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ORIGINAL RESEARCH

RETRACTED ARTICLE: CCDC7 Activates Interleukin-6 and Vascular Endothelial Growth Factor to Promote Proliferation via the JAK-STAT3 Pathway in Cervical Cancer Cells

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Objective: Tumor growth is one of the most lethal attributes of human malignancy. The expression of CCDC7, a novel gene which has multiple functions, has been shown to be associated with tumor growth and poor prognosis in patients with cancer. However, the specific functions of CCDC7 remain unclear. Here, we investigated the molecular mechanisms underlying the effects of CCDC7 on proliferation in cervical cancer.

Materials and Methods: The MTT and EdU assays were performed to evaluate the function of CCDC7. The immunohistochemical, quantitative real-time PCR (qRT-PCR), ELISA and Western blot assay were used to detect the gene and protein expression in tissues and cells. A xenograft test was conducted to detect the impact of CCDC7 on tumor development in vivo .

Results: In immunohistochemical analysis of 193 cases, normal cervical tissue and cervical cancer tissue showed that CCDC7 expression is closely correlated with the development of cervical cancer and was positively correlated with the clinical stage and histological grade. Overexpression or knockdown of CCDC7 affected cell proliferation in cervical cancer cells in vitro. In nude mouse xenograft model in vivo, knockdown of CCDC7 inhibited cell proliferation and tumor growth. Furthermore, CCDC7 overexpression upregulated interleukin (IL)-6 and vascular endothelial growth factor (VEGF) at mRNA and protein levels, and treatment with recombinant IL-6 or VEGF proteins also increased CCDC7 expression. In case set of 80 patients with cervical cancer, we found that CCDC7, IL-6, and VEGF affected patient prognosis. Finally, inhibition of various signaling pathways using specific inhibitors indicated that CCDC7 blocked the decrease in cell proliferation observed following suppression of the JAK-STAT3 pathway, suggesting that CCDC7 functioned via this critical signaling network.

Conclusion: Those findings indicated that CCDC7 may be a novel target for the treatment of cervical cancer and may have applications as a predictive marker for tumor growth in cervical carcinoma.

Keywords: CCDC7, IL-6, VEGF, tumor growth, cervical cancer, gene therapy

Introduction

Worldwide, carcinoma of the cervix is the second most common cancer among women and is one of the leading causes of cancer related deaths in women in both developed and developing countries.[1] Although patients with early cervical cancer may receive combined therapeutic approaches including surgery, radiotherapy, and chemotherapy, many patients still have poor prognoses.[2] A variety of clinical studies have suggested that tumor size, depth of invasion, and lymphatic vascular

space involvement are associated with prognosis in patients with cervical cancer.[3] Therefore, investigation of tumor growth-related regulatory molecules, molecular targets, and treatments for tumor growth in cervical cancer is necessary and may facilitate the development of novel therapies to improve survival in patients with cervical cancer.[3] Hence, many researchers have been carrying out studies aimed at identifying novel predictors or therapeutic targets for cervical carcinoma.

The coiled-coil domain containing 7 (*CCDC7*) gene[4] (Gene ID: 221016) encodes a protein with unknown functions. And it is the longest transcript of the CCDC7 gene family. Our previous studies have shown that members of this gene family play an important role in tumorigenesis, and may serve as the potential targets for biotherapy. In clinical studies by Real-time PCR experiments, we found that the mRNA expression level of members of CCDC7 gene family is higher in cancer tissues than the adjacent tissues in the contexts of cervical cancer, endometrial cancer, and colorectal cancer.[5–7] Moreover, the mRNA expression level of members of CCDC7 gene family is significantly increased with more advanced tumor stage in clinical specimens from patients with colorectal cancer.[6] Members of CCDC7 gene family exhibit higher expression in patients with early colorectal cancer, which is associated with a particularly poor prognosis.[8] However, while it is clear that expression of the *CCDC7* protein is associated with tumorigenesis, the function of CCDC7 and the mechanisms through which CCDC7 affects tumor growth are not clear.

Interleukin-6 (IL-6) is an important regulator of immune and inflammatory responses and functions as a growth factor for many types of tumor cells. IL-6 is a pleiotropic cytokine involved in tumor initiation, promotion, and progression and has been shown to play a central role in cancer-associated inflammation and the regulation of tumor growth.[9] One of the functions of this multifunctional cytokine is to activate target genes involved in cell proliferation. Moreover, IL-6 has been reported to be indispensable for oncogene-induced cell transformation and tumorigenesis, indicating the importance of IL-6 in tumor initiation. Several studies examining IL-6 expression in human gastric cancer tissues have shown that IL-6 expression is positively correlated with VEGF expression. However, the specific role of IL-6 in cervical cancer has not yet been clarified.

The IL-6 and Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway is activated in a variety of human cancers;[10] the JAK-STAT3 pathway is an important oncogenic signaling cascade that consists of the JAK family of non-receptor tyrosine kinases and the STAT family of transcription factors.[11] Persistent JAK-STAT3 signaling is implicated in many biological processes. STAT3 is known to be involved in both tumor-intrinsic and tumor-extrinsic processes, supporting tumor survival and metastasis.[12] However, in most malignancies, STAT proteins, in particular STAT3, is aberrantly activated (tyrosine phosphorylation) in the majority of cancers.[13] Importantly, the JAK-STAT3 signaling pathway has been regarded as a critical regulator of tumorigenesis, and the intensity of STAT3 activation within the tumor stroma is a major determinant of cytokine response and cellular functions promoting tumor growth.[14] Binding of cell surface receptors with ligands, such as IL-6, induces tyrosine phosphorylation of STAT3 protein by JAK and growth factor receptor tyrosine kinases.[15] Thus, abnormalities in the JAK/STAT signaling pathway are thought to be involved in the oncogenesis of several types of cancers.[16–18]

In this study, we aimed to elucidate the role of CCDC7 in promoting the tumorigenesis of cervical cancer using cell culture, mouse models, and clinical specimens.

Materials and Methods

Materials

The recombinant human proteins VEGF and IL-6 were purchased from Pepro Tech (Suzhou, China). Anti-STAT3 (1:1000), anti-JAK (1:1000), anti-phospho-STAT3 (Y705) (1:1000), and anti-phospho-JAK (Y1007/1008) (1:1000) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-CCDC7 (1:1000) antibody was purchased from Abcam (Cambridge, UK). Mouse anti-PCNA (1:1000) was obtained from BOSTER (China). The secondary antibody (goat anti-rabbit IgG) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). TransIT®-2020 Transfection Reagent was purchased from Mirus (USA). The total RNA kit was purchased from Tian Gen (China), and the first-strand cDNA synthesis kit and SYBR Premix Ex Taq were purchased from TaKaRa (Shiga, Japan). The ELISA kits for IL-6 and VEGF were from DAKEWE (China). Tumor microarrays were purchased from Alenabio (Shanxi, China). All other kits or reagents were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). The recombinant plasmid CCDC7-shRNA was obtained from Genechem

(Shanghai, China). The plasmid was prepared using an Endofree Plasmid Giga kit purchased from Qiagen (Chatsworth, CA, USA).

Cell Culture

Transient Transfections

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM L-Glu, and 5 mg/mL penicillin/streptomycin at 37 °C and in a humidified incubator containing 5% CO_2.

HeLa cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, 2.5 mL complete growth medium was added per well. Transfections were carried out using 250 μL of reduced-serum medium with 2.5 μg CCDC7 or siCCDC7 according to the protocol for the Trans IT-2020 reagent. Cells were incubated for 24–48 h before use.

MTT Assay

Cell proliferation was evaluated by MTT assay (Sigma, St. Louis, MO, USA). Cells were seeded at a density of 3×10^3 or 5×10^3 cells per well in 96-well plates (wells per treatment) in DMEM containing 10% FC in a final volume of 100 μL. Twenty-four h later, c were transfected with CCDC7 or siCC C7 as evious described. After transfection, cells w incu the presence of MTT (20 μL/well of 5 g/L s tion in sterile phosphate-buffered saline [P S] for 4 h at 7 °C. The medium was removed, and 150 μL f dimethylsulfoxide (DMSO) was used to ssolve the MT rmazan crystals. The absorbance fr each ell was measured at 570 nm using a microplat re er (Multiskan MK3, Thermo Labsystems nd).

EdU Cell Proliferation Assay

HeLa cells (10^4 cells/well) were seeded in 24-well plates (n = 3 wells pe reatment) and then transfected 24 h later with CCDC7 or siCCDC7. EdU assays were then carried out according to the manufacturer's protocol. Cells were imaged under an inverted fluorescence microscope.

