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ORIGINAL RESEARCH

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Overexpression of USP43 induces the growth and stem cell-like properties of cervical cancer by activating ERK1/2 through ZEB1

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Abstract

Cervical cancer (CC) is the most common type of gynecological malignancy in women, and targeting stem cells and inhibiting tumor stem cell-like properties of CC remains an important field of research as an attempt to improve treatment outcomes. This study focused on Ubiquitin-specific-processing Protease 43 (USP43), a member of the deubiquitinase (DUBs) family known to play a role in tumor progression, and analysis of the The Cancer Genome Atlas (TCGA) data and survival computations revealed that USP43 was highly expressed in CC and correlated with poor prognosis. However, the role of USP43 in CC has been under-reported, and its underlying mechanism remains unclear. To investigate the effect and mechanism of USP43, its expression in CC cells and tissues were examined, and the results showed that it was significantly upregulated. Subsequently, knockdown experiments revealed that reducing USP43 expression suppressed CC cell proliferation, and depleting USP43 inhibited the stem cell-like properties of CC cells and impaired their migration abilities. Further investigations indicated that USP43 promoted Zinc finger E-box binding protein 1 (ZEB1)-induced activation of Extracellular regulatory kinase 1/2 (ERK1/2) signaling in CC. Based on these findings, we propose that USP43 could serve as a promising target for CC.

Keywords

Cervical cancer (CC); USP43; ZEB1; ERK1/2; Stem cell-like properties

1. Introduction

Cervical cancer (CC) is the most common type of gynecological malignancy and a leading cause of morbidity among women worldwide [1]. It is a malignant tumor that primarily affects the cervical region, and despite recent advancements in medical treatments, achieving complete cure remains challenging [2]. In addition, although there might be several clinical treatment options for patients with CC, the overall survival rate remains relatively low [3–5]. The concept of cancer stem cells (CSCs) suggests that tumors often contain a subset of cells known as CSCs, which possess three distinct characteristics: self-renewal, pluripotency and expression of stem cell markers, which contribute to the malignant progression of the tumor [6]. Recent studies have identified the presence of CSCs in CC, and specific stem cell markers have been associated with cervical CSCs [7]. Consequently, addressing the targeting of stem cell markers and inhibiting the tumor stem cell-like properties of CC has become an urgent issue that needs to be resolved.

USP43 belongs to the deubiquitinase (DUBs) family, characterized by its intricate structure and cysteine cassette [8]. Extensive research has demonstrated that USP43 plays a regulatory role in tumor progression [8, 9]. For instance, in colorectal cancer (CRC), USP43 exhibits high expression in tumor tissues, promoting tumor growth, metastasis and increasing resistance to chemotherapy drugs [9]. Similarly, in breast cancer, USP43 has been shown to influence cell cycle and epithelial-mesenchymal transition (EMT), thereby facilitating tumorigenesis [8]. Additionally, high expression of USP43 has been observed in osteosarcoma tissues and correlated with poor prognosis [10]. In lung squamous cell carcinoma, increased USP43 expression has been associated with larger tumor size, deeper invasion depth and reduced overall survival in patients with positive lymph nodes [11]. However, there has been limited research on the role of USP43 in CC, and its underlying mechanism remains unclear.

Our preliminary investigations from TCGA and survival analysis revealed that USP43 exhibited significant upregulation in CC patients and was linked to unfavorable prognosis. Therefore, this study was designed to explore the impact and underlying mechanism of USP43 in CC. Our investigations show the involvement of USP43 in regulating stem cell characteristics in CC, primarily through its interaction with ZEB1, based on which we propose that targeting USP43 could hold potential as a therapeutic strategy for CC.

2. Materials and methods

2.1 Bioinformatics

Transcriptome and survival rate data were retrieved from The Cancer Genome Atlas database, and the expression levels in CC were analyzed in the Gene Expression Profiling Interactive Analysis (GEPIA) database.

