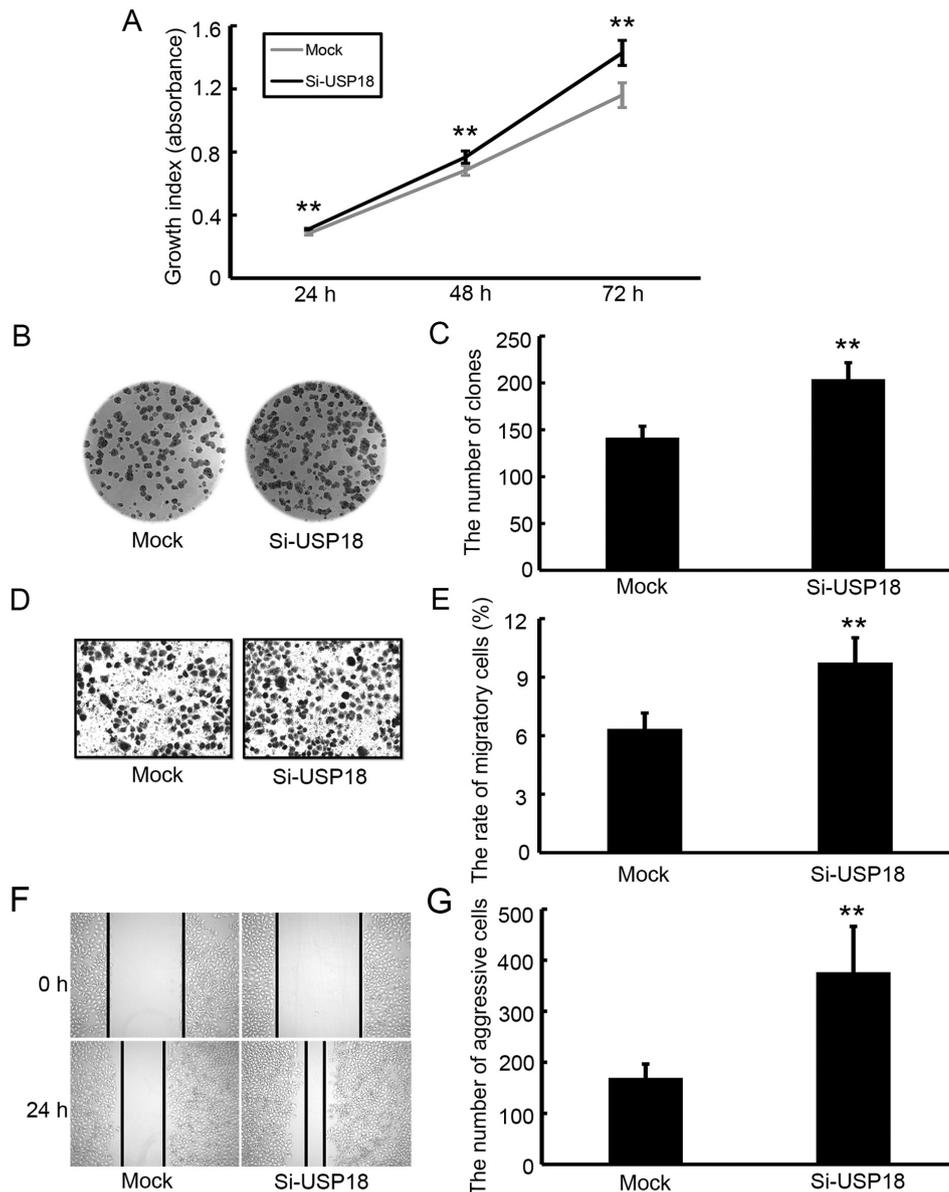


Figure 3. Roles of USP18-deficiency in HeLa cell malignant behaviors *in vitro*. (A) Proliferation analysis from mock- and USP18-siRNA-transfected HeLa cells using Cell Counting Kit-8 reagent. (B) Representative images of clonogenic analysis from mock- and USP18-siRNA-transfected HeLa cells. (C) Statistical analysis of colony formation ability. (D) Representative images of Transwell analysis from mock- and USP18-siRNA-transfected HeLa cells. Magnification, x200. (E) Statistical analysis of cell migratory capacity. (F) Representative images of wound healing assay from mock- and USP18-siRNA-transfected HeLa cells. Magnification, x100. (G) Statistical analysis of cell aggressiveness capacity. **P<0.01 vs. mock. USP18, ubiquitin-specific peptidase 18; siRNA, small interfering RNA.