Quantification of CCDC7, IL-6, and VEGF mRNA by qRT-PCR

Total RNA was extracted from the transfected cells with an RNA Simple Total RNA kit according to the manufacturer's instructions. Next, 1 μg of RNA was used to synthesize cDNA using a first-strand cDNA synthesis kit. qRT-PCR analysis was performed using SYBR Premix Ex Taq according to the manufacturer's protocol. Data were normalized according to the level of β-actin expression in each sample. For real-time PCR, a commercial power SYBR Green PCR master mix was used. Briefly, 1 μg of total RNA was used as the template and then reverse-transcribed using an oligo primer. The cDNA was further amplified in the qPCR assay using a universal reverse primer and a specific forward prim (Table S4). The reaction solution consisted of 2.0 diluted T-PCR product and 0.5 μM of each paired primer. The PCR protocol included initial denatur ion at °C for min and then 39 cycles of 95 °C f s, 59 °C (60 C) r 20 s, and 65°C for 5 s, and a final p at °C followed by melting curve analysis. The bsolute RNA le is in each sample were calculated rding to a s d curve created using serial dilutions of kn n amounts of specific templates against co nding cyc threshold (Ct) values. Each sample as tested in triplicate. Cycle threshold (Ct) values were btained gra ically for IL-6, CCDC7, VEGF, and β-actin. differen e in Ct values between β-actin and IL-6, CCD d VEGF are presented as ΔCt values.

Quantification of IL-6 and VEGF Secretion by ELISA

HeLa cells (1×10^5 cells/well) were seeded in 6-well plates. Twenty-four hours later, the cells were transfected with CCDC7 or siCCDC7. After incubation, the culture supernatants were collected, and the levels of IL-6 and VEGF were analyzed by human IL-6 and VEGF ELISA kits according to the manufacturer's protocol. The results were adjusted according to the total number of cells. Each sample was tested in triplicate.

Western Blotting

All protein samples for Western blot analysis were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and then transferred to nitrocellulose membranes. The membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% fat-free milk. All primary antibody incubations were performed for 18 h at 4 °C. Secondary antibody incubations were carried out at room temperature for 1 h. Secondary antibodies conjugated with horseradish peroxidase (HRP) were used for detection by

enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA) or ECL Plus (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions.

Immunohistochemical Staining

Tissues were dewaxed, and antigens were retrieved using high pressure. Then, the activity of endogenous peroxidases was blocked using 3% hydrogen peroxide for 10 min. After immersion in normal goat serum for 30 min, tissues were subjected to primary antibody incubation at 4 °C overnight. Subsequently, sections were washed with PBS and incubated with secondary antibodies that had been labeled with biotin for 30 min at 37 °C. After washing, the sections were incubated with the HRP complex for 30 min at 37 °C and visualized using diaminobenzidine (DAB).

All immunohistochemical images were obtained using an Olympus BX51 microscope equipped with a 40× objective lens (Olympus) and a DP 50 camera (Olympus). Images were processed using DPC controller software (Olympus).

The immunohistochemical staining was evaluated using a semiquantitative scoring method. The total CCDC7, IL-6 and VEGF staining was scored as per staining intensity: no staining (0), light positive staining (1), medium positive staining (2), and strong positive staining (3). The positively stained area was scored as: < 5% (0), 5%–25% (1), 26%–50% (2), 51%–75% (3), and > 75% (4). A final score was calculated by multiplying the intensity score by the expression score and the rating (from 0 to 12) was determined for each case.

Animal Studies

Four- to six-week-old nude BALB/c female mice were obtained from the IFK Bioscience (Beijing, China) and maintained at the Sichuan University, Chengdu. Animals were inoculated subcutaneously (s.c.) with of a total of 100 μL containing 1 × 10⁶ HeLa cells into the right shoulder. The interval between the first and the final inoculation into the mice was less than 1 h.

When the mean diameter of the tumors reached 5 mm (5 days after inoculation), the mice were separated at random into three groups (n = 8 per group): the NS group, receiving 100 μL saline; the control-shRNA group, receiving 10 μg of the HK plasmid and 50 μg liposome complexes (final volume = 100 μL); and the CCDC7-shRNA group, receiving 10 μg of the shRNA plasmid and 50 μg liposome complexes (final volume = 100 μL). Nude mice were treated once daily for 10 days via injections around the tumor.

Tumor size was monitored by measuring the largest diameter and the diameter perpendicular to the largest diameter using a caliper once every 3 days. Tumor volume was calculated according to the following formula: $V = \text{length} \times \text{width}^2 \times 0.52$.[19] All of the data are represented as means ± standard errors of the means (SEMs). After 25 days of treatment, the mice were sacrificed, and tumors were isolated and fixed in 4% neutral formalin for histological analysis.

Statistical Analysis

The significance of differences between samples was determined using Student's *t*-test or one-way analysis of variance (ANOVA) implemented in GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Data are presented as means ± SEMs. Each experiment was performed with triplicate samples, and all experiments were repeated at least twice. Statistical significance was defined as $P < 0.05$.

Results

CCDC7 Expression in Cervical Tissues

In order to study the clinical significance of CCDC7 in cervical cancer tissue, we performed immunohistochemical analysis of CCDC7 expression in 193 cases of cervical tissue in a microarray (Table 1). The results showed that CCDC7 expression was significantly higher in cancer tissues than in normal cervical tissues. Indeed, while CCDC7 expression in normal cervical tissues was negative or weakly positive (Figure 1A), significantly increased expression was observed with increasing grade and pathological stage of cervical cancer(Figure 1B and C). Thus, CCDC7 may be involved in regulating the growth of cervical cancer.

Overexpression of CCDC7 Promoted the Proliferation of HeLa Cells in vitro

To investigate the function of CCDC7 in cervical cancer cells, we transfected HeLa cells with *CCDC7*. As shown using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), the expression of *CCDC7* increased eight-fold when compared with that in the control cells after 24, 36, and 48 h of growth (Figure S1A). To study the effects of CCDC7 on proliferation in HeLa cells, we used EdU assays to measure cell numbers and found a 62% increase in growth compared with the control (Figure 2A and B). Importantly, no differences in growth rates were observed between

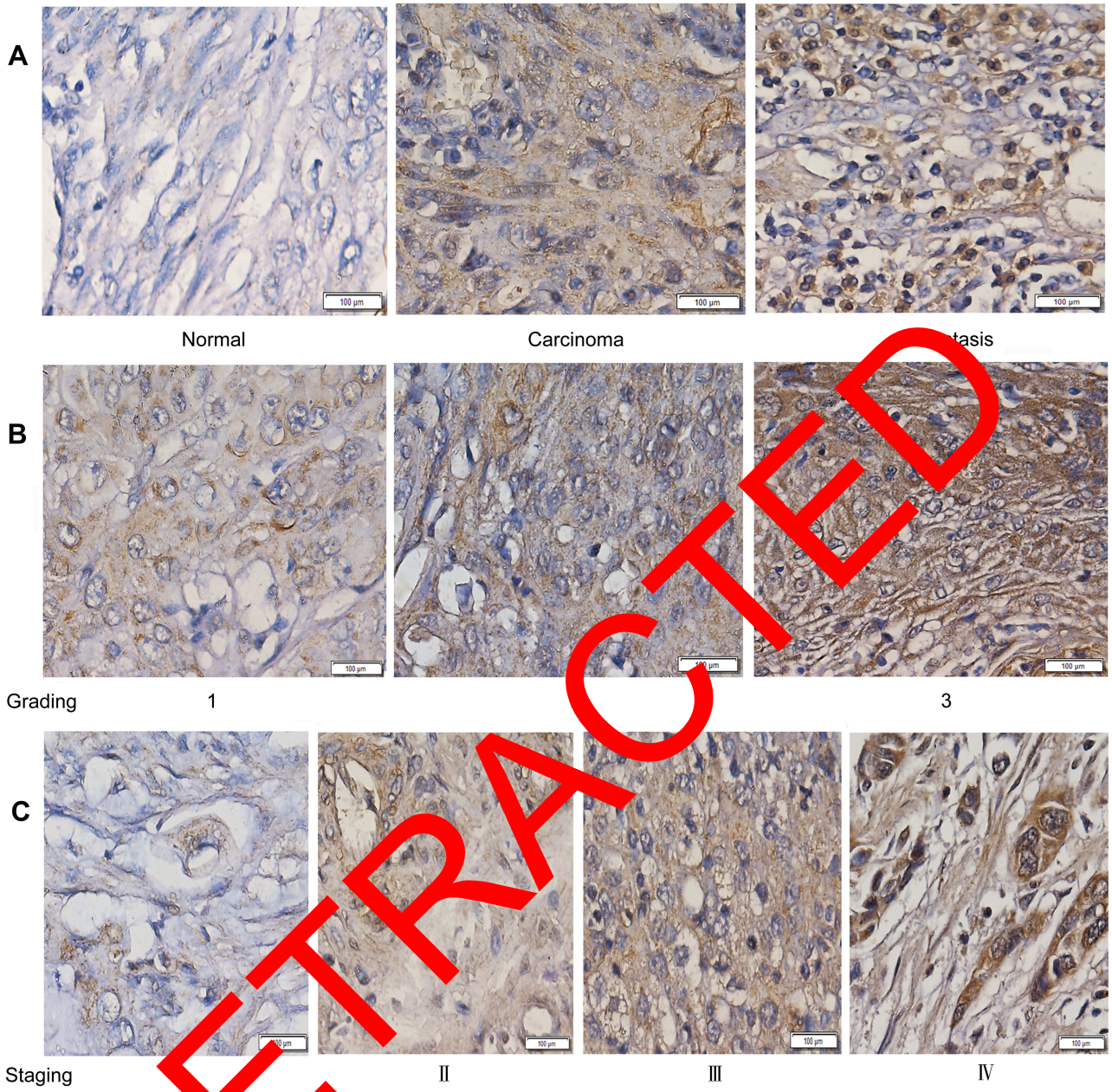

Figure 1 CCDC expression in cervical tissues. (**A**) CCDC7 expression in normal tissues, cervical cancer tissues, and metastatic cancer tissues. (**B**) CCDC7 expression in cervical cancer tissues of different types and classifications. (**C**) CCDC7 expression in cervical cancer tissues of different stages.