2.2 Cell culture

The cell lines in this study, including CC cell lines Hela, Ca-Ski, SiHa and normal human cervical epithelial cells H8, were all bought from American Type Culture Collection (ATCC, USA). The CC cell lines Hela, Ca-Ski, SiHa were human papillomavirus (HPV)-positive, whereas H8 was HPV-negative. Keratinocyte serum-free medium (Gibco, CA, USA) was used to culture H8 cells, while Eagle's Minimum Essential Medium (Gibco) was used to culture Hela, Ca-Ski and SiHa cell lines.

2.3 Cell transfection

Briefly, a total amount of 600 ng of the vector was transfected into Hela and Ca-Ski cells using Lipofectamine 3000 (Thermo Fisher Scientific, L3000001, Waltham, MA, USA) for 24 hours. The vector used to target USP43 (sh-USP43#1 and sh-USP43#2) and its corresponding negative control (sh-NC), as well as the USP43 overexpression plasmids, were all designed by GenePharma (Shanghai, China).

2.4 RT-qPCR

After RNA isolation using the Trizol kit (R0016, Beyotime, Shanghai, China), reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara, RR037A, Osaka, Japan). For RT-qPCR analysis, SYBR Premix Ex Taq™ II (Takara, RR820A, Osaka, Japan) was employed, and the amplification was detected using the Bio-Rad CFX-96 system (Bio-Rad, Hercules, CA, USA). The thermocycling conditions were as follows: an initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension step was performed at 4 °C for 30 minutes.

2.5 Immunoblot

The cells were lysed using a buffer composed of 1% Triton X-100, 150 mM NaCl and 50 mM Tris (pH 7.5), and the protein concentration was determined using the bicinchoninic acid (BCA) assay method. Subsequently, the proteins were separated (20 μg/lane) using Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 8% gels, transferred onto polyvinylidene difluoride membranes (Millipore-Sigma), blocked with a solution of 0.2% Tween 20 and 5% non-fat milk in Tris-buffered saline at room temperature for 2 hours, followed by incubation with primary antibodies USP43 (Abcam, ab196796; 1:500), cluster of differentiation (CD) 44 (Abcam, ab254530; 1:1000), CD133 (Abcam, ab222782; 1:500), SOX2 (Abcam, ab92494; 1:1000), ZEB1 (Abcam, ab203829; 1:1000), ERK1/2 (Abcam, ab184699; 1:1000), p-ERK1/2 (Abcam, ab278538; 1:500) and β-actin (Abcam, ab8226; 1:3000), then secondary antibodies for 1 hour. Lastly, the blots were photographed after chemiluminescence. All reagents used in this experiment were purchased from Wuhan Google Co., LTD.

2.6 Cell counting kit-8 (CCK-8) assay

Cell viability was assessed using the CCK-8 kit (C0039, Beyotime, Shanghai, China), for which approximately 10,000 cells were seeded into each well of a 96-well plate, then incubated with the CCK-8 solution for 1.5 hours. Lastly, the absorbance of the wells was measured at 450 nm using a microplate reader.

2.7 Colony formation assay

The cells were seeded into 6-well plates and cultured in media containing 10% Fetal bovine serum (FBS) for 14 days at 37 °C. Following the incubation, the cells were fixed with PFA for 15 minutes and stained with 0.1% crystal violet for 20 minutes. Images of the cells were captured using an Axio Observer light microscope (Carl Zeiss AG, Axio Observer 3.0, Oberkochen, Batenfuburg Oblast, Germany), and the number of colonies formed was quantified using ImageJ v9.0 (National Institutes of Health, Bethesda, MD, USA). The colony was defined as follows. Each colony contains more than 50 cells and is between 0.3 and 1.0 mm in size.

