

CBX7 is involved in the progression of cervical cancer through the ITG β 3/TGF β 1/AKT pathway

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Received May 22, 2023; Accepted October 2, 2023

DOI: 10.3892/ol.2023.14147

Abstract. The chromobox protein homolog 7 (CBX7) serves a tumor-suppressive role in human malignant neoplasias. The downregulation of CBX7 is associated with the poor prognosis and aggressiveness of various human cancers. However, the biological functions and underlying mechanisms of CBX7 in cervical cancer remain unclear. The present study investigated the role and mechanism of CBX7 in cervical cancer. Lentivirus and siRNA were used to construct cervical cancer cells with stable CBX7 knockdown and SiHa xenograft models. The

cell growth, migration, invasion and apoptosis were observed through *in vivo* and *in vitro* experiments. The expression levels of CBX7, integrin β 3 (ITG β 3), transforming growth factor β 1 (TGF β 1), phosphatidylinositol-3-kinase (PI3K), AKT, E-cadherin (E-cad) and vimentin (VIM) were detected by western blot analysis and reverse transcription-quantitative PCR. The correlation between CBX7 and these genes was analyzed. TGF β 1 was also silenced through shRNA in cells with stable CBX7 knockdown to detect its effect on cell growth, invasion and apoptosis, and on pathway-related gene expression. It was revealed that knockdown of CBX7 promoted the proliferation, migration, and invasion of cervical cancer cells, and inhibited apoptosis. In addition, CBX7 knockdown promoted tumor growth *in vivo*. Correlation analysis demonstrated that CBX7 was negatively correlated with ITG β 3, TGF β 1, PI3K, AKT, phosphorylated AKT and VIM, but positively correlated with E-cad. Moreover, the knockdown of TGF β 1 reversed the promotion of cell proliferation and inhibition of apoptosis induced by CBX7 knockdown and attenuated the increase of ITG β 3, TGF β 1, PI3K, AKT and VIM caused by CBX7 knockdown. In conclusion, the findings of the present study indicated that the downregulation of CBX7 enhances cell migration and invasion while inhibiting cell apoptosis in cervical cancer by modulating the ITG β 3/TGF β 1/AKT signaling pathways.

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Abbreviations: CBX7, chromobox homolog 7; E-cad, E-cadherin; HPV, human papillomavirus; ITG β 3, integrin β 3; PRC1, polycomb repressive complex 1; TGF β 1, transforming growth factor β 1

Key words: cervical cancer, chromobox homolog 7, integrin β 3, transforming growth factor β 1

Introduction

Cervical cancer is one of the most common gynecological tumors and its incidence ranks fourth worldwide in gynecological tumors (1). Although its mortality rate has been declining in recent years, its morbidity and the number of overall

cancer-related deaths are on the rise. There are still >500,000 new cases and >250,000 cancer-related deaths worldwide each year, and the 5-year survival rate is only 64.2% (2). In addition, given that certain women have exceeded the recommended age for the human papillomavirus (HPV) vaccine, and not all cases of cervical cancer are attributed to HPV infection, certain patients still experience a progressive development into advanced cancer (3). Therefore, it is necessary to further explore the mechanism of cervical cancer and identify novel therapeutic targets.

Chromobox homolog 7 (CBX7) belongs to the polycomb repressive complex 1 (PRC1) family (4). The expression of CBX7 in tumors varies, and it plays several functions in tumor occurrence and development. The level of CBX7 is low in some tumors such as breast cancer, bladder cancer, colon cancer and glioma, indicating that it may be a tumor suppressor gene (5-8). *In vitro* and *in vivo* experiments have revealed that overexpression of CBX7 could inhibit the progression of glioma (9,10). In pancreatic cancer, CBX7 was revealed to negatively regulate the survival, drug resistance and migration of pancreatic cancer cells (11). In lung cancer, CBX7 was demonstrated to enhance the sensitivity of lung cancer cells to chemotherapeutic drugs (12). However, in gastric cancer, lymphoma and prostate, and ovarian cancer CBX7 is an oncogene (13-16). For example, Ni *et al* (13) found that CBX7 overexpression in gastric cancer cells promotes tumor progression. However, the effect of CBX7 on cervical cancer remains unclear.

Gene ontology map of 44 genes revealed 13 physiologically important cellular pathways, such as integrin (ITG), transforming growth factor β 1 (TGF β 1), phosphatidylinositol-3-kinase (PI3K), cadherin and other signaling pathways (17). Rapisarda *et al* revealed that downregulation of CBX7 expression promoted the expression of ITG, which further activated TGF β (18). TGF β 1 can reduce the expression of E-cadherin (E-cad) and induce epithelial-mesenchymal transition (EMT) (19,20). The integrin β 3 (ITG β 3)/AKT signaling pathway can promote the growth of platelet-induced hemangioendothelioma (21). Ni *et al* determined that the mechanism of CBX7 underlying its inhibitory effect on cell growth may involve the AKT signaling pathway (11). A previous study by the authors also demonstrated that low CBX7 expression was positively correlated with poor prognosis in patients with cervical cancer (22). By overexpressing CBX7, it was also revealed that CBX7 promoted apoptosis in cervical cancer cells (23).

In the present study, the effects of CBX7 on the metastasis and apoptosis of cervical cancer cells as well as the underlying mechanisms were further studied.

Materials and methods

Subjects and human cervical cancer samples. A total of 120 patients with cervical cancer admitted to the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) from January 2018 to Dec 2019 were enrolled in the present study. The patients were aged from 28 to 72 years (mean, 56.88 \pm 0.92 years). All patients were diagnosed using histopathological examination of tissue samples by experts in gynecological pathology. The tumor samples were obtained during high resolution digital colposcopy, cervical colonization

and hysterectomy. The matching adjacent non-tumorous tissues were collected and used as controls. Prior written and informed consent was obtained from every patient and the study was approved (approval no. 20120220-01) by the Ethics Review Board of Xinjiang Medical University (Urumqi, China).

Immunohistochemistry. The expression levels of CBX7, ITG β 3, TGF β 1, PI3K, AKT, phosphorylated (p)-AKT, vimentin (VIM) and E-cad in the tissue were determined by immunohistochemistry, using a rabbit polymer method detection system (cat. no. PV6001; OriGene Technologies, Inc.). The tissue samples were fixed with 4% paraformaldehyde for 24 h at room temperature, dehydrated with graded alcohol series, embedded in paraffin, and cut into 4- μ m serial sections. Tissue slides (4 μ m) were then deparaffinized at 60°C, followed by treatment with 100% xylene for 20 min and rehydration in graded series of ethanol at room temperature. Followingly, the sections were incubated in 3% H₂O₂ (cat. no. 1301; Dezhou Dexinkang Disinfection Products Co., Ltd.) at room temperature for 20 min. The antigen retrieval was performed with 0.01 M citrate buffer at 95°C for 10 min. After blocking with normal sheep serum (1:100; cat. no. SAP-9100; OriGene Technologies, Inc.) at room temperature for 30 min, the section was incubated with anti-CBX7 (1:200; cat. no. ab21873; Abcam), anti-ITG β 3 (1:500; cat. no. ab7166; Abcam), anti-TGF β 1 (1:200; cat. no. BA0290; Boster Biological Technology; <https://www.boster.com.cn/index/products/productslist?catid=all&keyword=s=BA0290>), anti-PI3K (1:100; cat. no. E-AB-22165; Elabscience Biotechnology, Inc.), anti-AKT (1:1,000; cat. no. ab8805; Abcam), p-AKT (1:100; cat. no. 4060; CST Biological Reagents Co., Ltd.), anti-E-cad (1:500; cat. no. ab40772; Abcam) and anti-VIM (1:300; cat. no. ab92547; Abcam) monoclonal antibodies, respectively, at 4°C overnight. The section was then incubated with HRP-conjugated anti-rabbit En Vision system (1:200; cat. no. PV-6001; OriGene Technologies, Inc.) at 37°C for 20 min, followed by staining for 3-5 min with diaminobenzidine tetrahydrochloride (cat. no. ZLI-9018; OriGene Technologies, Inc.). The sections were then counterstained with hematoxylin for 20 s at room temperature. Finally, the sections were observed under an IX71 inverted light microscope (Magnification: 200x; Olympus, Tokyo, Japan). Immunohistochemistry was assessed using a semiquantitative approach, whereby the intensity of staining and the percentage of positive cells were combined. The staining intensity was categorized as absent (0 points), weak (1 point), moderate (2 points) or strong (3 points). The mean percentage of positively stained cells was scored as follows: i) 0 points (<25%), ii) 1 point (25-50%), iii) 2 points (51-75%) and iv) 3 points (76-100%). The total score was calculated by multiplying the staining intensity and the percentage of positive cells, ranging from 0 to 9. The scores \leq 1 were classified as negative expression, while scores >1 were classified as positive expression.