on the malignant behavior of HeLa cells. Following incubation for 24, 48 and 72 h, the results demonstrated that the proliferative ability of siRNA-transfected HeLa cells was significantly increased compared with mock-transfected HeLa cells (Fig. 3A). The results of the colony formation, migration and wound healing assays demonstrated that silencing of USP18 in HeLa cells significantly increased their colony forming (Fig. 3B and C), migratory (Fig. 3D and E) and aggressive abilities (Fig. 3F and G).

USP18-knockdown alters ERK-signaling pathway-specific protein expression. The potential molecular mechanism by which USP18 regulates the malignant process of HeLa cells was assessed via western blotting. The results demonstrated that the

protein expression levels of Bcl-2, STAT3 (Fig. 4A and B) and p-ERK (Fig. 4C and D) were significantly increased following USP18-knockdown in HeLa cells, suggesting the involvement of ERK signaling in the malignant phenotypes of UCC cells.

USP18 silencing-induced malignant behaviors of HeLa cells depend on ERK signaling. To further clarify whether the roles of USP18 silencing on HeLa cells were mediated by ERK signaling, the ERK1/2 blocker PD98059 was used. The results demonstrated that there were no significant differences in cell proliferation at 24 or 48 h (Fig. 5A) and cell migration at 24 h (Fig. 5C and D) between siRNA- and mock-transfected cells following treatment with PD98059. Although the proliferative rate of siRNA-transfected cells was significantly higher

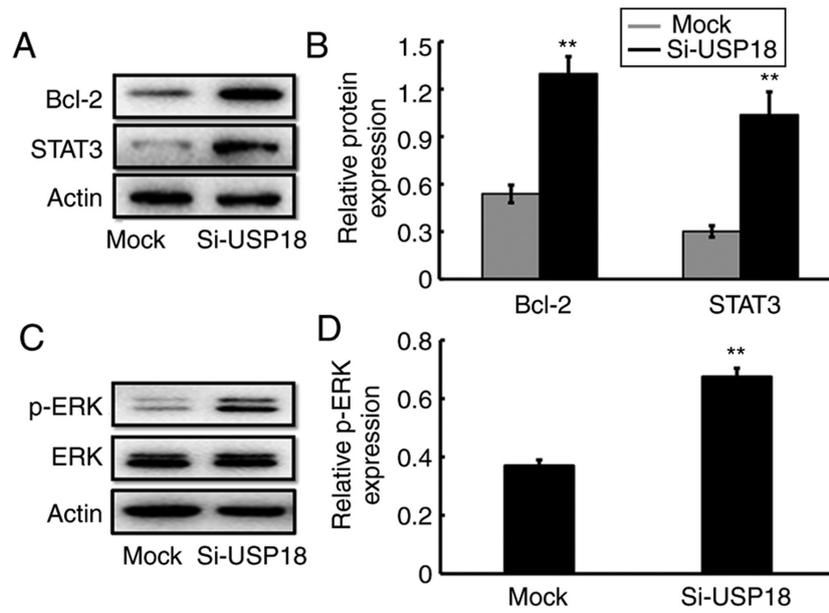


Figure 4. Effects of USP18-deficiency on regulating ERK-associated gene expression levels in HeLa cells. (A) Representative western blotting images of Bcl-2 and STAT3 from mock- and USP18-siRNA-transfected HeLa cells. (B) Quantification of Bcl-2 and STAT3 protein expression. (C) Representative western blotting images of ERK and p-ERK from mock- and USP18-siRNA-transfected HeLa cells. (D) Quantification of p-ERK levels. ** $P < 0.01$ vs. mock. USP18, ubiquitin-specific peptidase 18; siRNA, small interfering RNA; p, phosphorylated.