2.8 Transwell assay

The cells were placed in the Transwell and allowed to migrate for 24 hours. After incubation at 37 °C for 24 hours, the cells that had invaded the underside of the Transwell were fixed with 4% paraformaldehyde at room temperature for 25 minutes, stained with 2% crystal violet at room temperature for 25 minutes, and imaged using light microscopy.

2.9 Formation of tumor spheroids

Hela and Ca-Ski cells were sorted and cultured in a medium supplemented with 20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) for 1–2 weeks, during which the medium was changed every two days. After incubation, the formed spheroids were detached using 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) and observed under a microscope. A spheroid was defined as cells with a diameter greater than 75 μm.

2.10 Statistics

Data analysis was performed using GraphPad v8.0 (GraphPad Software, Inc., National Institutes of Health, Bethesda, MD, USA). *In vitro* experiments were repeated thrice, and the error bars represent their mean ± Standard deviation (SD). The unpaired Student's *t*-test was used to determine the statistical significance between the two groups. A *p*-value of less than 0.05 ($p < 0.05$) was considered indicative of a statistically significant difference.

3. Results

3.1 USP43 is overexpressed in CC

We first investigated the potential impact of USP43 on CC and found that USP43 was highly expressed in CC tissues, which was confirmed on the GEPIA website (Fig. 1A). In addition, we evaluated the mRNA and protein expression levels of USP43 in CC cell lines (Hela, Ca-Ski and SiHa) and compared them to those observed in normal H8 cells (Fig. 1B,C), and the results indicated that USP43 exhibited higher expression in both CC tissues and CC cell lines.

3.2 Loss of USP43 blocks the proliferation of CC cells

To elucidate the biological roles of USP43 in CC, we first downregulated its expression by transfecting sh-USP43#1 and sh-USP43#2 into Hela and Ca-Ski cells (Fig. 2A). Then, we assessed the impact of USP43 knockdown using CCK-8 and colony formation assays, and the results revealed that Hela and Ca-Ski cells exhibited reduced proliferation upon silencing of USP43 (Fig. 2B,C). These findings collectively indicate that the knockdown of USP43 inhibits the malignant behaviors of CC cells.

3.3 USP43 silencing inhibits CSCs-like characteristics of CC cells

Given the significant role of CSCs in cancer development, we investigated the potential impact of USP43 on the CSC-like characteristics of CC cells. Western blot analysis revealed that the protein levels of CSC markers, including CD44, CD133 and SOX2, were decreased upon USP43 depletion (Fig. 3A), and tumor spheroid formation assay demonstrated that the ability of CSCs to form spheroids was decreased after knocking down USP43 (Fig. 3B). Overall, these findings suggest that USP43 knockdown suppresses the stem cell-like properties of CC cells.

3.4 USP43 depletion inhibits the migration of CC cells

Next, we investigated the effects of USP43 on the motility of CC cells. Transwell migration and invasion assays showed that USP43 knockdown in Hela and Ca-Ski cells inhibited cellular invasion and migration (Fig. 4A,B), confirming that USP43 depletion inhibited the motility of CC cells.

3.5 USP43 activates ZEB1-dependent ERK1/2 pathway in CC

To elucidate the mechanism underlying the impact of USP43 knockdown on CC cells, we assessed the levels of USP43 in Hela and Ca-Ski cells using Western blot and observed that the downregulation of USP43 reduced ZEB1 protein expression (Fig. 5A). Furthermore, we explored whether USP43 modulates the ERK1/2 pathway through ZEB1. The results demonstrated that the knockdown of USP43 resulted in decreased phosphorylation levels of ERK1/2 (Fig. 5A). Moreover, the overexpression of USP43 reversed the decrease of ZEB1 expression and ERK1/2 phosphorylation levels caused by USP43 depletion in Hela and Ca-Ski cells (Fig. 5B). Collectively, these results suggested that USP43 regulates ERK1/2 pathway through ZEB1 in CC cells.