Cell lines. HeLa (cat. no. ZQ0068) and SiHa (cat. no. ZQ0129) cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. These cell lines were incubated in DMEM (cat. no. 11965-092; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat. no. 10091-148; Gibco; Thermo Fisher Scientific, Inc.),

100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (30-002-CI; Cellgro; Corning, Inc.). The cells were maintained at 37°C and 95% humidity in a 5% CO₂ atmosphere. Once the cells reached 80-90% confluency, they were subcultured.

Establishment of stable CBX7 knockdown in cervical cancer cells. To stably knockdown CBX7 expression in cervical cancer cells, four CBX7-specific siRNAs were designed and synthesized by Beijing CorreGene Biotechnology Co., Ltd. Briefly, the plasmids, including LentiCRTM-spKO-CBX7-sg1 (10 μg), LentiCRTM-spKO-CBX7-sg2 (10 μg), LentiCRTM-spKO-CBX7-sg3 (10 μg), LentiCRTM-spKO-CBX7-sg4 (10 μg), and LentiCRISPR E (10 μg), as well as the auxiliary plasmids including psPAX2 (10 μg) and pVSVG (5 μg) were packaged into lentiviruses and co-transfected into 293T cells using Lipofiter™ (Invitrogen; 11668-019). The culture medium was changed after 18 h. The transfection was performed for 48 h. On days 3, 4 and 5, the lentiviruses were collected and centrifuged at 4°C, 2,000 x g for 10 min. After removing cell debris, the supernatant was collected and centrifuged at 4°C, 9,000 x g for 120 min. Next, low-passage SiHa and HeLa cells were seeded in 10-cm culture dishes and infected with LentiCRTM-spKO-CBX7-sg1, LentiCRTM-spKO-CBX7-sg2, LentiCRTM-spKO-CBX7-sg3, LentiCRTM-spKO-CBX7-sg4 and LentiCRISPR E viruses for 24 h at 37°C and 5% CO₂ at a multiplicity of infection of 30. The control cells were not infected. The cells were then incubated overnight at 37°C and 5% CO₂. At 24-48 h after infection, cells were cultured in the medium containing 1 $\mu\text{g}/\text{ml}$ of puromycin. Every day, the DMEM containing 1 $\mu\text{g}/\text{ml}$ puromycin was replaced. After 7 days, the resistant cells were collected and seeded into 25 cm² culture flasks for further cultivation. The transduction efficiency was confirmed using 24 h after transduction. The method of constructing HeLa cell CBX7 gene-knockout cell lines is the same as that for SiHa cells. The knockdown efficiency of CBX7-shRNA lentivirus was determined using western blot analysis. LentiCRTM-spKO-CBX7-sg1 (GGAGCCAGAAGAGCA CATCT) and LentiCRTM-spKO-CBX7-sg4 (gcTGCCGA GTGGGCACGTCA) were selected as the lentiviruses with the optimal knockdown efficiency for HeLa and SiHa, respectively. HeLa and SiHa cells were divided into 3 groups: the SiCBX7 group (transfected with LentiCRTM-spKO-CBX7-sg), the SiCBX7-EVC group (transfected with LentiCRISPR E), and the control (CTRL) group (non-transfected cells).

Knockdown of TGF β . TGF β -specific shRNAs (TGF β 1-HOMO-875, TGF β 1-HOMO-1158, and TGF β 1-HOMO-1408) and control shRNA were designed and synthesized by Beijing Kerui Biotechnology Co., Ltd. shRNA sequences used for the knockdown of TGF β : TGF β 1-HOMO-875 forward, 5'-GCU ACCGCUGCUGGCUATT-3' and reverse, 5'-UAGCCA CAGCAGCGGUAGCTT-3'; TGF β 1-HOMO-1158 forward, 5'-GAGGUCACCCGCGUGCUAATT' and reverse, 5'-UUA GCACGCGGGUGACCUCTT-3' and TGF β 1-HOMO-1408 forward, 5'-GCGACUCGCCAGAGUGGUUTT' and reverse, 5'-AACCACUCUGGCGAGUCGCTT-3'. The sequence of control shRNA was forward, 5'-UUCUCCGAACGUGUCACG UTT-3' and reverse, 5'-ACGUGACACGUUCGAGAATT-3'. The transfection of control shRNA or TGF β shRNAs into

SiHa and HeLa cells was carried out using Lipofectamine 2000 transfection reagent (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. Briefly, cells in the logarithmic phase of growth and in good condition were used. The shRNAs (4 μg) and Lipofectamine 2000 (5 μl) were separately diluted in a 100 μl serum-free medium and incubated at room temperature for 20 min. They were then mixed and incubated with cells at 37°C and 5% CO₂. After incubation for 5 h, the culture medium was replaced with a complete medium containing 10% fetal bovine serum, and the cells were further cultured for 48 h. The RT-qPCR determined the TGF β shRNA with the optimal knockdown efficiency (24). The cells were divided into 6 groups: siCBX7 + NC, siCBX7-EVC + NC, CTRL + NC group, siCBX7 + shRNATGF β 1, siCBX7-EVC + shRNATGF β 1 and CTRL+ shRNATGF β 1 group.

MTT viability assay. HeLa and SiHa cells with knockdown of CBX7 or TGF β 1 (1.0x10⁴ cells/well) were plated in 96 well plates. At the following timepoints: 0, 12, 24, 48, 72 and 96 h, 20 μl of MTT (cat. no. BS186; Biosharp Life Sciences) was added and incubated at 37°C for 4 h. The cells were then treated with 150 μl of DMSO (cat no. D8370; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min under agitation in a light-protected environment. The absorbance was subsequently measured at 570 nm using a microplate reader from Thermo Fisher Scientific, Inc.

Wound healing assay. HeLa and SiHa cells with stable knock-down of CBX7 or TGF β 1 (5x10⁵ cells/well) were seeded in six-well plates and placed in a 37°C cell culture incubator. The cells were cultured until cell confluence reached >90%. A wound was then created vertically using a 1-ml pipette tip. After washing with PBS, cells were incubated with fresh DMEM without FBS. The wound was photographed at 0, 24 and 48 h of culture using an IX71 inverted light microscope (magnification, x100x, Olympus Corporation). The distance of the wound was measured using Image J software (version 1.50; National Institutes of Health), and the results were expressed as the percentage of the remaining wound area. This assay was performed in triplicate.

Cell migration assay. Cell migration ability was analyzed using a Transwell chamber (cat. no. 353097; Corning, Inc.). After serum-starved culture overnight, a total of 1x10⁴ HeLa and SiHa cells with stable knockdown of CBX7 or TGF β 1 were plated in the upper chambers of Transwell plates in 100 μl of serum-free medium. The lower chamber was filled with a medium containing 10% fetal bovine serum, which served as the chemoattractant. Following incubation at 37°C for 24 h, cells that did not migrate through the pores were carefully wiped out with cotton wool. The migratory cells were fixed with pure methanol for 30 min, stained with 0.1% crystal violet for 20 min at room temperature, and observed with an IX71 inverted light microscope (Olympus Corporation).

Cell apoptotic analysis by flow cytometry. Cell apoptotic analysis was performed using an Annexin-V/PI assay kit (cat. no. KGA1026; Nanjing KeyGen Biotech Co., Ltd.). Briefly, HeLa and SiHa cells (1x10⁶/ml) with stable knock-down of CBX7 or TGF β 1 were cultured for 24 h, the cells

Table I. Primer sequences of reverse transcription-quantitative PCR.