the control group and the vector-transfected cells ($P > 0.05$). Moreover, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays indicated that transfection with CCDC7 increased the proliferation of HeLa cells by 55% compared to that of the control (Figure 2C). Additionally, the expression of PCNA protein, a marker of proliferation, was increased following transfection with CCDC7 (Figure 2D). Thus, taken together, these results indicated that CCDC7 expression promoted the proliferation of HeLa cells.

Knockdown of CCDC7 Suppressed HeLa Cell Proliferation in Vitro and Vivo

Next, we analyzed the effects of CCDC7 knockdown via siRNA on HeLa cell proliferation using MTT and EdU assays. As shown in Figure 3, we found that cell proliferation was inhibited in a time-dependent manner in HeLa cells transfected with siRNA for 24–72 h, as compared with the negative control group (Figure S1B).The EdU assay detected that the cell proliferation rate decreased to 55.27% (Figure 3A and B) and the inhibition rate was

Table 1. Relationship Between CCDC7 Expression and Indicators of Cervical Cancer Clinical Pathology

Factors	Number of Patients	Average Score	*p* value
Group			0.001
Normal	29	3.17 ± 0.33	
Adenocarcinoma	156	6.48 ± 0.25	
Metastatic	8	9.50 ± 0.98	
Age			0.6460
<60 years	91	7.46 ± 0.63	
≥60 years	78	7.15 ± 0.35	
Differentiation			0.1467
Well	30	5.46 ± 0.68	
Moderate	72	7.15 ± 0.35	
Poor	25	8.24 ± 0.53	0.01
Clinical stage			
I	45	5.53 ± 0.50	
II	75	6.22 ± 0.32	
III	29	8.10 ± 0.58	0.001
IV	7	8.71 ± 1.04	
T			
T1	65	5.43 ± 0.43	
T2	55	6.27 ± 0.40	
T3	3	9.00 ± 1.73	0.04
T4	2	10.50 ± 1.50	
N			0.01
N0	103	5.21 ± 0.31	
N1	23	7.30 ± 0.63	
M			[illegible]
M0	121	5.3[illegible] 0.30	
M1	5	[illegible] 0.97	

Note: Clinical and TNM stagings were according to Ame[illegible]n Joint Committee on Cancer: AJCC Cancer Staging Manual[illegible]h edition.

reduced from 64.20% to [illegible]3% (Figure 3C). To further assess the function of CCDC7, we also examined the expression of PCNA. As expected, PCNA expression decreased following transfection with siRNA for CCDC7 (Figure 3D). These findings suggested that knockdown of CCDC7 inhibited the growth of HeLa cells in vitro.

Because of these dramatic effects of CCDC7 knockdown in cells, we next used a mouse xenograft model to investigate the effects of CCDC7 inhibition in tumors. Mice bearing HeLa-derived tumors were treated daily with normal saline, Lip-Control-shRNA, or Lip-CCDC7-shRNA, and tumor size was analyzed. Importantly, tumors in the CCDC7-shRNA group were significantly smaller than those in the control group after 2 (Figure 4A) and 10 (Figure 4B) days of treatment. Moreover, animals treated with Lip-CCDC7-shRNA showed a significant decrease in the average tumor volume when compared with the controls ($p < 0.05$; Figure 4C). Tumor wet weights were also reduced after 30 days (0.76 ± 0.041 and 0.64 ± 0.031 g in the control and vector groups, respectively, versus 0.34 ± 0.025 g in the CCDC7-sh group; Figure 4D; $p < 0.0001$). Thus, these data supported that knockdown of CCDC7 by RNA interference suppressed the proliferation and oncogenic potential of cervical cancer cells and decreased tumor formation in nude mouse xenografts.

IL-6 and VEGF Were Up[illegible]egulated Following Overexp[illegible]ression of CCDC7

Next, because IL-6 and VEGF are important cytokines involved in a variety of cancer-related phenotypes, we examined the effects of CCDC7 overexpression on IL-6 and VEGF in HeLa cells by qRT-PCR and enzyme-linked immunosorbent assay (ELISA). As shown in Figure 5, when compared with the negative control, CCDC7 transfection increased the expression of IL-6 and VEGF mRNA and protein. Specifically, *IL-6* mRNA expression was increased by 73.86%, 80.85%, and 71.11% (Figure 5A.a), and IL-6 protein expression was increased by 22.9[illegible], 49.01%, and 48.73% (Figure 5A.b) in CCDC7-transfected cells at 24, 36, and 48 h after transfection, respectively. VEGF mRNA expression was increased by 57.83%, 66.50%, and 40.38% (Figure 5A.c), while VEGF protein expression was increased by 35.1%, 47.78%, and 71.37% (Figure 5A.d) in CCDC7-transfected cells at 24, 36, and 48 h after transfection, respectively. These data indicated that IL-6, VEGF, and CCDC7 were all achieving the highest expression after CCDC7 transfection 48 h, moreover, they had the same consistency in dynamic expression.

CCDC7 RNAi Significantly Inhibited IL-6 and VEGF Expression in HeLa Cells

Next, we examined whether the opposite effect could be observed, ie, whether knockdown of CCDC7 resulted in decreased expression of IL-6 and VEGF. As shown in Figure 5, when compared with the negative control group, CCDC7 siRNA transfection decreased the expression of IL-6 and VEGF mRNA and protein. Specifically, *IL-6* mRNA expression was reduced by 82%, 90%, and 97% (Figure 5B.a), while IL-6 protein expression was reduced by 42%, 55%, and 65%