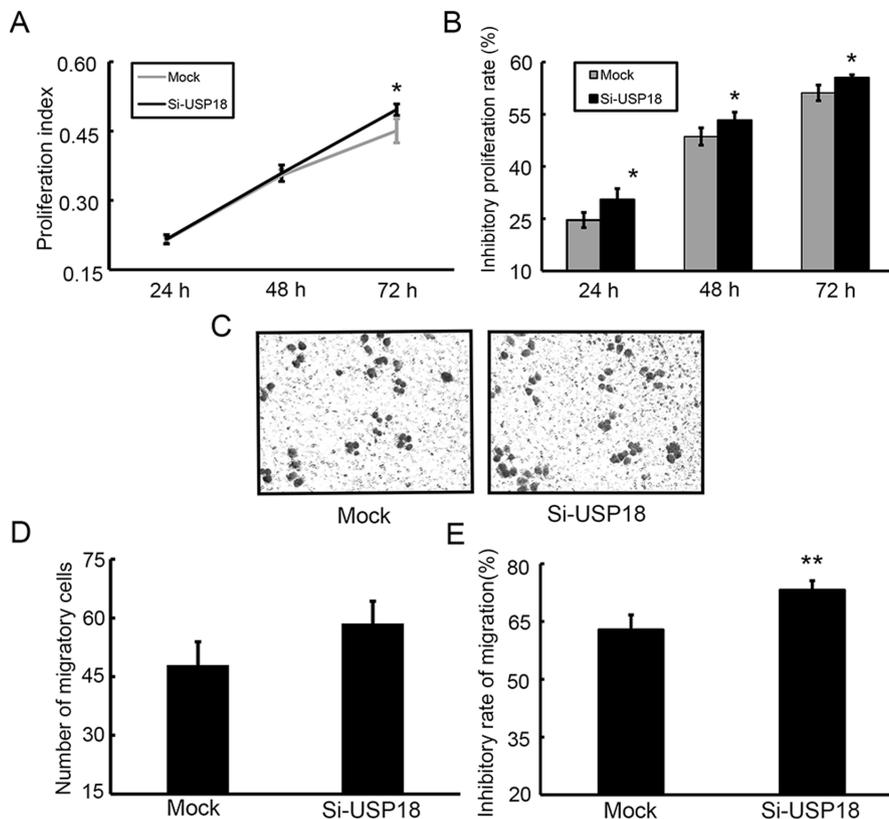


Figure 5. Involvement of ERK signaling in USP18-deficient-mediated malignant behaviors in HeLa cells. (A) Proliferation analysis after mock- and USP18-siRNA-transfected HeLa cells treatment with PD98059. (B) Proliferation inhibitory activity analysis after mock- and USP18-siRNA-transfected HeLa cells treatment with PD98059. (C) Representative images of Transwell analysis from mock- and USP18-siRNA-transfected HeLa cells treatment with PD98059. Magnification, $\times 200$. (D) Statistical analysis of cell migratory capacity. (E) Migration inhibitory activity analysis after mock- and USP18-siRNA-transfected HeLa cells treatment with PD98059. * $P < 0.05$ and ** $P < 0.01$ vs. mock. USP18, ubiquitin-specific peptidase 18; siRNA, small interfering RNA.

compared with that of mock-transfected cells after 72 h of exposure to PD98059 ($P < 0.05$; Fig. 5A), the difference between

the two groups was slightly lower compared with that between the two groups treated without PD98059 (Fig. 3A). Following

treatment with PD98059, the inhibition rates exerted by PD98059 on the proliferation (Fig. 5B) and migration (Fig. 5E) of siRNA-transfected HeLa cells were significantly higher compared with those in mock-transfected HeLa cells. Overall, the present results suggested that USP18 silencing-induced malignant responses in HeLa cells depend on the activation of the ERK signaling pathway.