Gene	Primer	Sequence	Size
Homo β -actin	Forward	5'-AGCGAGCATCCCCAAAGTT-3'	285 bp
	Reverse	5'-GGGCACGAAGGCTCATCATT-3'	
Homo TGF β 1	Forward	5'-CAGCAACAATTCCTGGCGATACCT-3'	140 bp
	Reverse	5'-CGCTAAGGCGAAAGCCCTCAAT-3'	
Homo ITG β 3	Forward	5'-TGGGGCTGATGACTGAGAAG-3'	206 bp
	Reverse	5'-ACGCACTTCCAGCTCTACTT-3'	
Homo AKT	Forward	5'-ACACCAGGTATTTTGTATGAGGA-3'	143 bp
	Reverse	5'-TCAGGCCGTGCCGCTGGCCGAGTAG-3'	
Homo VIM	Forward	5'-TGAGTACCGGAGACAGGTGCAG-3'	119 bp
	Reverse	5'-TAGCAGCTTCAACGGCAAAGTTC-3'	
Homo CBX7	Forward	5'-CGGAAGAAGCGCGTGCAGGAAGGGT-3'	372 bp
	Reverse	5'-GCGGAGCGGGAAGGGCAGGGTGGG-3'	
Homo PI3K	Forward	5'-GACTCAAAAAGGTGTTTCGG-3'	311 bp
	Reverse	5'-ACAAGTTATAGGGCTCGGC-3'	
Homo E-cad	Forward	5'-ACGCATTGCCACATACACT-3'	149 bp
	Reverse	5'-CCATGACAGACCCCTTAAA-3'	

TGF β 1, transforming growth factor β 1; ITG β 3, integrin β 3; VIM, vimentin; CBX7, chromobox protein homolog 7; PI3K, phosphatidylinositol-3-kinase; E-cad, E-cadherin; bp, base pairs.

were collected and washed. Next, the cells were stained with Annexin-V-FITC/PI at room temperature in the dark for 10~15 min. Finally, the samples were analyzed on a Cyto FLEX flow cytometer (Beckman Coulter, Inc.). The data were analyzed using FlowJo v10 (FlowJo LLC).

Animals. Female BALB/c nude mice (age, 4-6 weeks old, weighing 18 \pm 2g, n=36) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. They were housed under strict pathogen-free conditions with a temperature of 20-24°C, a relative humidity of 50%, and a 12-h light-dark cycle. All mice were provided food and water ad libitum. All animal experiment procedures were approved (approval no. 20120220-01) by the Ethics Committee of Xinjiang Medical University.

Xenograft tumor model. SiHa control cells and SiHa cells with stable transfection of siCBX7-EVC and siCBX7 (5 \times 10⁶ cells in 100 μ l PBS) were injected subcutaneously into the right flank of each nude mouse (7 weeks old, n=12/group). The tumors were measured every three days using a vernier caliper and the volumes of tumors were calculated using the following formula: Volume (mm³)=length x width² x 0.5. After 30 days of tumor formation, tumor measurements were conducted every three days. On day 45 post-inoculation, the mice were euthanized by intraperitoneal injection of 60 mg/kg pentobarbital, followed by rapid cervical dislocation. To confirm death, the absence of breathing and nerve reflexes were used as criteria. The tumors were dissected and examined using a stereoscopic microscope.

Western blotting. Total protein was extracted from HeLa and SiHa cells (with stable knockdown of CBX7 or

TGF β 1 knockdown) and tumor tissues using RIPA lysis buffer (cat. no. 20188; Sigma-Aldrich; Merck KGaA). The protein concentration was determined using a BCA Protein Assay kit. Proteins (50 μ g/lane) were then subjected to 10% SDS-PAGE electrophoresis and PVDF membrane transfer. The membranes were blocked with 5% skimmed milk and 0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature. After blocking, the membrane was incubated with the corresponding primary antibodies at 4°C overnight. The primary antibodies included anti-TGF β 1 (1:300; BA0290; Boster Biological Technology), anti-CBX7 (1:500 dilution; cat. no. ab21837; Abcam), anti-vimentin (1:2,000; cat. no. 10366-1-AP; Proteintech Group, Inc.), anti-p-AKT (1:2,000; cat. no. 4060; CST Biological Reagents Co., Ltd.), anti-PI3K p85 (1:2,000; cat. no. bs-3332R; BIOSS), anti-AKT1 (1:1,000; cat. no. ab8805; Abcam), anti-ITG β 3 (1:1,000; cat. no. ab7166; Abcam), anti-E-cad (1:1,000; cat. no. ab40772; Abcam) and anti- β -actin (1:500; cat. no. BM0627; Boster Biological Technology). Finally, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. BA1054; Boster Biological Technology) for 2 h at room temperature. The signal was visualized using ECL western blotting detection reagent (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.). The blots were analyzed with a gel imaging analysis system (Bio-Rad Laboratories, Inc.). Relative protein expression levels were quantified using Quantity One 1-D software (version 4.62; Bio-Rad Laboratories, Inc.).

RT-qPCR. Total RNA was extracted from cells and tumor tissues using Trizol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA using