4. Discussion

CC is the most prevalent type of gynecological malignancy, with most cases occurring in developing countries and accounting for approximately 85% of all CC cases worldwide [3]. Despite advancements in CC screening, the clinical treatment outcomes for advanced and complex cases remain unsatisfactory, leading to a poor prognosis [12, 13]. In recent years, targeted therapy and immunotherapy have emerged as promising approaches for the treatment of advanced or recurrent CC due to advancements in modern molecular biology and genomics [6, 14]. To further enhance the therapeutic efficacy of CC, the identification of new therapeutic targets and targeted drugs is crucial [15]. In this regard, our study revealed that USP43 promotes tumor proliferation and enhances the stem cell-like properties of CC cells by facilitating ZEB1-induced activation of the ERK1/2 pathway. Based on these findings, we propose that USP43 could serve as a potential therapeutic target for CC.

The bioinformatic analysis results further confirmed the overexpression of USP43 in CC patients. The data obtained from colony formation and Transwell assays substantiated the impact of USP43 on the proliferation, invasion and stem cell-like properties of CC cells. Thus, it could be concluded that USP43 plays a significant role in the progression of CC. Importantly, the role of USP43 in the development of various other tumors has been extensively investigated. For instance, USP43 was found to influence prognosis in pancreatic cancer by affecting the proliferation and infiltration of immune cells in the surrounding microenvironment [16]. Similarly, in osteosarcoma, USP43 was reported to be highly expressed in the cancerous tissues and was associated with poor prognosis [10]. Furthermore, in lung cancer, USP43 protein expression positively correlated with tumor size and lymph node metastasis [11]. Notably, USP43 was demonstrated to mediate ZEB1 expression, thereby affecting the proliferation and metastasis of colorectal cancer [9]. In line with these findings, our study revealed that USP43 mediates ZEB1 expression and contributes to the progression of CC. It was also shown to promote tumorigenesis in breast cancer by mediating cell cycle progression and epithelial-mesenchymal transition (EMT) [8]. Collectively, these studies, in conjunction with our findings, emphasize the potential of USP43 as a promising therapeutic target in CC.

USP43, as a member of the deubiquitinase (DUBs) family, has been found to interact with ZEB1 protein, leading to deubiquitination modifications and stabilization of ZEB1 protein expression [9, 17, 18]. ZEB1 is a crucial member of the ZEB transcription factor family, known for promoting tumor proliferation, migration and other malignant phenotypes [17, 18]. Previous studies have shown that ZEB1 can facilitate the activation of ERK1/2 phosphorylation, thereby promoting the proliferation and invasion of prostate cancer [19]. ERK1/2 belongs to the mitogen-activated protein kinase (MAPK) family and plays a significant role in cellular processes such as growth, apoptosis and malignant transformation [20]. The ERK1/2 pathway receives signals from various mitogens and