Discussion

USP18 is a major isopeptidase, which was initially identified based on its role to efficiently deconjugate interferon-stimulated gene 15 (ISG15), a two-domain ubiquitin-like protein, from ISGylation (9). In addition to ISG15, USP18 is highly induced by type I and III interferons, and it has been proposed that USP18 is a vital blocker of the type I interferons signaling pathway (22). Furthermore, a study demonstrated that type III interferons may induce USP18 production (23). In the presence of USP18, type III interferons acquire higher properties to weaken type I interferons-mediated actions by repressing JAK-STAT signaling (23).

Increasing evidence has suggested that USP18 is implicated in a variety of physiological and pathological processes in different tissues and cells, including cell development, viral infection, viral replication and antibacterial response (10,24,25). However, a vast expansion in the understanding of USP18 expression and its association with tumor biology has occurred. It has been reported that USP18 is frequently overexpressed in different types of cancer, including breast cancer, bladder cancer and hepatocellular carcinoma, and its overexpression is positively associated with several pathological tumor characteristics (15-17). For example, a recent study indicated that USP18 methylation is predominantly downregulated, whereas its expression is upregulated in breast cancer, which is positively associated with increasing TNM stage, worse disease-free survival rate and HER2⁺ patients, but negatively associated with apoptosis (17). Accordingly, it has been suggested that USP18 may be used as a predictive marker for poor prognosis in muscle invasive bladder cancer, since high USP18 expression is a significant risk factor for cancer-specific death, and decreased USP18 expression is markedly associated with longer cancer-specific survival (16). USP18 has also been the focus of investigations evaluating its functions in tumorigenesis. USP18 silencing in a mouse model for breast cancer exhibited a significant decrease in tumor growth, and USP18-deficiency in breast cancer MCF-7 cells *in vitro* triggered an increase in the induction of apoptosis (18,19). In addition, downregulation of USP18 expression in glioblastoma cells may protect against tumor cell invasion and migration by repressing EMT (14), an essential event for cancer metastasis, by which tumor cells obtain increased motility and invasiveness. Gain-of-function assays *in vitro* have demonstrated that overexpression of USP18 has an important role in regulating tumor progression due to its contribution in enhancing breast cancer tumor cell proliferation, colony formation and cell cycle progression (17). However, studies have also revealed that USP18 may exert an opposing role in the control of cancer development. For example, ectopic USP18 expression in B16 melanoma cancer cells may suppress tumorigenesis, restraining cancer cell-mediated inhibition of T-cell proliferation and activation, thus facilitating cancer cells

to specific immune responses (20). In human leiomyosarcoma, downregulation of USP18 is associated with a poor clinical outcome, and USP18-deficient mice exhibited an enhanced ability to develop these sarcomas (26).

Although a recent study demonstrated that USP18 is a critical regulator for the tumorigenicity of cervical cancer CaSki and SiHa cells (27), the results of the present study demonstrated that USP18 expression was downregulated in UCC compared with in normal tissues. USP18 expression was knocked down in HeLa cells, and the malignant behaviors in cells, including proliferation, colony formation and migration, were enhanced. Mechanistically, p-ERK expression was significantly upregulated following USP18-knockdown in HeLa cells. Previous studies have reported that several signaling pathways, such as the PTEN/AKT (28), AKT/Skp2 (16) and JAK/STAT (29) signaling pathways, are implicated in USP18 associated-biological roles. To the best of our knowledge, the present study was the first to illustrate an involvement of ERK signaling in the function of USP18 in tumorigenesis. ERKs are a family of protein-serine/threonine kinases, which serve vital roles in the control of diverse cell functions, such as cell differentiation, proliferation and survival, by phosphorylating several substrates including transcription factors, protein kinases and phosphatases (30). Increasing evidence has suggested that amplification or activation of ERK signaling frequently occurs in several malignant tumors, such as gastric adenocarcinoma and lung cancer (31,32), which results in increased cell proliferation, promotion of cell cycle progression and repressed apoptosis of tumor cells (33). Given that the ERK signaling pathway participates in several aspects of tumorigenesis by regulating the expression of its downstream signaling molecules, such as NF- κ B (34), Bcl-2 (35) and STAT3 (36), the present study assessed whether the expression levels of these genes were affected following USP18-knockdown. The results demonstrated that Bcl-2 and STAT3 expression was upregulated, whereas NF- κ B expression remained unchanged (data not shown) following USP18-knockdown in HeLa cells. Bcl-2 is a novel gene encoding a unique apoptosis inhibitor that efficiently suppresses apoptosis induced by the p53 tumor suppressor protein (37). STAT3 is an important proto-oncogene essential for modulating the transition from the G₁ to S phase of the cell cycle (38), and most cancer cases arise due to proliferating cells losing control of cell cycle regulation, in which loss of the G₁/S-phase transition checkpoint is a major cause of cancer (39).