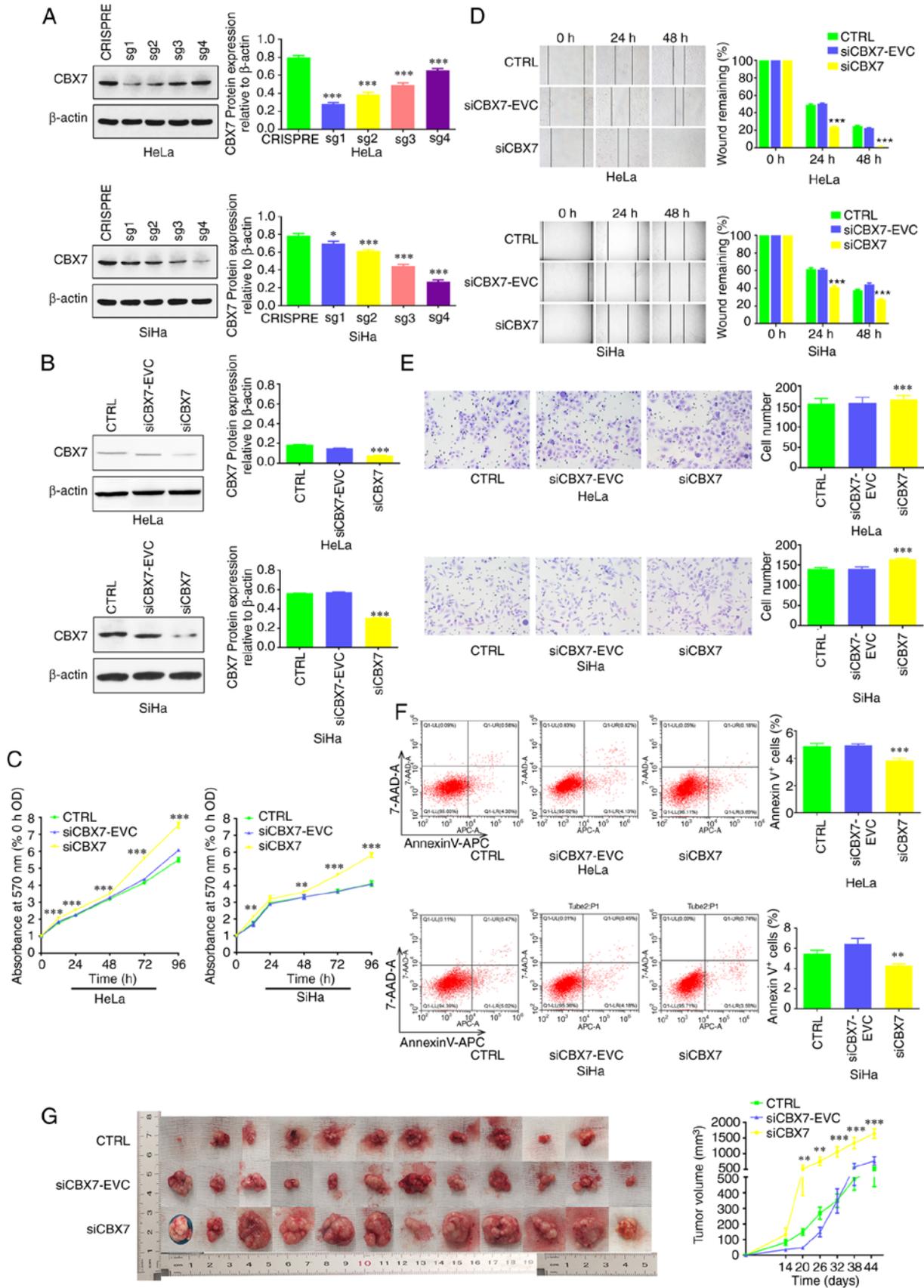


Figure 1. Low expression of CBX7 promotes the growth, migration, invasion and apoptosis inhibition of cervical cancer cells. (A) Expression of CBX7 in cervical cancer cells with stable knockdown of CBX7 was verified by western blotting. (B) The expression of CBX7 in stably transfected cervical cancer cells was verified by western blotting. (C) The effect of CBX7 on the proliferation of HeLa and SiHa cells was detected by MTT assay. (D) The effect of CBX7 on the migration of HeLa and SiHa cells was detected by wound healing assay (Magnification: 100x). (E) The effect of CBX7 on the invasion of HeLa and SiHa cells was detected by Transwell assay (Magnification: 200x). (F) The effect of CBX7 on the apoptosis of cervical cancer cells was detected by flow cytometry. (G) The effect of CBX7 on the growth of cervical carcinoma-transplanted tumors in nude mice. Error bars in all panels represent the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001 compared with the control and siCBX7-EVC. CBX7, chromobox protein homolog 7; CTRL, control; OD, optical density.

Table II. Expression of CBX7, ITGβ3, TGFβ1, PI3K, AKT, p-AKT, E-cad and VIM in cervical cancer and adjacent non-tumorous cervical tissues (N, %).

Groups	CBX7		ITGβ3		TGFβ1		PI3K	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Cervical cancer tissue	71 (59.2)	49 (40.8)	51 (42.5)	69 (57.5)	18 (15.0)	102 (85.0)	29 (24.2)	91 (75.8)
Adjacent non-tumorous cervical tissues	51 (42.5)	69 (57.5)	97 (80.8)	23 (19.2)	60 (50.0)	60 (50.0)	66 (55.0)	54 (45.0)
χ^2	6.018		37.297		33.504		23.852	
P-value	0.014		<0.001		<0.001		<0.001	

Groups	AKT		p-AKT		E-cad		VIM	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
CC	59 (49.2)	61 (50.8)	56 (46.7)	64 (53.3)	89 (74.2)	31 (25.8)	27 (22.5)	93 (77.5)
CTRL	87 (72.5)	33 (27.5)	66 (60)	44 (40)	8 (6.2)	122 (93.8)	102 (85.0)	18 (15.0)
χ^2	13.71		4.096		118.710		101.950	
P-value	<0.001		0.043		<0.001		<0.001	

CBX7, chromobox protein homolog 7; ITGβ3, integrin β3; TGFβ1, transforming growth factor β1; p-, phosphorylated; E-cad, E-cadherin; VIM, vimentin; CC, cervical cancer; CTRL, control.

a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). The reaction was conducted at 42°C for 18 min and 98°C for 5 min. The levels of mRNA were examined using the SYBR Green Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.). The primer sequences are presented in Table I. The thermocycling conditions were as follows: Initial denaturation at 50°C for 2 min, 95°C for 10 min, 95°C for 30 sec and 60°C for 30 sec for 40 cycles. The internal control was β-actin. The relative mRNA level was calculated with the $2^{-\Delta\Delta C_q}$ method (24).

Statistical analysis. All statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and GraphPad software (v.7.0; GraphPad Software; Dotmatics). Data are presented as the mean ± SD of three independent experiments. The differences in the ITGβ3, TGFβ1, PI3K, AKT, p-AKT, VIM and E-cad expression levels between the cervical cancer tissues and adjacent non-tumorous cervical tissues were analyzed by the χ^2 test. The correlation of contingency paired data of CBX7 with other indexes was analyzed by ϕ coefficient analysis. One-way ANOVA followed by Tukey's post hoc test or χ^2 method were used for multiple groups or pairwise comparison of multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CBX7 knockdown promotes the proliferation and metastasis but inhibits the apoptosis of cervical cancer cells. CBX7 was first knocked down in cervical cancer cell lines. The results demonstrated that clones of HeLa-CBX7sg1 and

SiHa-CBX7sg4 had the lowest CBX7 expression (Fig. 1A). These clones were used in the subsequent experiments. CBX7 expression in these clones was further verified by western blot analysis, which revealed that CBX7 was successfully knocked down (Fig. 1B). Subsequently, to determine the effects of CBX7 on cell proliferation, an MTT assay was performed. The results showed that the OD_{570} of the siCBX7 group significantly increased compared with that of the siCBX7-EVC and CTRL groups, indicating that CBX7 knockdown resulted in significantly increased proliferation ($P < 0.05$; Fig. 1C). The wound healing assay revealed that the wound area of the siCBX7 group was significantly smaller than the other two groups at 48 h, indicating that downregulation of CBX7 significantly promoted cell migration ($P < 0.05$; Fig. 1D). Next, a Transwell assay was performed and revealed that cell migration was also significantly increased by CBX7 knockdown. There was a significant increase in the number of migrated cells in the siCBX7 group ($P < 0.001$; Fig. 1E). Furthermore, flow cytometry revealed that the proportion of apoptotic cells in the siCBX7 group was significantly lower than that in the siCBX7-EVC and CTRL groups ($P < 0.05$; Fig. 1F). Additionally, a xenograft tumor model was subsequently established by injecting infected siCBX7 SiHa cells into nude mice to investigate whether knockdown of CBX7 exerts similar growth-promoting effects *in vivo*. As revealed in Fig. 1G, one nude mouse in the control group did not develop tumors, while the tumor incidence rate for the other two groups was 100%. The largest tumor was observed in the siCBX7 group, with a tumor volume of 1,812.54 mm³. Statistically, the tumor volume was increased in the siCBX7 group compared with that in the siCBX7-EVC and CTRL ($P < 0.05$; Fig. 1G), demonstrating that