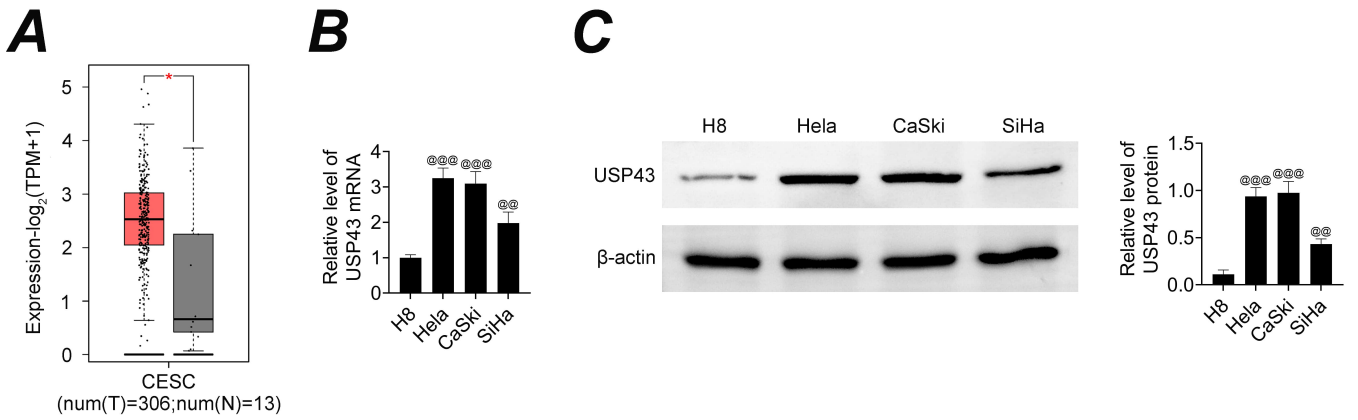

FIGURE 1. Overexpression of USP43 in CC. (A) USP43 expression in CC was predicted using GEPIA. (B) USP43 mRNA levels in CC cell lines (Hela, Ca-Ski and SiHa) and the normal cell line H8 were assessed by RT-qPCR (repetition = 3). (C) USP43 protein levels in CC cell lines (Hela, Ca-Ski and SiHa) and the normal cell line H8 were assessed by Immunoblot (repetition = 3). Error bars indicate SD. @@$p < 0.01$, @@@$p < 0.001$. CESC: cervical cancer; TPM: transcripts per million; USP43: Ubiquitin-specific-processing Protease 43; TPM: Transcript per million.