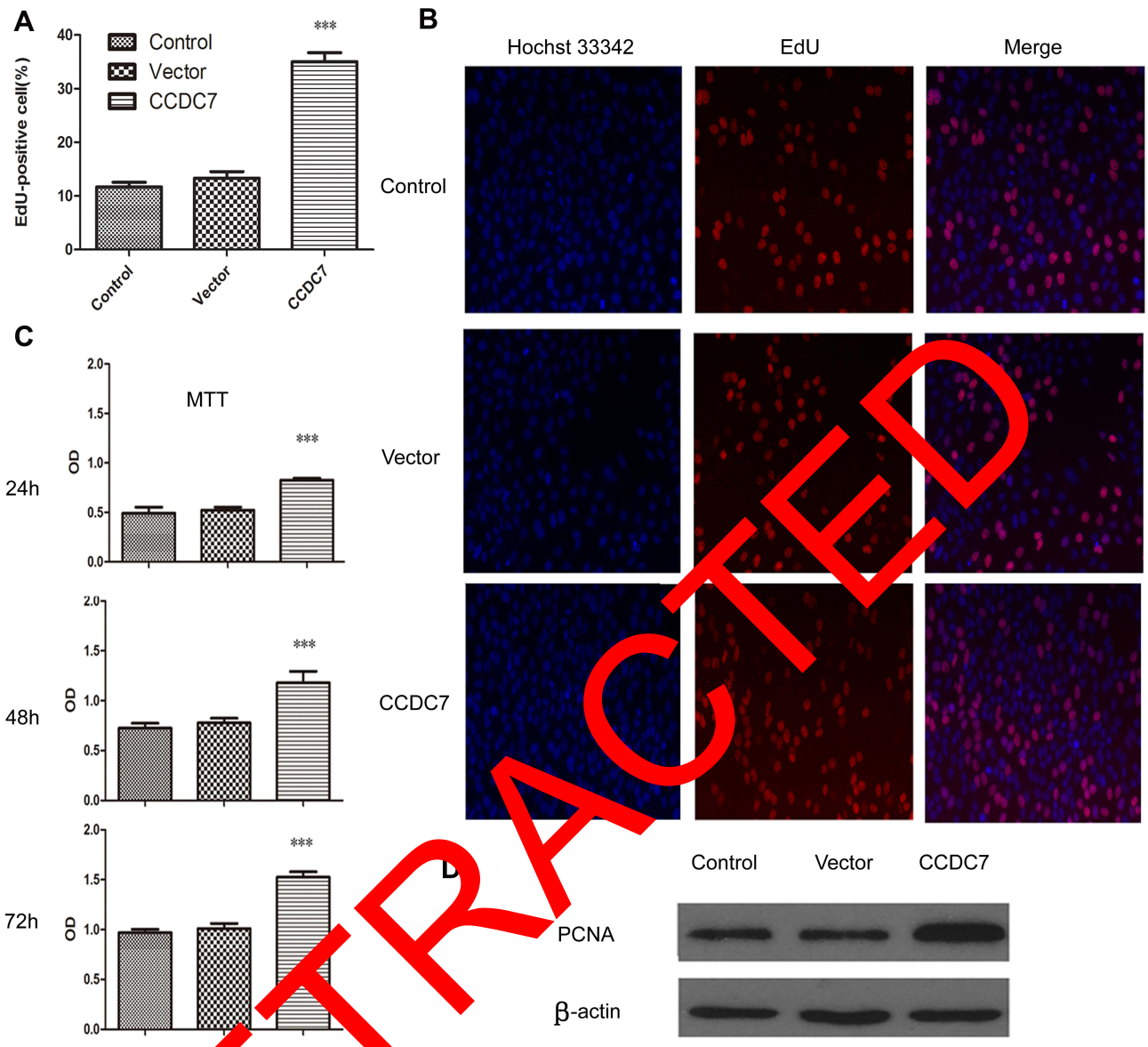

Figure 2 Overexpression of the *CCDC7* gene promoted HeLa cell proliferation in vitro. (**A**) EdU-positive cells. Each bar represents the mean ± SD of three independent experiments. ***$p < 0.00$ compared with control group cells. (**B**) HeLa cells were transfected with CCDC7, and cell viability was measured 24 h later using EdU assays. Newly grown HeLa cells were marked by combined Edu and Hoechst 33258 staining. EdU stained cells (red) were observed under a fluorescence microscope (200×), and cells stained with Hoechst 332 (blue) were counted as a proliferation control (%)(**C**) MTT assays were used to determine cell proliferation in HeLa cells transfected with CCDC7. Left, plating cell count 000 (HeLa cells were transfected with CCDC7 for 48 h, and Western blotting was then used to determine the levels of PCNA. Equal amou of total cellular protei μg) were resolved by 12% SDS-PAGE. β-Actin was used as loading control.

(Figure 5B.b) in CCDC7 siRNA-transfected cells at 24, 36, and 48 h after transfection, respectively. *VEGF* mRNA expression was reduced by 83%, 89%, and 92% (Figure 5B.c), while VEGF protein expression was reduced by 54%, 75%, and 78% (Figure 5B.d) in CCDC7 siRNA-transfected cells at 24, 36, and 48 h after transfection, respectively. These data indicated that knockdown *CCDC7* can downregulate IL-6 and VEGF. They also had the same consistency in dynamic expression.

Treatment with Recombinant IL-6 or VEGF Protein Enhanced CCDC7 Expression

IL-6 has been reported to promote VEGF-dependent angiogenesis in human cancer cells.[20] However, little is known about the relationships among IL-6 and VEGF expression levels in cervical cancer. Therefore, we next examined the expression of CCDC7, IL-6, and VEGF after treatment of cells with recombinant VEGF proteins and IL-6 proteins

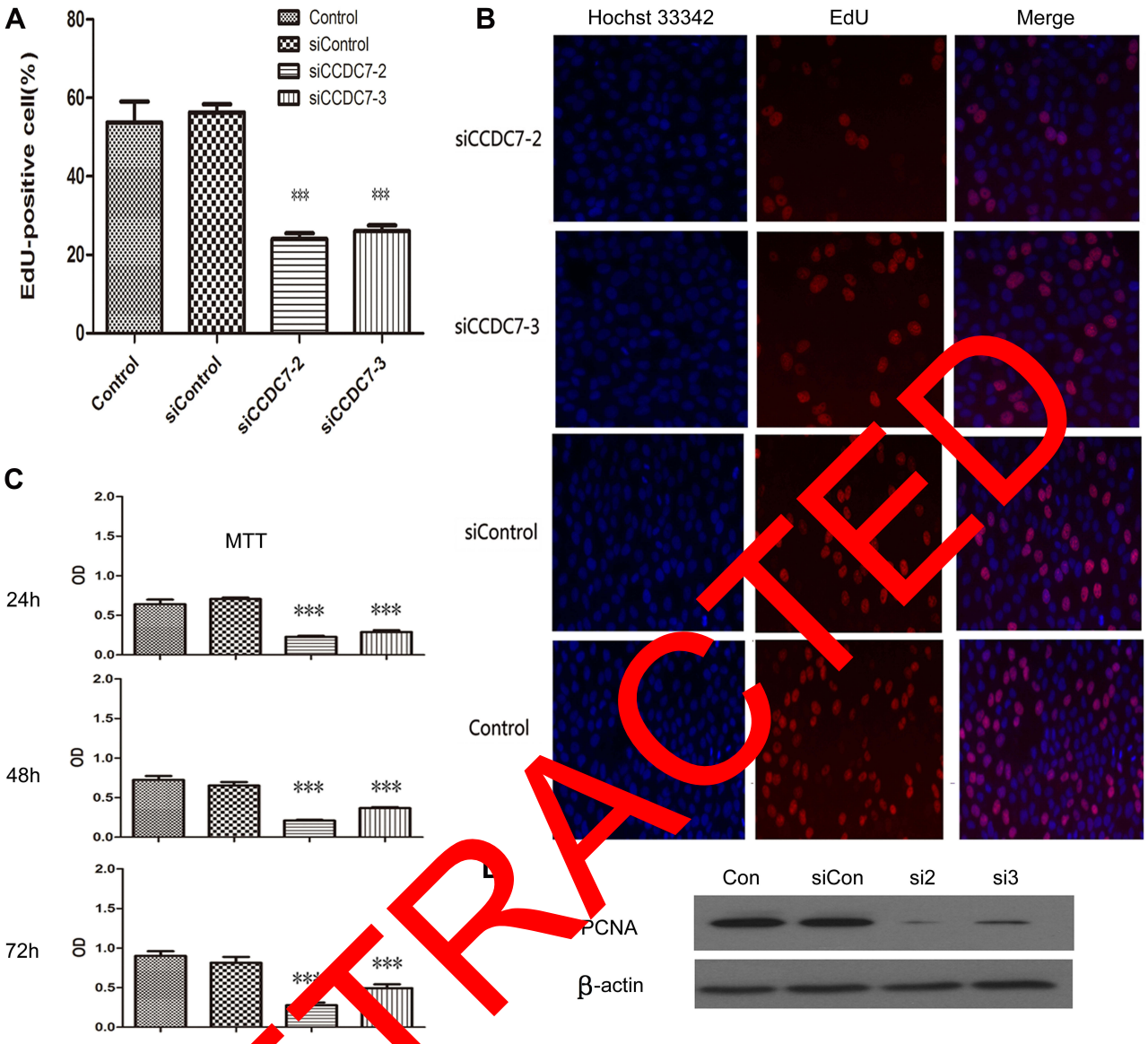

Figure 3 Knockdown of CCDC7 suppressed HeLa cell proliferation in vitro. (**A**) EdU-positive cells. Each bar represents the mean ± SD of three independent experiments. ***$p < 0.0001$, compared with control group cells. (**B**) At 24 h after transfection with siCCDC7, cell viability was estimated by EdU assay. Newly grown HeLa cells were stained by combined EdU and Hoechst 33258 staining. EdU stained cells (red) were observed under a fluorescence microscope (200×), and cells stained with Hoechst 33258 (blue) were counted as a proliferation control (%) (**C**) MTT assays were used to determine cell proliferation at 24 h after transfection with CCDC7. Left, starting cell counts. (**D**) HeLa cells were transfected with CCDC7 for 48 h, and Western blotting was then used to determine the levels of PCNA. Equal amounts of total cellular protein (50 μg) were resolved by 12% SDS-PAGE. β-Actin was used as loading control.

respectively. The results showed that VEGF proteins treatment increased the expression of *CCDC7* mRNA in a concentration-dependent manner, as shown in Figure 6A. On the other hand, stimulation with VEGF also upregulated IL-6 mRNA and protein expression in the Hela cells (Figure 6B, C). Moreover, that IL-6 proteins treatment increased the expression of *CCDC7* mRNA in a concentration-dependent manner, as shown in Figure 6D. Interestingly, stimulation with IL-6 also increased the expression of VEGF mRNA and protein in the Hela cells (Figure 6E, F). These data suggested that IL-6 promoted VEGF and CCDC7 expression, while VEGF also promoted IL-6 and CCDC7 expression in HeLa cells.