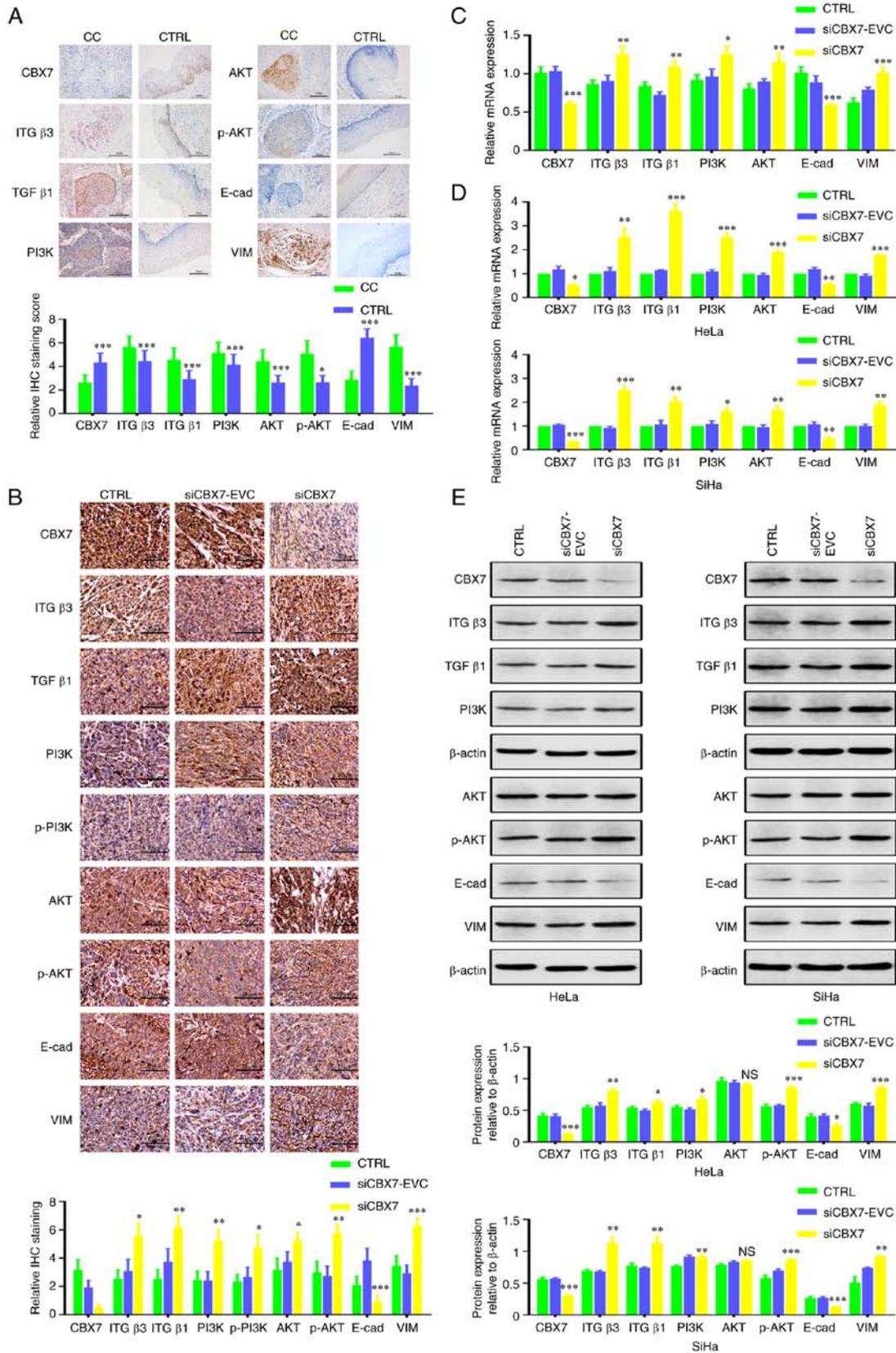


Figure 2. Effect of CBX7 on the expression of ITGβ3, TGFβ1, PI3K, AKT, p-AKT, VIM, and E-cad. (A) Representative images and statistical analysis of IHC detection of CBX7, ITGβ3, TGFβ1, PI3K, AKT, p-AKT, E-cad and VIM protein expression in CC and CTRL tissues (magnification: 200x). (B) Representative images and statistical analysis of IHC detection of CBX7, ITGβ3, TGFβ1, PI3K, AKT, p-AKT, E-cad and VIM protein in transplanted tumor tissues (Magnification: 200x). (C) The effect of CBX7 on *CBX7*, *ITGβ3*, *TGFβ1*, *PI3K*, *AKT*, *p-AKT*, *E-cad* and *VIM* mRNA in nude mouse-transplanted tumor tissues by reverse transcription-quantitative PCR. (D) The effect of CBX7 on *CBX7*, *ITGβ3*, *TGFβ1*, *PI3K*, *AKT*, *p-AKT*, *E-cad* and *VIM* mRNA in HeLa and SiHa cells. (E) Expression of CBX7, ITGβ3, TGFβ1, PI3K, AKT, p-AKT, E-cad and VIM protein in HeLa and SiHa cells. Error bars in all panels represent the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with the control and siCBX7-EVC. CBX7, chromobox protein homolog 7; ITGβ3, integrin β3; TGFβ1, transforming growth factor β1; PI3K, phosphatidylinositol-3-kinase; p-, phosphorylated; VIM, vimentin; E-cad, E-cadherin; CC, cervical cancer; CTRL, control; IHC, immunohistochemistry.

Table III. Correlation of CBX7 with ITGβ3, TGFβ1, PI3K, AKT, p-AKT, E-cad and VIM in cervical cancer tissues (N, %).

CBX7	ITGβ3 frequency (%)			TGFβ1 frequency (%)					
	Negative	Positive	Total	Negative	Positive	Total			
Negative	21 (17.5)	50 (41.7)	71	5 (4.2)	66 (55.0)	71			
Positive	30 (25.0)	19 (15.8)	49	13 (10.8)	36 (30.0)	49			
Total no.	51	69	120	18	102	120			
Phi (φ)	-0.315			-0.268					
P-value	0.001			0.003					
CBX7	PI3K frequency (%)			AKT frequency (%)			p-AKT frequency (%)		
	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total
Negative	6 (5.0)	65 (54.2)	71	26 (21.7)	45 (37.5)	71	13 (10.8)	58 (48.3)	71
Positive	23 (19.2)	26 (21.7)	49	33 (27.5)	8 (13.3)	49	43 (35.8)	6 (5.0)	49
Total no.	29	91	120	59	61	120	56	64	120
Phi (φ)	-0.442			-0.302				-0.684	
P-value	<0.001			0.001				<0.001	
CBX7	E-cad frequency (%)			VIM frequency (%)					
	Negative	Positive	Total	Negative	Positive	Total			
Negative	62 (51.7)	9 (7.5)	71	6 (5.0)	65 (52.4)	71			
Positive	27 (22.5)	22 (18.3)	49	21 (17.5)	28 (23.3)	49			
Total no.	89	31	120	27	93	120			
φ	0.362			-0.405					
P-value	<0.001			<0.001					

CBX7, chromobox protein homolog 7; ITGβ3, integrin β3; TGFβ1, transforming growth factor β1; p-, phosphorylated; E-cad, E-cadherin; VIM, vimentin.

Table IV. Comparison of positive expression rates of key genes in the ITGβ3/TGFβ1 signaling pathway and EMT markers in three groups of nude mice with xenograft tumors (N, %).

Groups	N	CBX7		ITGβ3		TGFβ1		PI3K		p-PI3K	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
CTRL	11	4 (36.4)	7 (73.6)	4 (36.4)	7 (73.6)	3 (27.3)	8 (72.7)	4 (36.4)	7 (73.6)	3 (27.3)	8 (72.7)
siCBX7-EVC	12	6 (50.0)	6 (50.0)	5 (28.6)	7 (71.4)	5 (28.6)	7 (71.4)	5 (28.6)	7 (71.4)	5 (41.7)	7 (58.3)
siCBX7	12	11 (91.7)	1 (8.3)	1 (8.3)	11 (91.7)	2 (16.7)	10 (83.3)	2 (16.7)	10 (83.3)	3 (25.0)	9 (75.0)
χ ²		4.216		4.274		1.874		2.038		0.886	
P-value		0.040		0.118		0.392		0.383		0.642	

Groups	N	AKT		p-AKT		E-cad		VIM	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
CTRL	11	3 (27.3)	8 (72.7)	4 (36.4)	7 (73.6)	6 (45.5)	6 (54.5)	3 (27.3)	8 (72.7)
siCBX7-EVC	12	3 (25.0)	9 (75.0)	5 (28.6)	7 (71.4)	4 (33.3)	8 (66.7)	4 (33.3)	8 (66.7)
siCBX7	12	1 (8.3)	11 (91.7)	1 (8.3)	11 (91.7)	11 (91.7)	1 (8.3)	0 (0.0)	12 (100.0)
χ ²		1.757		4.274		10.485		6.861	
P-value		0.415		0.118		0.005		0.032	

CBX7, chromobox protein homolog 7; ITGβ3, integrin β3; TGFβ1, transforming growth factor β1; PI3K, phosphatidylinositol-3-kinase; p-, phosphorylated; E-cad, E-cadherin; VIM, vimentin; CTRL, control.

the downregulation of CBX7 may promote xenograft tumor growth in nude mice.

Effect of CBX7 on the expression of key genes in the ITGβ3/TGFβ1 signaling pathway and EMT markers. Immunohistochemistry was performed to detect the protein expression of CBX7, ITGβ3, TGFβ1, PI3K, AKT, p-AKT, VIM and E-cad in cancer tissues from humans and mice. As illustrated in Fig. 2A and Table II, the expression of CBX7 and E-cad in human cervical cancer tissues was significantly lower, whereas the expression of ITGβ3, TGFβ1, PI3K, AKT, p-AKT and VIM was significantly higher than those of adjacent tissues (P<0.05). Correlation analysis demonstrated that CBX7 and E-cad expression were positively correlated (φ=0.362; P<0.001); however, CBX7 was negatively correlated with other proteins (all P<0.05; Table III). In xenograft tumor tissues of mice, knockdown of CBX7 increased the level of VIM and decreased expression of E-cad significantly (Fig. 2B; Table IV; P<0.01). In addition, RT-qPCR revealed that CBX7 knockdown significantly increased *VIM*, *ITGβ3*, *TGFβ1*, *PI3K* and *AKT* mRNA levels, and reduced *E-cad* mRNA expression in xenograft tumor tissues (P<0.05) (Fig. 2C).