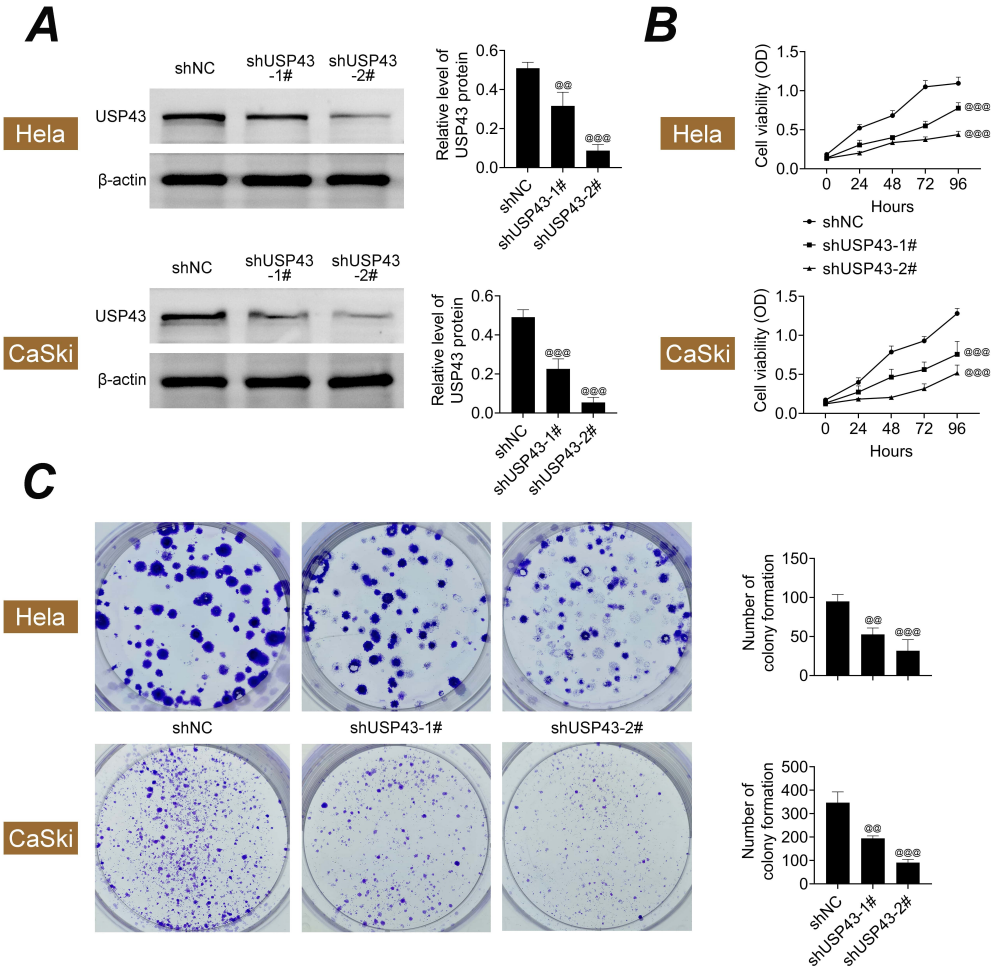

FIGURE 2. Loss of USP43 blocks the proliferation of CC cells. (A) USP43 expression in Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was tested by Immunoblot assay. (B) Cell proliferation of Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was analyzed by CCK-8 test and the corresponding OD450 measurements. (C) Cell proliferation of Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was analyzed by colony formation assay. Error bars indicate SD. @@$p < 0.01$, @@@$p < 0.001$. NC, negative control. USP43: Ubiquitin-specific-processing Protease 43.