In conclusion, the results of the presents study demonstrated that USP18 expression was downregulated in UCC tissues, and USP18-knockdown facilitated tumor cell proliferation and migration by affecting the expression levels of genes associated with the ERK signaling pathway. Overall, the current results provide a novel mechanism for USP18-deficiency, which may serve a crucial role in UCC progression in an ERK-dependent manner.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81272854), Key

Projects of Natural Science Foundation of Heilongjiang Province (grant no. ZD2019H008), Excellent Innovation Team Construction Project of Basic Scientific Research Business Fee of Provincial Colleges and Universities in Heilongjiang Province (grant no. 2019-KYYWF-1334), Double First-class Discipline Construction Project in Heilongjiang Province (grant name. northern medicine and functional food), Young innovative talents training project of regular undergraduate colleges and universities in Heilongjiang Province (grant no. UNPYSCT-2020054), Youth Academic Backbone Support Program for Institution of Common Higher Education in Heilongjiang Province (grant no. 1252G059), Personnel Training Project of Basic Scientific Research Business Expenses of Department of Education in Heilongjiang Province (grant no. 2019-KYYWF-1338), Science and Innovation Team Foundation of Jiamusi University (grant no. cxtD-2016-03) and Biology Team Project of Jiamusi University (grant no. jdxktD-2019003).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AP and YL participated in cell experiments and drafted the manuscript. JG and PZ participated in statistical analyses. YH, LS and JRW performed immunohistochemistry assay. CZ, YC, and QR participated in cell transfection and cell experiments. SL, SF and TZ performed western blot analyses. AP, YL and JTW confirm the authenticity of all the raw data. WW and JTW designed the study and performed the revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The medical ethics committee at Jiamusi University (Jiamusi, China) approved all procedures performed in the present study involving animals and human participants, which were in accordance with ethical standards, and all patients provided written informed consent prior to participation in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Van Hede D, Langers I, Delvenne P and Jacobs N: Origin and immunoescape of uterine cervical cancer. *Presse Med* 43: e413-e421, 2014.
2. Bray F, Ferlay J, Colombet M, Soerjomataram I, Siegel RL, Torre A and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68: 394-424, 2018.
3. Arbyn M, Weiderpass E, Bruni L, de Sanjosé S, Saraiya M, Ferlay J and Bray F: Estimates of incidence and mortality of cervical cancer in 2018: A worldwide analysis. *Lancet Glob Health* 8: e191-e203, 2020.
4. Oikonomaki M, Bady P and Hegi ME: Ubiquitin specific peptidase 15 (USP15) suppresses glioblastoma cell growth via stabilization of HECTD1 E3 ligase attenuating WNT pathway activity. *Oncotarget* 8: 110490-110502, 2017.
5. Baker RT, Wang XW, Woollatt E, White JA and Sutherland GR: Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. *Genomics* 59: 264-274, 1999.
6. Wilkinson KD: Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* 11: 1245-1256, 1997.
7. Chung CH and Baek SH: Deubiquitinating enzymes: Their diversity and emerging roles. *Biochem Biophys Res Commun* 266: 633-640, 1999.