Similar results were obtained in stable cells with knockdown of CBX7 (Fig. 2D). In HeLa and SiHa cells after CBX7 knockdown, RT-qPCR results showed that knockdown of CBX7 reduced *E-cad* mRNA (57.0%) and increased the mRNA levels of *VIM* (179.5%), *ITGβ3* (253.3%), *TGFβ1* (364.5%), *PI3K* (251.4%) and *AKT* (191.2%) (Fig. 2D). Similarly, the results of western blotting demonstrated that knocking down CBX7 in HeLa and SiHa cells promoted the expression of VIM, ITGβ3, TGFβ1, PI3K and AKT, and reduced the level of E-cad (Fig. 2E and F).

The aforementioned results indicated that CBX7 may regulate the ITGβ3/TGFβ1/AKT signaling pathway and may be involved in the EMT process.

Knockdown of CBX7 promotes cervical cancer progression through the ITGβ3/TGFβ1/AKT signaling pathway. To verify the mechanism of CBX7 in cervical cancer, TGFβ1 of the TGFβ1 pathway was further knocked down and its effect on the proliferation, metastasis and apoptosis of cervical cancer cells was explored. The expression of *TGFβ1* mRNA after transfection of TGFβ1-HOMO-875, TGFβ1-HOMO-1158 and TGFβ1-HOMO-1408 was 0.51, 0.31 and 0.47, respectively, significantly lower than that in the control group (P<0.001) (Fig. 3A). The TGFβ1-HOMO-1158 fragment had the highest knockdown efficiency and was therefore used in subsequent experiments. TGFβ1 expression in control cells or cells with stable knockdown of CBX7 was further verified by RT-qPCR. The results revealed that TGFβ1 was successfully knocked down in each group (Fig. 3B). The MTT assay and Transwell assay demonstrated that the downregulation of CBX7 increased cell proliferation (Fig. 3C) and migration (Fig. 3D) compared with siCBX7-EVC and CTRL groups. Furthermore, the proliferation and migration ability of cells with knockdown of CBX7 and TGFβ1 decreased and were lower than the cells with CBX7 knockdown only at 12, 24, 48 and 72 h. Flow cytometry revealed that the downregulation of CBX7 decreased apoptosis compared with siCBX7-EVC and CTRL groups. Additionally, the apoptosis ability of cells with knockdown of CBX7 and TGFβ1 increased and was higher than the cells with CBX7 knockdown-only (Fig. 3E). Collectively, TGFβ1 silencing reversed the increase in cell proliferation, and migration and reversed the decrease in apoptosis. The aforementioned results

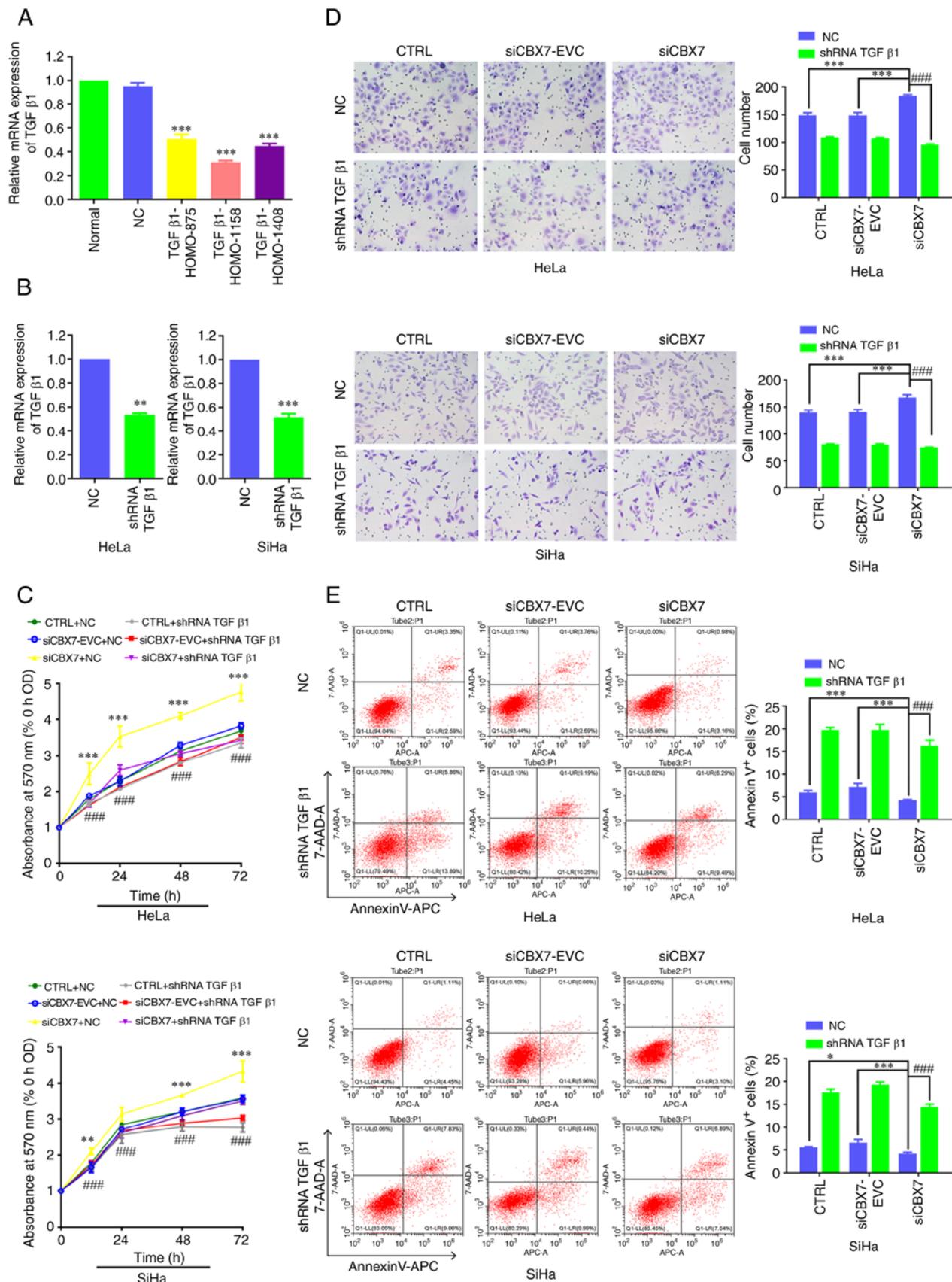


Figure 3. Knockdown of TGF β 1 reverses the effects of CBX7 knockdown on cervical cancer cell proliferation, migration and apoptosis. HeLa and SiHa cells with stable expression of CBX7 knockdown were transfected with TGF β 1 shRNAs. (A) TGF β 1 mRNA was assessed with reverse transcription-quantitative PCR to determine the TGF β shRNA with the optimal knockdown efficiency. (B) TGF β 1 mRNA expression in each group after transfection with TGF β 1 shRNA. (C) The proliferation ability of HeLa and SiHa cells was detected by MTT assay. (D) The migration ability of HeLa and SiHa cells was analyzed by Transwell assay (Magnification: 200x). (E) The apoptosis of HeLa and SiHa cells was assessed using flow cytometry. Error bars in all panels represent the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001, siCBX7 compared with the control and siCBX7-EVC; ### P <0.001, siCBX7 + shRNA TGF β 1 compared with siCBX7. TGF β 1, transforming growth factor β 1; CBX7, chromobox protein homolog 7; shRNA, short hairpin RNA; NC, negative control; CTRL, control.