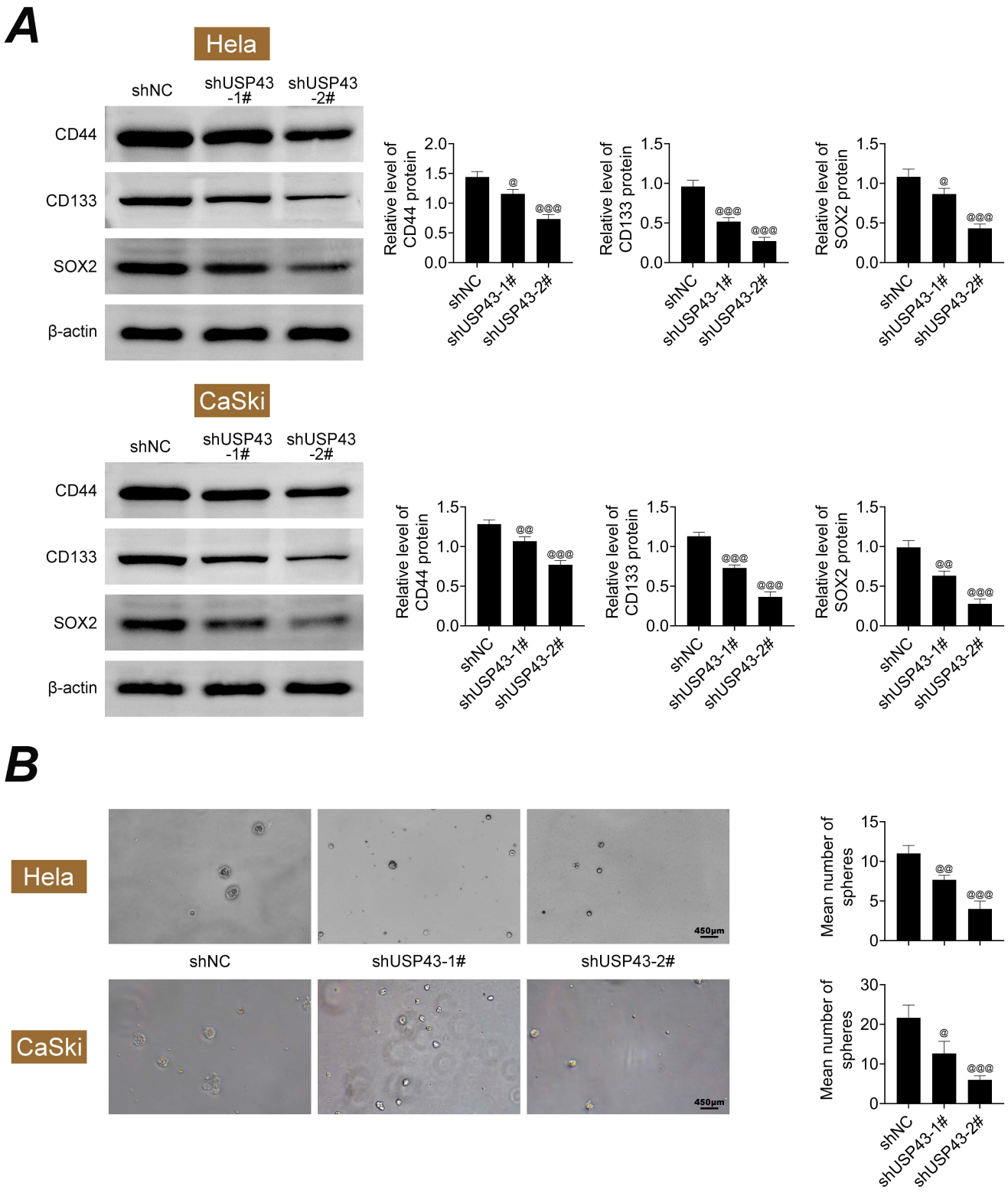

FIGURE 3. USP43 silencing inhibits CSCs-like characteristics of CC cells. (A) Protein levels of CD44, CD133 and SOX2 in Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was tested by Immunoblot. (B) Cell self-renewal ability of Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was detected by spheroid formation test. Error bars indicate SD. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$. NC: negative control. USP43: Ubiquitin-specific-processing Protease 43; CD44: Cluster of differentiation 44; SOX2: Sex Determining Region Y Box Protein 2.

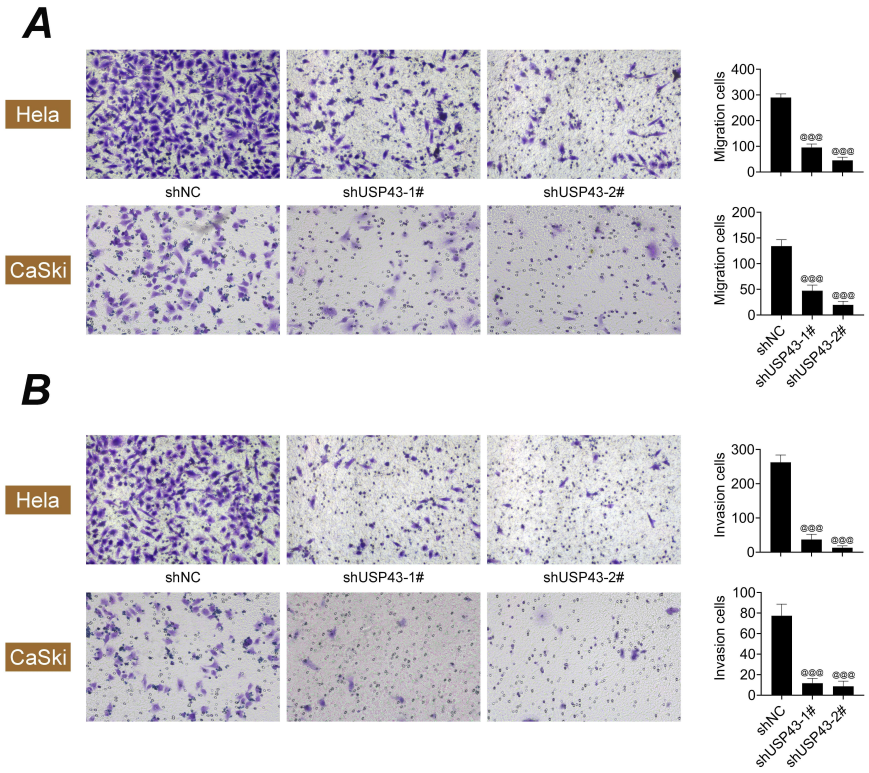

FIGURE 4. USP43 depletion inhibits the migration of CC cells. (A) Cell migration of Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was tested by Transwell migration assay. (B) Cell invasion of Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 was tested by Transwell-invasion assay (repetition = 3). Error bars indicate SD. @@@$p < 0.001$. NC, negative control. USP43: Ubiquitin-specific-processing Protease 43.