Expression of CCDC7, IL-6, and VEGF in Xenograft Tumors and Human Cervical Cancer Samples

The expression levels of CCDC7, IL-6, and VEGF were next analyzed in xenograft tumors from our nude mouse model and in human cervical cancer samples. Tumor sections from the

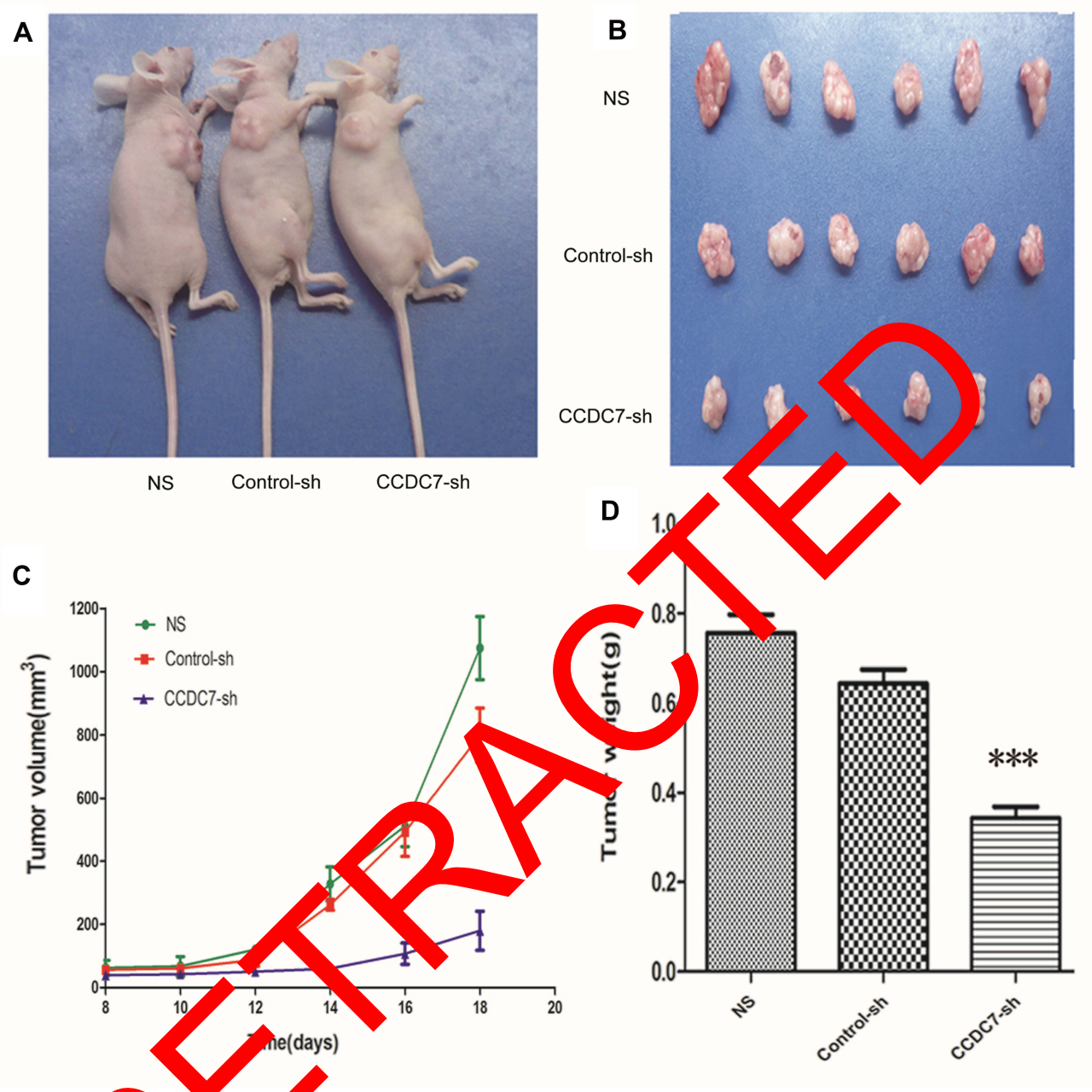

Figure 4 Kn[illegible]down o[illegible]CDC7 su[illegible]d HeLa cell proliferation in vivo. Nude BALB/c mice were transplanted with cervical cancer tumors as described in the Materials and Meth[illegible]. Mice wer[illegible]hen treated [illegible]ith sh-CCDC7 for 10 days. Representative images shown in (**A**) is treated with sh-CCDC7 2 days. (**B**) Tumor sizes in different groups of h[illegible] (**C**) G[illegible]owth. Mice were treated with normal saline (NS), control-shRNA, or CCDC7-shRNA, and tumors were measured as described in the Materials and [illegible]ds. Tumor volumes are shown as the means ± standard errors (SEs). (**D**) Tumor weights. Mice were sacrificed, and tumors were collected and weighed immediately. Dat[illegible]ow the means ± SEs; ***$p < 0.0001$, compared with the control groups.

CCDC7-shRNA group displayed significantly decreased of positive cells when compared with the NS and control-sh treatment groups ($P < 0.05$; Figure 7A). Moreover, Western blotting analysis revealed that the expression of CCDC7, IL-6, VEGF, and PCNA were significantly reduced in the CCDC7-sh group compared with the NS group (Figure 7B). To further confirm the expression patterns of CCDC7, IL-6, and VEGF in cervical cancer, we used two clinical cervical cancer samples (Figure 7C) of different clinical stages with the corresponding pericarcinoma tissues. The left one was stage 1, and the right one was stage 3. The results were similar to those observed in tumors from our xenograft model. Therefore, these data suggested that CCDC7 was clinically relevant in the context of cervical cancer.

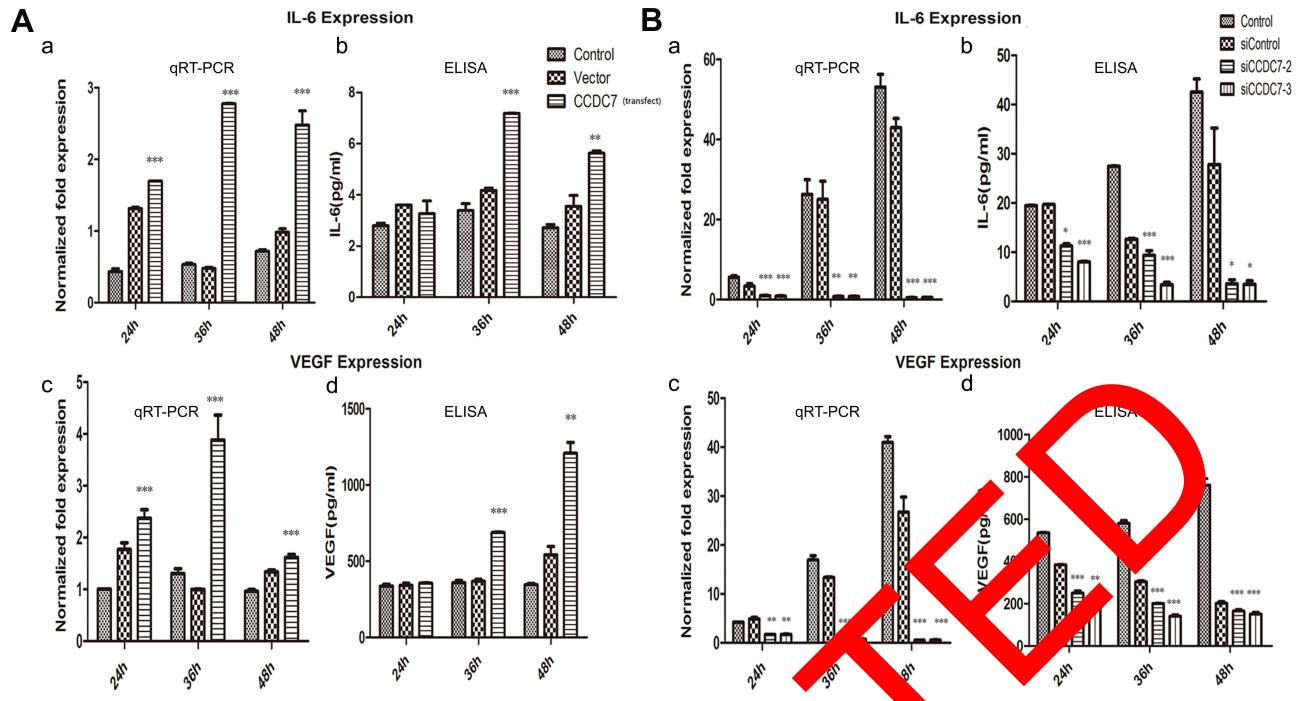

Figure 5 IL-6 and VEGF were upregulated following overexpression of CCDC7 and CCDC7 RNA significantly inhibited IL-6 and VEGF expression in HeLa Cells. (**A.a,c**) qRT-PCR analysis of *IL-6* and *VEGF* mRNA levels in HeLa cells transfected with CCDC7. (**A.b,d**) ELISA of IL-6 and VEGF levels in the supernatants of HeLa cells transfected with CCDC7. (**B,a,c**) qRT-PCR analysis of *IL-6* and *VEGF* mRNA levels in cells transfected with siCCDC7. (B,b,d) ELISA of *IL-6* and *VEGF* levels in cells transfected with siCCDC7. Data are means ± SDs from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ compared with the control and vector groups.