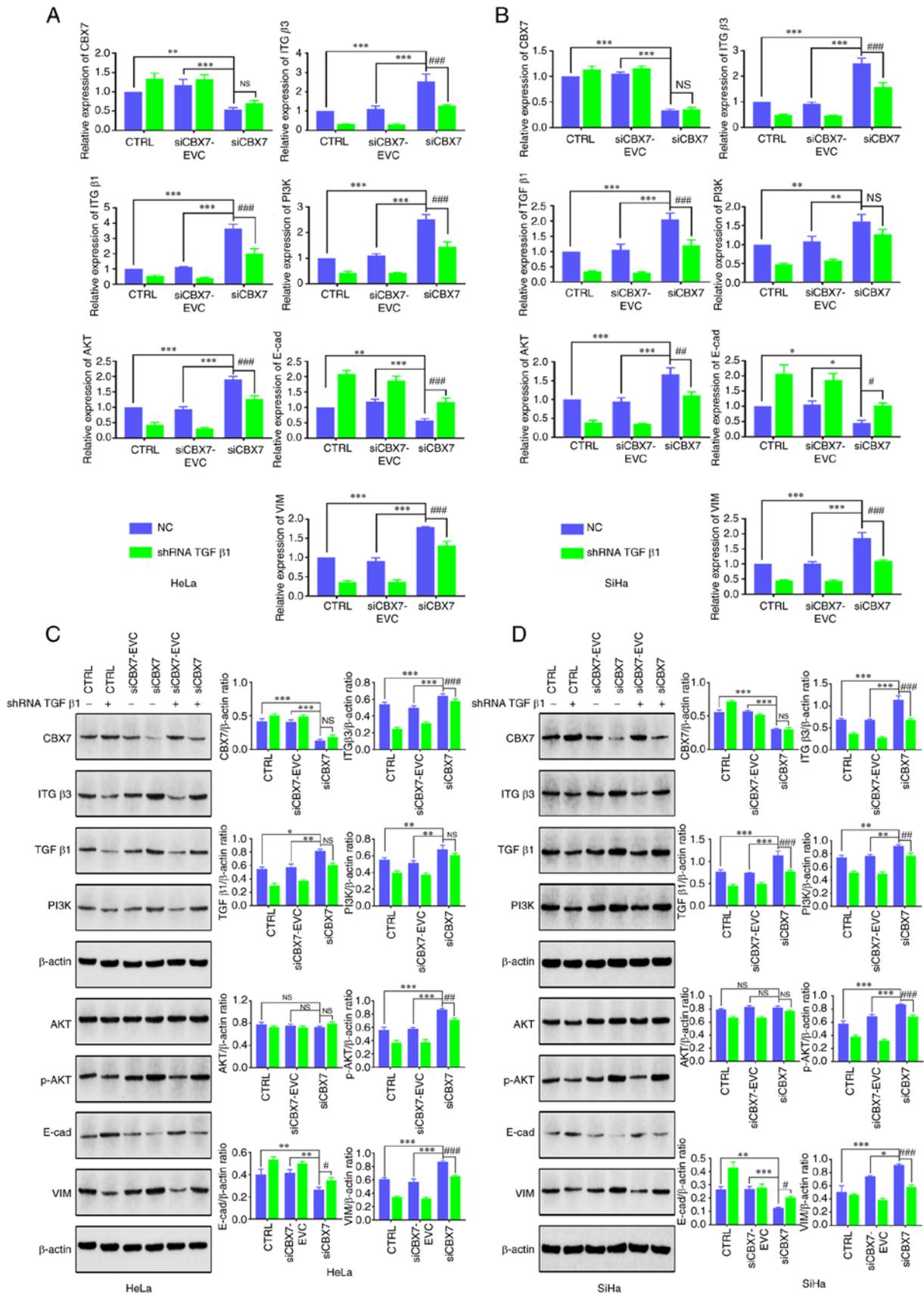


Figure 4. Effect of TGF β 1 silencing and CBX7 knockdown on the expression of key genes in the PI3K/AKT and ITG β 3/TGF β 1 signaling pathways. HeLa and SiHa cells with stable expression of CBX7 knockdown were transfected with silencing plasmids of TGF β 1. The mRNA expression of genes in the PI3K/AKT and ITG β 3/TGF β 1 signaling pathways in (A) HeLa and (B) SiHa cells was detected by reverse transcription-quantitative PCR. The protein expression of genes in the PI3K/AKT and ITG β 3/TGF β 1 signaling pathways in (C) HeLa and (D) SiHa cells was detected by western blot analysis. Error bars in all panels represent the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001, siCBX7 compared with the control and siCBX7-EVC; # P <0.05, ## P <0.01 and ### P <0.001, siCBX7 + shRNA TGF β 1 compared with the siCBX7. TGF β 1, transforming growth factor β ; CBX7, chromobox protein homolog 7; PI3K, phosphatidylinositol-3-kinase; ITG β 3, integrin β 3; shRNA, short hairpin RNA; CTRL, control; NS, not significant; E-cad, E-cadherin; VIM, vimentin; NC, negative control.

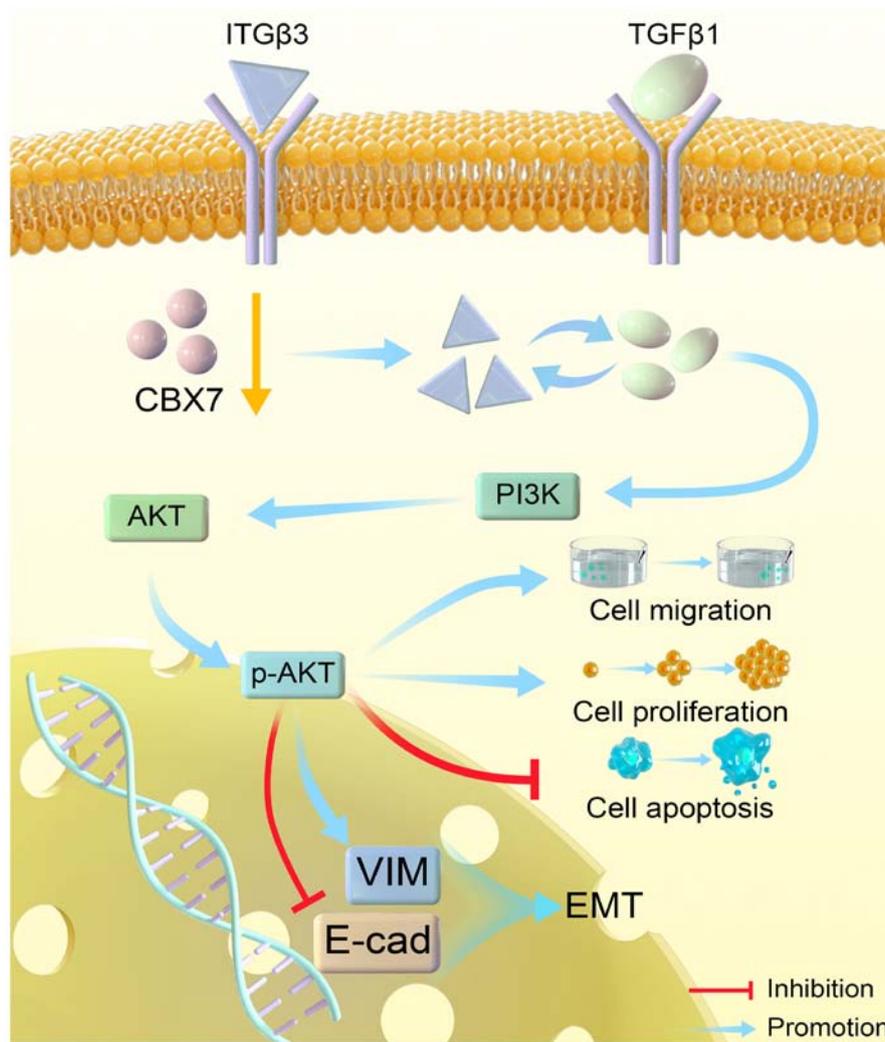


Figure 5. Schematic diagram illustrating the mechanism of CBX7 in cervical cancer. In cervical cancer, downregulation of CBX7 may induce EMT, promote cell proliferation, migration and inhibit cell apoptosis through the ITGβ3/TGFβ1/AKT signaling pathway. CBX7, chromobox protein homolog 7; EMT, epithelial-mesenchymal transition; ITGβ3, integrin β3; TGFβ1, transforming growth factor β1; PI3K, phosphatidylinositol-3-kinase; p-, phosphorylated; VIM, vimentin; E-cad, E-cadherin.

indicated that CBX7 may regulate the proliferation, metastasis and apoptosis of cervical cancer cells through the TGFβ1 signaling pathway. In addition, the expression of key genes of the ITGβ3/TGFβ1 signaling pathway and the EMT process were also analyzed. The RT-qPCR results revealed that the expression levels of *CBX7* and *E-cad* were significantly lower in the siCBX7 group compared with the siCBX7-EVC and CTRL groups, whereas *ITGβ3*, *TGFβ1*, *PI3K*, *AKT* and *VIM* exhibited higher expression levels ($P < 0.05$). No differences in CBX7 expression were observed between the cells with silenced and unsilenced TGFβ1. Silencing TGFβ1 in cells resulted in decreased mRNA levels of *ITGβ3*, *PI3K*, *AKT*, and *VIM*, whereas *E-cad* mRNA levels increased (Fig. 4A and B). Western blot analysis demonstrated that silencing TGFβ1 resulted in varying degrees of decrease in ITGβ3, p-PI3K, p-AKT and VIM, except CBX7 and AKT, while E-cad expression increased (Fig. 4C and D). Thus, knockdown of TGFβ1 had no significant effect on CBX7 itself, but partially reversed the activation of the ITGβ3/TGFβ1/AKT signaling pathway and EMT-related genes caused by CBX7 knockdown. These results indicated that knocking down CBX7 may regulate

the EMT of cervical cancer cells through the downregulated TGFβ1 signaling pathway.