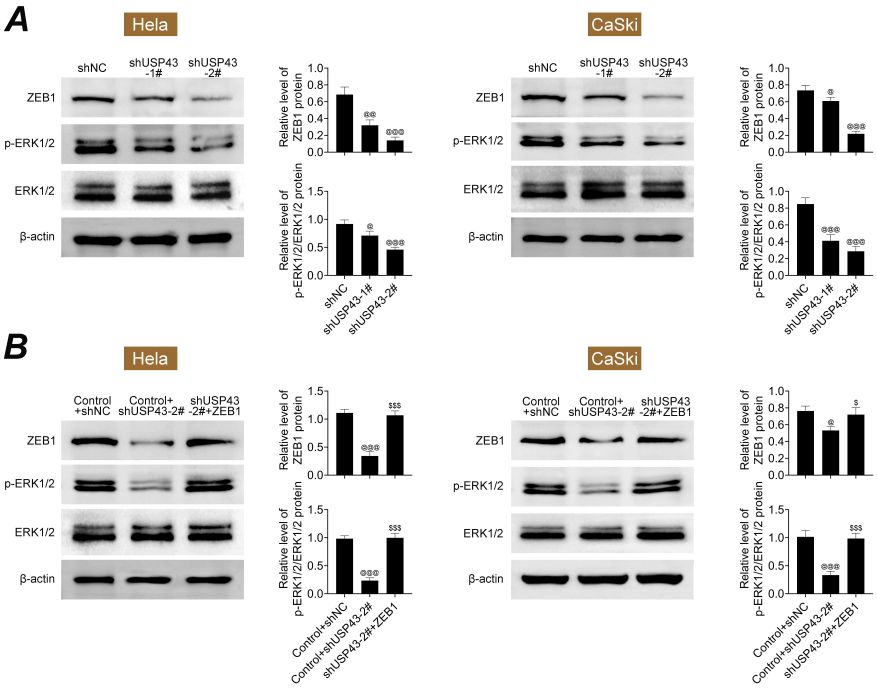

FIGURE 5. USP43 activates ZEB1-dependent ERK1/2 pathway in CC. (A) Protein levels of ZEB1 and ERK1/2 and the phosphorylation levels in Hela and Ca-Ski cells after transfection with sh-USP43#1 and sh-USP43#2 were tested by Immunoblot. (B) Protein levels of ZEB1 and ERK1/2 and the phosphorylation levels in Hela and Ca-Ski cells after the indicated transfection were assessed by Immunoblot. Error bars indicate SD. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$, shUSP43 *vs.* shNC. $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$, shUSP43 + USP43 *vs.* shUSP43. NC, negative control. ZEB1: Zinc finger E-box binding protein 1; ERK1/2: Extracellular regulatory kinase 1/2; USP43: Ubiquitin-specific-processing Protease 43.

stress factors, leading to the activation of ERK1/2 substrates and regulation of cell proliferation, apoptosis and invasion [21]. Activation of ERK1/2 phosphorylation has also been linked to promoting stem cell-like properties in tumors [22]. Intriguingly, our study uncovered that USP43 promotes ZEB1-induced activation of ERK1/2 in CC cells. However, the precise mechanism underlying this interaction requires further investigation.

5. Conclusions

In summary, our findings highlight the role of USP43 in inducing growth and stem cell-like properties in CC through its interaction with ZEB1 and activation of the ERK1/2 pathway. Taken together, the data obtained by this present study further support the notion that USP43 could serve as a potential target for CC treatment.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

QLX, XLL—designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors. Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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