8. Liu LQ, Ilaria R Jr, Kingsley PD, Iwama A, van Etten RA, Palis J and Zhang DE: A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation. *Mol Cell Biol* 19: 3029-3038, 1999.
9. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ and Zhang DE: UBP43 (USP18) specifically removes ISG 15 from conjugated proteins. *J Biol Chem* 277: 9976-9981, 2002.
10. Honke N, Shaabani N, Zhang DE, Hardt C and Lang KS: Multiple functions of USP18. *Cell Death Dis* 7: e2444, 2016.
11. Friedrich SK, Schmitz R, Bergerhausen M, Lang J, Cham LB, Duhan V, Häussinger D, Hardt C, Addo M, Prinz M, *et al.*: Usp18 expression in CD169⁺ macrophages is important for strong immune response after vaccination with VSV-EBOV. *Vaccines (Basel)* 8: 142, 2020.
12. Liu X, Li H, Zhong B, Blonska M, Gorjestani S, Yan M, Tian Q, Zhang DE, Lin X and Dong C: USP18 inhibits NF- κ B and NFAT activation during Th17 differentiation by deubiquitinating the TAK1-TAB1 complex. *J Exp Med* 210: 1575-1590, 2013.
13. Działałek-Macioszczyk P, Haraźna J and Stompór T: Versatility of USP18 in physiology and pathophysiology. *Acta Biochim Pol* 66: 389-392, 2019.
14. Cai X, Feng S, Zhang J, Qiu W, Qian M and Wang Y: USP18 deubiquitinates and stabilizes Twist1 to promote epithelial-mesenchymal transition in glioblastoma cells. *Am J Cancer Res* 10: 1156-1169, 2020.
15. Tong HV, Hoan NX, Binh MT, Quyen DT, Meyer CG, Hang DT, Hang DT, Son HA, Van Luong H, Thuan ND, *et al.*: Upregulation of Enzymes involved in ISGylation and Ubiquitination in patients with hepatocellular carcinoma. *Int J Med Sci* 17: 347-353, 2020.
16. Kim YH, Kim WT, Jeong P, Ha YS, Kang HW, Yun SJ, Moon SK, Choi YH, Kim IY and Kim WJ: Novel combination markers for predicting survival in patients with muscle invasive bladder cancer: USP18 and DGCR2. *J Korean Med Sci* 29: 351-356, 2014.
17. Tan Y, Zhou G, Wang X, Chen W and Gao H: USP18 promotes breast cancer growth by upregulating EGFR and activating the AKT/Skp2 pathway. *Int J Oncol* 53: 371-383, 2018.
18. Burkart C, Arimoto K, Tang T, Cong X, Xiao N, Liu YC, Kotenko SV, Ellies LG and Zhang DE: Usp18 deficient mammary epithelial cells create an antitumour environment driven by hypersensitivity to IFN- λ and elevated secretion of Cxcl10. *EMBO Mol Med* 5: 1035-1050, 2013.
19. Potu H, Sgorbissa A and Brancolini C: Identification of USP18 as an important regulator of the susceptibility to IFN-alpha and drug-induced apoptosis. *Cancer Res* 70: 655-665, 2010.
20. Hong B, Li H, Lu Y, Zhang M, Zheng Y, Qian J and Yi Q: USP18 is crucial for IFN- γ -mediated inhibition of B16 melanoma tumorigenesis and antitumor immunity. *Mol Cancer* 13: 132, 2014.
21. Wu S, Shang H, Cui L, Zhang Z, Zhang Y, Li Y, Wu J, Li RK and Xie J: Targeted blockade of interleukin-8 abrogates its promotion of cervical cancer growth and metastasis. *Mol Cell Biochem* 375: 69-79, 2013.
22. François-Newton V, Magno de Freitas Almeida G, Payelle-Brogard B, Monneron D, Pichard-Garcia L, Piehler J, Pellegrini S and Uzé G: USP18-Based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon a response. *PLoS One* 6: e22200, 2011.
23. Fan W, Xie S, Zhao X, Li N, Chang C, Li L, Yu G, Chi X, Pan Y, Niu J, *et al.*: IFN- λ 4 desensitizes the response to IFN- α treatment in chronic hepatitis C through long-term induction of USP18. *J Gen Virol* 97: 2210-2220, 2016.