Discussion

PRC1 is actively expressed in various cancers and participates in tumor progression. As a member of PRC1, CBX7 is a tumor suppressor involved in a variety of cancers (4). Previous studies have revealed that inhibition of CBX7 promotes the migration of gliomas and tumor invasion (10,25). In addition, *in vitro* experiments have confirmed that inhibition of CBX7 promotes the growth of pancreatic cancer (11) and breast cancer (5). CBX7 has also been confirmed to inhibit the growth of liver cancer cells *in vivo* and *in vitro* (26). Recently, in CBX7 knockout mice (9), CBX7 had an inhibitory effect on tumors. CBX7 is involved in tumor growth (12), invasion (6) and migration (27). CBX7 overexpression in urinary bladder cancer cells inhibits tumorigenicity, whereas CBX7 depletion promotes tumor development (28). Moreover, miR-19 affects the migration and cell cycle of lung cancer cells by inhibiting CBX7 expression (29). Consistently, the findings of the

present study revealed that knockdown of CBX7 promoted the migration and invasion of cervical cancer cells *in vitro* and inhibited cell apoptosis. Therefore, low expression of CBX7 may promote tumor progression of cervical cancer.

By analyzing the expression of genes and proteins in human cervical cancer tissues, transplanted tumors in nude mice and cervical cancer cells, downregulation of CBX7 was found to increase the expression of VIM, ITG β 3, TGF β 1, PI3K and AKT, and decrease the expression of E-cad. Furthermore, correlation analysis demonstrated that CBX7 was positively correlated with E-cad and negatively correlated with VIM. Federico *et al* reported that CBX7 positively regulated E-cad expression by interacting with the histone deacetylase 2 protein (30). E-cad and VIM are epithelial and mesenchymal markers of EMT, respectively (31). E-cad can identify cell-cell junctions and apicobasal polarity, limit the migratory potential of epithelial cells and maintain the integrity of tissue structure and function (32,33). The downregulation of E-cad can lead to the destabilization of adherent junctions and promote the transformation of epithelial cells to mesenchymal cells (34). A previous study has shown that the reduction of epithelial phenotype and the increase of mesenchymal phenotype may promote tumor progression (35). Additionally, it has been reported that CBX7 upregulation enhanced E-cad expression (30). In the present study, consistently, it was revealed that knockdown of CBX7 inhibited E-cad and promoted VIM, suggesting that low expression of CBX may promote cervical cancer progression through regulation of EMT. Furthermore, CBX7 can upregulate E-cad (30) and plays a key role in the occurrence of EMT and the progression of advanced cancer (28). Previous research conducted by the authors revealed that CBX7 was downregulated in cervical cancer and its downregulation was associated with a poor prognosis in patients with cervical cancer (22,23). Moreover, it was revealed that CBX7 overexpression inhibited cell proliferation and migration in cervical cancer cells (22,23). Furthermore, the knockdown of CBX7 expression in the present study significantly promoted the migration and invasion of cervical cancer cells. These results strongly suggest that CBX7 may function as a tumor suppressor in the development of cervical cancer. Moreover, it was preliminarily inferred that the low expression of CBX7 involved in the occurrence of cervical cancer EMT may be related to ITG β 3, TGF β 1, PI3K, and AKT signaling pathways, which all play an important role in tumor cell migration and invasion (36,37).

Furthermore, TGF β 1 expression was silenced in cells with stable knockdown of CBX7. TGF β 1 silencing was identified to reduce the migration and invasion of stably transfected HeLa and SiHa cells and increase cell apoptosis. Rapisarda *et al* found that downregulation of CBX7 promoted the expression of ITG β 3 (18). IT5 β 3/AKT can promote the progression of hemangioendothelioma. Furthermore, research has revealed that TGF β 1 induces the upregulation of integrin alpha V, leading to the promotion of EMT in ovarian cancer cells (38). Intercellular adhesion molecule 1 was demonstrated to promote the migration of triple-negative breast cancer cells through an integrin-mediated mechanism dependent on TGF- β /EMT (39). There is an interaction between TGF β 1 and ITG β 3 (40,41). CBX7 has been revealed to activate PTEN, inhibit the downstream PI3K/AKT pathway, and suppress the

proliferation, metastasis and invasion of pancreatic cancer cells (11). In addition, CBX7 was demonstrated to enhance the sensitivity of bladder cancer cells to cisplatin treatment through the inactivation of the PI3K/AKT signaling pathway (42). The TGF β 1 and PI3K/AKT signaling pathways are crucial in regulating diverse cellular responses, such as proliferation, apoptosis and migration (43). PI3K serves as a vital intracellular kinase in organisms, while AKT acts as its most significant downstream factor. The inhibition of TGF β 1 suppresses the occurrence of EMT in cervical cancer cells, thereby restraining cell migration and invasion (44). In the present study, it was found that silencing of TGF β 1 reversed the effects of CBX7 knockdown on the expression of key genes in the ITG β 3/TGF β 1 signaling pathway and the EMT process. Therefore, CBX7 may regulate the progression of cervical cancer through ITG β 3/TGF β 1/AKT signaling pathway (Fig. 5).

The present study has some limitations. Firstly, a subcutaneous xenograft model of cervical cancer cells in nude mice was constructed. However, this model does not accurately replicate the conditions within the nude mice. In the future, the orthotopic transplantation tumor model, which involves transplantation to anatomically relevant sites and exhibits stronger biological relevance to cervical cancer, will be used. Secondly, the present study validated the impact of CBX7 on proliferation, metastasis and apoptosis. However, the factors related to proliferation, metastasis and apoptosis were not examined. The inclusion of these factors in the analysis can enhance the credibility of the conclusions.

In summary, the present study show that low expression of CBX7 is associated with high proliferation and metastasis and low apoptosis of cervical cancer cells. In addition, low expression of CBX7 may affect the PI3K/AKT signaling pathway by upregulating ITG β 3/TGF β 1 and promoting the proliferation of cervical cells and the occurrence of EMT. The findings of the present study provide insights into the mechanisms of cervical cancer and might help the development of potential therapeutic targets for cervical cancer treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia Fund (grant nos. SKL-HIDCA-2020-WF1 and SKL-HIDCA-2022-GJ2), the Postdoctoral Science Foundation of China (grant no. 2019M663963XB), the Xinjiang Autonomous Region Collaborative Innovation Program (grant no. 2019E0282) and the Xinjiang Medical University Student Innovation and Entrepreneurship Training Program (grant no. CX2021046).

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

PT, JD and RL designed the study. PT and JD wrote the manuscript. CM, AM, LD and GM performed experiments. QY, YaL, HM, YuL, CZ and JR collected and analyzed data. All authors have read and approved the final manuscript. PT and RL confirm the authenticity of all raw data.

Ethics approval and consent to participate

The present study was conducted following the Declaration of Helsinki and approved (approval no. 20120220-01) by the Institutional Review Board of Xinjiang Medical University (Urumqi, China). Written informed consent was obtained from all subjects involved in the study. The animal study protocol was approved (approval no. 20120220-01) by the Ethics Committee of Xinjiang Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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