Expression of CCDC7, VEGF, and IL-6 Was Associated with Tumor Growth

Next, we analyzed the roles of CCDC7, IL-6 and VEGF in cervical cancer using three tissue microarrays of cervical cancer specimens. Statistical analysis indicated that CCDC7, IL-6, and VEGF expression levels were significantly lower in normal cervical tissue than in squamous carcinoma tissues ($P < 0.01$; Tables S1–3). Significant differences were observed between well, moderately, and poorly differentiated carcinoma tissues; different clinical stages showed also significant differences in expression (Tables S1–3). As shown in Figure 8, levels of CCDC7, IL-6, and VEGF were also significantly different between squamous carcinoma tissues classified as stages I, II, and III, as compared with noncancerous tissues, which suggesting that the expression of CCDC7, IL-6, and VEGF was altered with tumor growth. This further supported that tumor growth was associated with CCDC7 expression in cervical carcinoma, Moreover, CCDC7, IL-6, and VEGF had the same trend in different pathological grading and clinical staging. So there may be a mechanism among the three molecules, which effect the occurrence and development of cervical cancer.

CCDC7 Regulated IL-6 and VEGF Though JAK-STAT3 Pathway

Next, we sought to examine the mechanisms through which CCDC7 may affect cervical cancer growth using an array of signaling pathway inhibitors. In our analysis, the phosphoinositol 3 kinase (PI3K), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), MAP kinase kinase (MEK1/2), and JAK pathways were considered to have the effect of promoting cell proliferation. We found that AG490, which was the inhibition of JAK pathways was linked to the effect of CCDC7 on cell proliferation. We used MTT assay (Figure S3A) and EdU assay (Figure S2 and S3B) to measure the proliferation of HeLa cells. Compared with control groups, the cell number of the JAK group decreased to 53%, at the same time, the other pathways did not effect the proliferation of HeLa cells. After the treatment of AG490 in HeLa cells, we transfect vector and CCDC7 respectively, 24 h later, the number of cells increased rapidly again (Figure 9A). Thus, these data suggested that CCDC7-dependent HeLa cell proliferation may involve the JAK pathway.

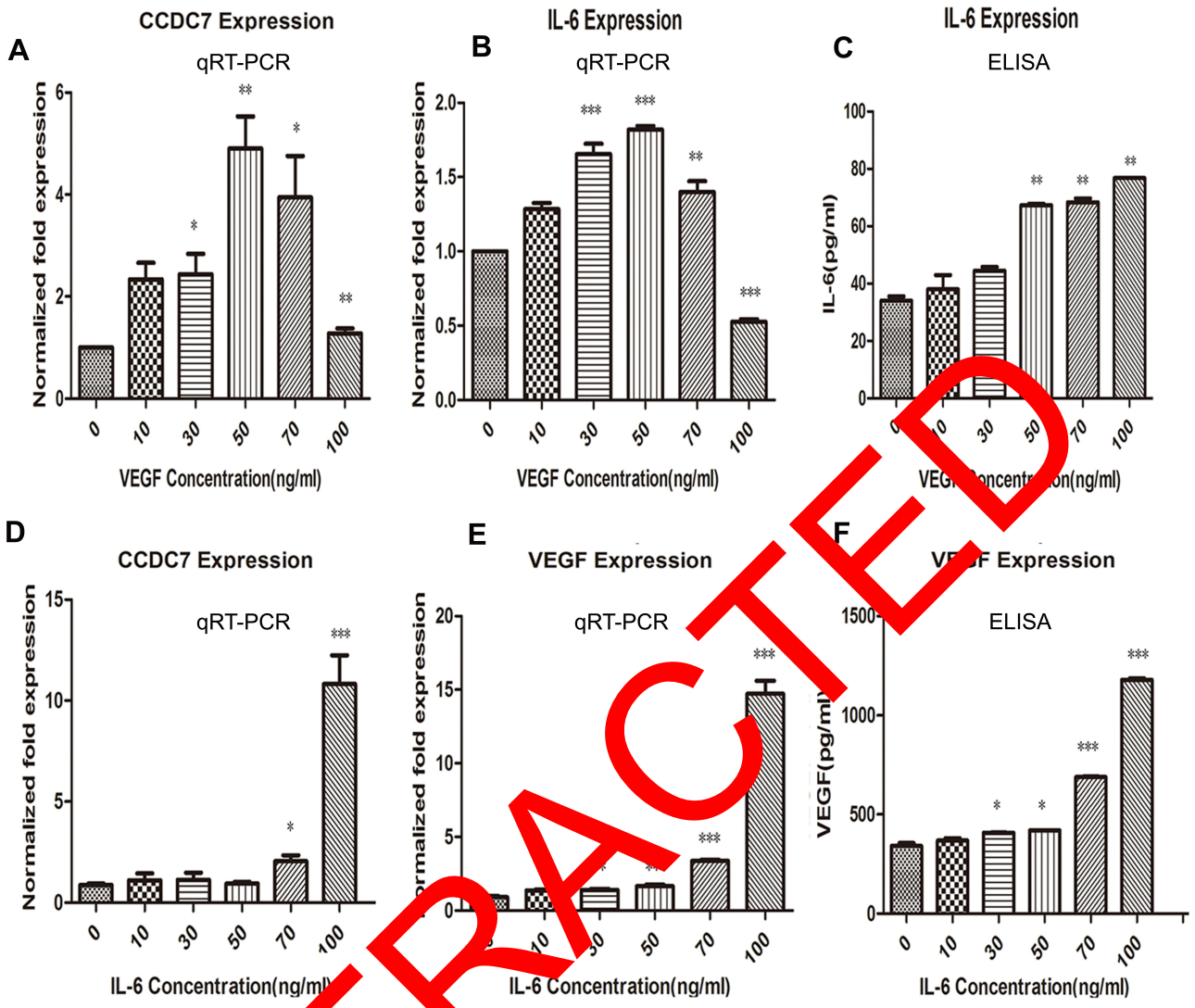

Figure 6 Treatment with recombinant IL-6 or VEGF protein enhanced CCDC7 expression. (**A,B,D,E**) qRT-PCR analysis *IL-6*, *VEGF*, and *CCDC7* mRNA levels in HeLa cells treated with recombinant VEGF or IL-6. (**C,F**) ELISA of IL-6 and VEGF levels in the supernatants of HeLa cells treated recombinant VEGF or IL-6.Data are means ± SDs from three independent experiments. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$ compared with the control and vector groups.

JAK-STAT3 activation has been reported to regulate the expression of a variety of important genes.[21,22] Therefore, we next examined whether JAK-STAT3 was involved in cells transfected with CCDC7 or CCDC7 siRNA by measuring the phosphorylation of JAK and STAT3. JAK activation, as indicated by phosphorylation at the activation loop Tyr1007/1008 and STAT3 activation at the activation loop Tyr705. When transfected with CCDC7, P-JAK and P-STAT3 was increased, while P-JAK and P-STAT3 was reduced in cells transfected with CCDC7 siRNA as compared with control cells (Figure 9B).