24. Dagenais-Lussier X, Loucif H, Cadorel H, Blumberger J, Isnard S, Bego MG, Cohen EA, Routy JP and van Grevenynghe J; Montreal Primary Infection Study Group: USP18 is a significant driver of memory CD4 T-cell reduced viability caused by type I IFN signaling during primary HIV-1 infection. *PLoS Pathog* 15: e1008060, 2019.
25. Kang JA and Jeon YJ: Emerging roles of USP18: From biology to pathophysiology. *Int J Mol Sci* 21: 6825, 2020.
26. Chinyenetere F, Sekula DJ, Lu Y, Giustini AJ, Sanglikar A, Kawakami M, Ma T, Burkett SS, Eisenberg BL, Wells WA, *et al*: Mice null for the deubiquitinase USP18 spontaneously develop leiomyosarcomas. *BMC Cancer* 15: 886, 2015.
27. Diao W, Guo Q, Zhu C, Song Y, Feng H, Cao Y, Du M and Chen H: USP18 promotes cell proliferation and suppressed apoptosis in cervical cancer cells via activating AKT signaling pathway. *BMC Cancer* 20: 741, 2020.
28. Mustachio LM, Kawakami M, Lu Y, Rodriguez-Canales J, Mino B, Behrens C, Wistuba I, Bota-Rabassedas N, Yu J, Lee JJ, *et al*: The ISG15-specific protease USP18 regulates stability of PTEN. *Oncotarget* 8: 3-14, 2017.
29. Gu T, Lu L, An C, Zhang Y, Wu X, Xu Q and Chen G: Negative regulation of the RLR-mediated IFN signaling pathway by duck ubiquitin-specific protease 18 (USP18). *J Cell Physiol* 234: 3995-4004, 2019.
30. Yoon S and Seger R: The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors* 24: 21-44, 2006.
31. Bang YJ, Kwon JH, Kang SH, Kim JW and Yang YC: Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinoma. *Biochem Biophys Res Commun* 250: 43-47, 1998.
32. Tang Q, Wu J, Zheng F, Hann SS and Chen Y: Emodin increases expression of insulin-like growth factor binding protein I through activation of MEK/ERK/AMPK α and interaction of PPAR γ and Sp1 in lung cancer. *Cell Physiol Biochem* 41: 339-357, 2017.
33. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y and Hu LL: ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med* 193: 1997-2007, 2020.
34. Lin CW, Shen SC, Chien CC, Yang LY, Shia LT and Chen YC: 12-O-tetradecanoylphorbol-13-acetate-induced invasion/migration of glioblastoma cells through activating PKC α /ERK/NF-kappaB-dependent MMP-9 expression. *J Cell Physiol* 225: 472-481, 2010.
35. Yang T, Xu F, Sheng Y, Zhang W and Chen Y: A targeted proteomics approach to the quantitative analysis of ERK/Bcl-2-mediated anti-apoptosis and multi-drug resistance in breast cancer. *Anal Bioanal Chem* 408: 7491-7503, 2016.
36. Mu X, Shi W, Xu Y, Xu C, Zhao T, Geng B, Yang J, Pan J, Hu S, Zhang C, *et al*: Tumor-derived lactate induces M2 macrophage polarization via the activation of the ERK/STAT3 signaling pathway in breast cancer. *Cell Cycle* 17: 428-438, 2018.
37. Miyake H, Hanada N, Nakamura H, Kagawa S, Fujiwara T, Hara I, Eto H, Gohji K, Arakawa S, Kamidono S and Saya H: Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adenoviral-mediated p53 gene transfer. *Oncogene* 16: 933-943, 1998.
38. Luo J, Yan R, He X and He J: Constitutive activation of STAT3 and cyclin D1 overexpression contribute to proliferation, migration and invasion in gastric cancer cells. *Am J Transl Res* 9: 5671-5677, 2107.
39. Ragkousi K and Gibson MC: Epithelial integrity and cell division: Concerted cell cycle control. *Cell Cycle* 17: 399-400, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.