Discussion

Cervical cancer is the third leading cause of cancer-related death in women worldwide;[23] hence, a number of prognostic and predictive indicators are used to guide therapy in patients with cervical carcinoma. Previous studies have shown that cervical cancer, colon cancer, and endometrial cancer are associated with the mRNA expression of CCDC7 gene family.[8] In this study, we analyzed the role and clinical significance of CCDC7 gene in cervical cancer. Our results demonstrated that CCDC7 expression was associated with the progression of cervical cancer and that CCDC7 regulated cell proliferation via the JAK-STAT3

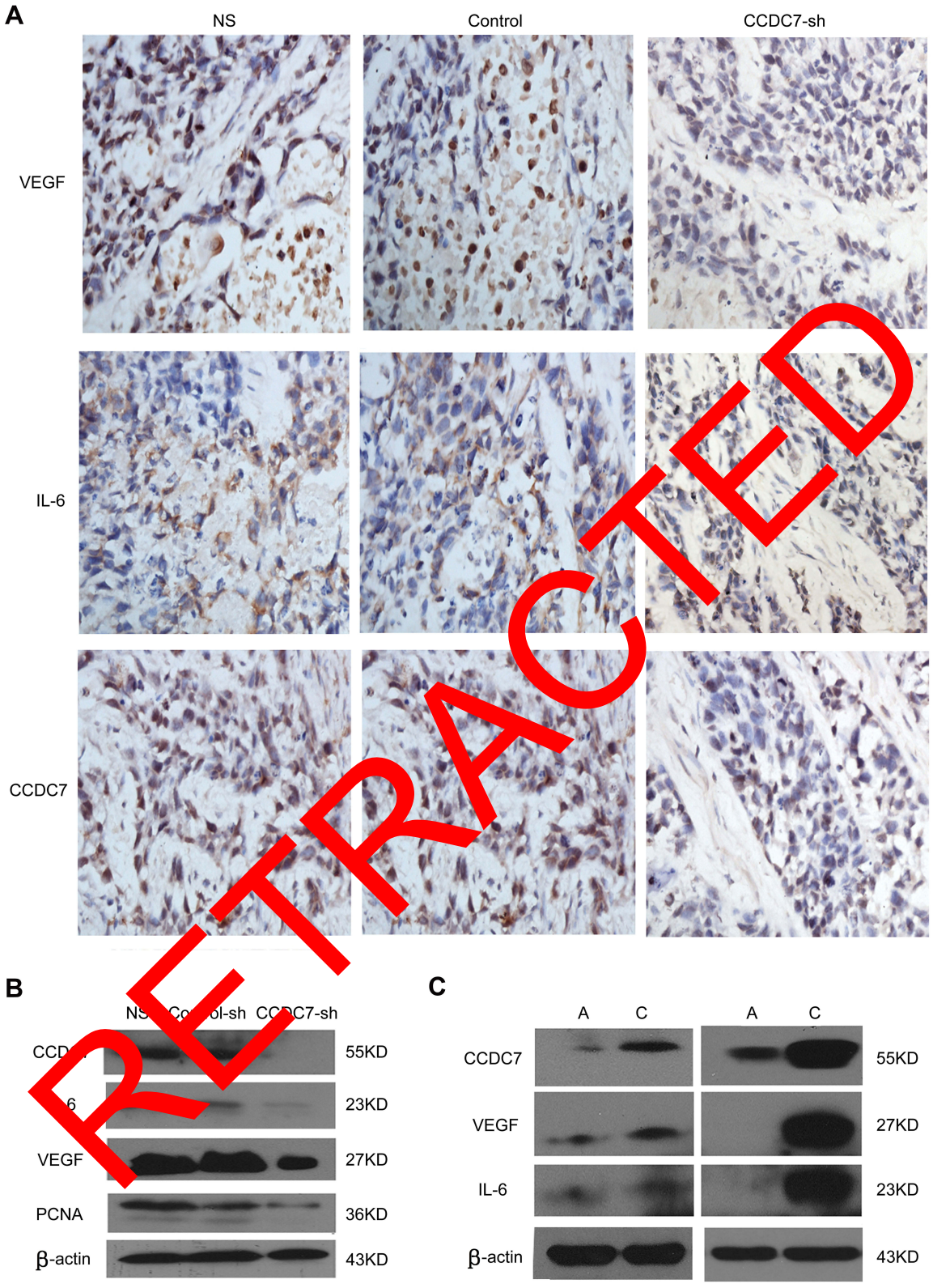

Figure 7 **(A)** The expression levels of CCDC7, IL-6, and VEGF exhibited similar patterns in tumors from nude mice and in human cervical cancer samples. Immunohistochemical staining of CCDC7, IL-6, and VEGF (brown) in paraffin-embedded sections of nude mice's tumors. Typical images were taken under a light microscope (magnification 400×). (**B**) Western blotting was used to analyze the expression of CCDC7, IL-6, VEGF, and PCNA in nude mice with HeLa cell-derived tumors. (**C**) Western blotting was used to analyze the expression of CCDC7, IL-6, and VEGF in human clinical cervical cancer samples,left: stage 1; right: stage 3. β-Actin was used as a loading control.

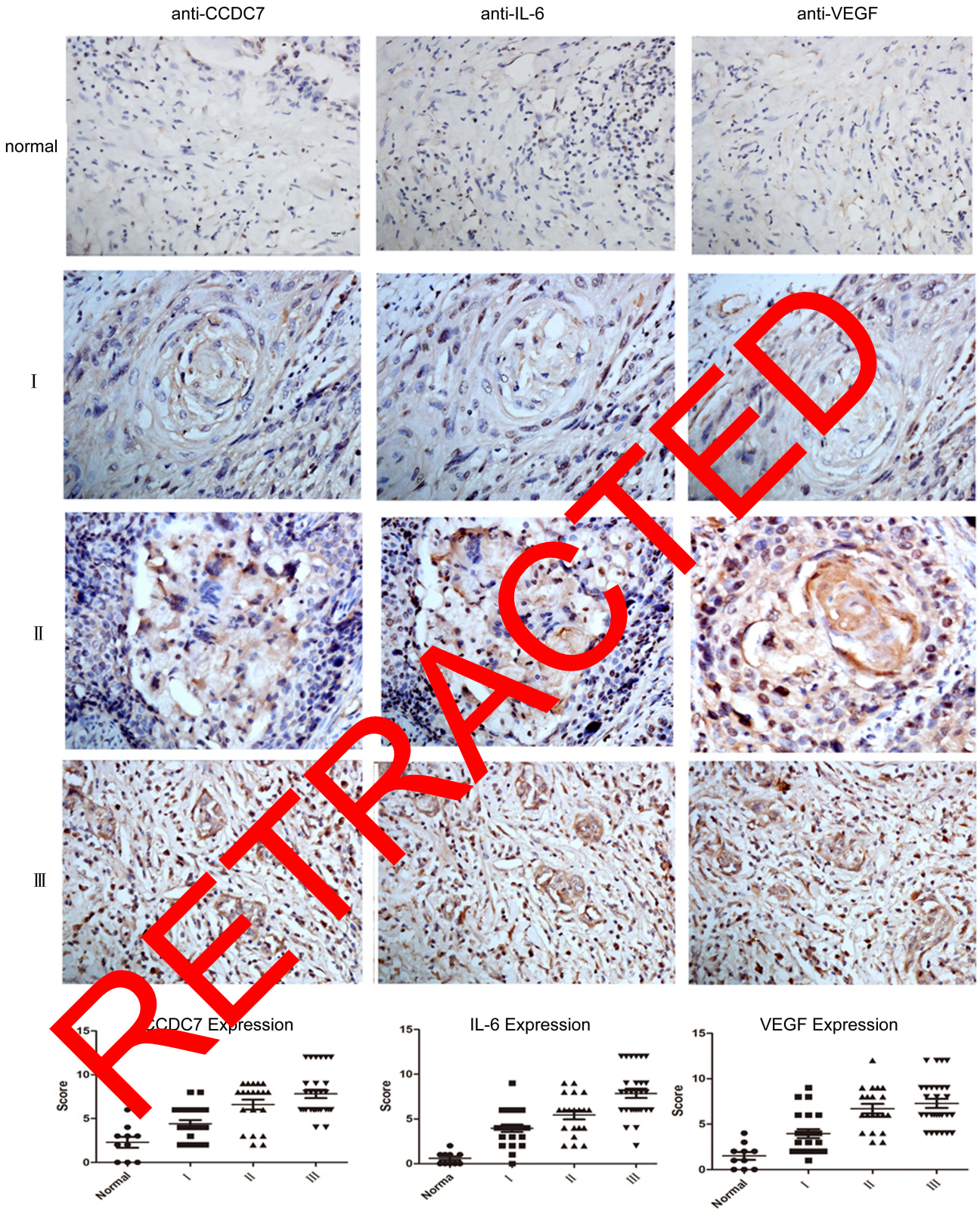

Figure 8 Expression of CCDC7, VEGF, and IL-6 was associated with tumor growth. (Upper) CCDC7, IL-6, and VEGF expression in normal cervical tissues and squamous carcinoma tissues. CCDC7 (left), IL-6 (middle), and VEGF (right) expression was investigated in the same three tissue microarrays of clinical cervical cancer samples. (Lower) The immunohistochemical staining score was analyzed using GraphPad Prism 5.0.

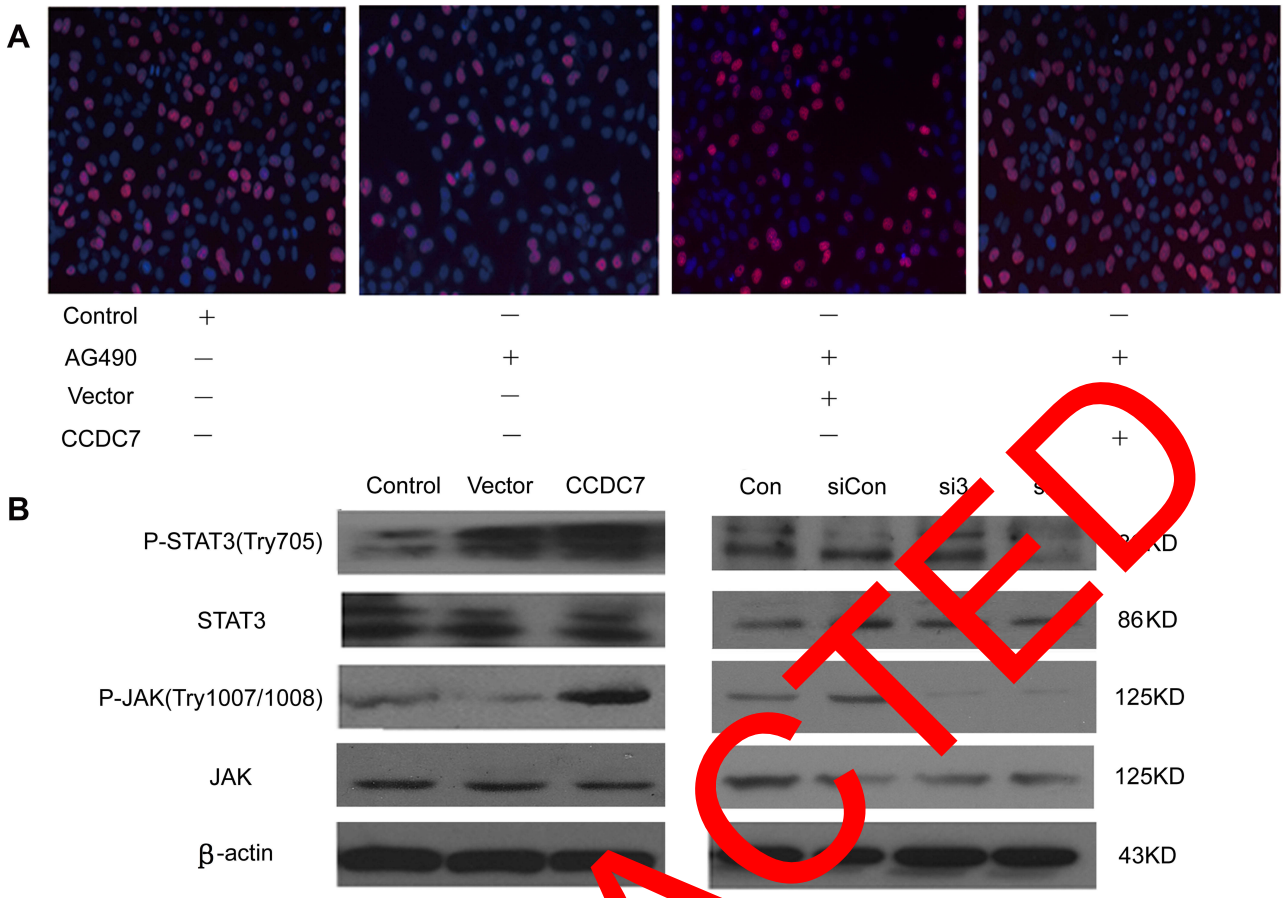

Figure 9 CCDC7 regulated IL-6 and VEGF though the JAK-STAT3 pathway. Cells were treated with inhibitors of PI3K, MAPK, JNK, MEK1/2, and JAK pathways (MAPK: 1, SB20538; MEK1/2: 2, U0126; PI3K: 3, LY29402 and 4, wortmannin; JAK: AG490; and JNK: 6, SP600125). (**A**) Inhibitors were added, and cells were transfected 24 h later with CCDC7 or vector. EdU assays were then performed to analyze cell viability. Newly grown HeLa cells were stained by combined EdU and Hoechst 33258 staining. EdU stained cells (red) were observed under a fluorescence microscope (200×) and cells stained with Hoechst 33258 (blue) were counted as a proliferation control (%). (**B**) HeLa cells were transfected with CCDC7 for 48 h, and Western blotting was used to determine the levels of STAT3, P-STAT3, JAK, and P-JAK in overexpression of CCDC7 (left) and knockdown of CCDC7 (right). Equal amounts of total cellular protein (50 μg) were resolved by 12% SDS-PAGE. β-Actin was used as loading control.

pathway and the expression of IL-6 and VEGF cytokines. These data provide important insights into the role of CCDC7 in cervical cancer.

The proliferative capacity of tumor cells plays an important role in invasiveness and metastasis of specific cancers.[2] In this study, we found that the expression of CCDC7 was closely associated with the proliferation of HeLa cervical cancer cells. Indeed, when we manipulated the expression of CCDC7 in HeLa cells, including in vitro and in vivo experiment, we observed that overexpression of CCDC7 promoted cell proliferation while knockdown of CCDC7 expression inhibited cell proliferation. In the model of cervical cancer in nude mice, we observed that knockdown of CCDC7 expression can therapy the mice tumor and inhibit tumor growth. Thus, CCDC7 was shown to be a potent oncogenic protein and a new therapeutic target in cervical cancer.

IL-6 is a pleiotropic inflammatory cytokine that induces the growth and differentiation of immune cells as well as the expression of many cytokines. IL-6 is also a representative marker of clinical correlation and a common prognostic factor in patients with cancer.[25] Various studies have suggested that IL-6 is important in cervical carcinogenesis because it is a central pro-inflammatory cytokine involved in female genital infection and is abundant in the microenvironment of cervical cancer.[26] Moreover, the association of IL-6 with angiogenesis has been reported to depend on its ability to induce the production of VEGF, a potent angiogenic agent.[20] In gastric carcinoma, IL-6 induces VEGF expression by increasing angiogenesis and therefore may be a marker of tumor angiogenesis and disease status.[27,28] Furthermore, recent studies have revealed that IL-6 and VEGF can increase tumor growth capacity and are targets

for the diagnosis, treatment, and prognosis of tumors. Consistent with this, in our study, we found that CCDC7 expression was positively correlated with IL-6 and VEGF expression; overexpression of CCDC7 upregulated IL-6 and VEGF, while knockdown of CCDC7 downregulated these two cytokines. Moreover, addition of recombinant IL-6 or VEGF also caused CCDC7 expression to increase. Consistent patterns of expression were also observed in tumors from nude mice and in clinical specimens of cervical cancer. Thus, our data demonstrated that these three molecules were interconnected in promoting the progression of cervical cancer. However, it is not clear whether these molecules interact directly; further studies are required to investigate these effects.

IL-6 has been reported to regulate the JAK/STAT, Ras/MAPK, and PI3K/Akt pathways.[29] Inappropriate activation of JAK/STAT signaling occurs with high frequency in human cancers and is associated with cancer cell survival and proliferation. STAT3 regulates fundamental biological processes, including cell proliferation, survival, and development. Recently, accumulating evidence has indicated that abnormalities in the JAK/STAT signaling pathway are involved in the oncogenesis of several cancers.[30] STAT proteins are transcription factors that participate in normal cellular events, such as proliferation, apoptosis, and angiogenesis, and STAT3 has been shown to be required in many aspects of tumorigenesis.[31] In our study, we showed that specifically blocking the JAK/STAT pathway by AG490 could reduce HeLa cell proliferation, and this effect was abrogated by expression of CCDC7. These data confirmed that the effects of CCDC7 on HeLa cell proliferation were regulated though the JAK-STAT pathway. Moreover, as STAT3 phosphorylation and nuclear translocation are required for STAT3 transcriptional activity, we also analyzed the phosphorylation of JAK2 and STAT3 in HeLa cells overexpressing CCDC7. Importantly, overexpression CCDC7 promoted phosphorylation of these targets, while CCDC7 knockdown significantly suppressed phosphorylation of these targets, thereby supporting that CCDC7 was involved in activation of the JAK-STAT3 pathway.

Conclusion

In conclusion, we demonstrated, for the first time, that CCDC7 played an important role in the regulation of cervical cancer proliferation through modulation of IL-6 and VEGF expression. Interestingly, IL-6 and VEGF also mediated CCDC7 expression. In the model of cervical cancer in nude mice, the CCDC7-shRNA therapeutic experiments confirmed that the interference of endogenous CCDC7 could inhibit the cell proliferation and tumor growth, which had the effect of therapeutic of cervical cancer. These findings have clinical applications for the treatment and diagnosis of patients with cervical cancer and suggest that CCDC7 may be an important therapeutic target in cervical cancer. Additionally, CCDC7 was identified as an oncogenic protein in cervical cancer and maybe function via the JAK-STAT3 pathway. However, further research is still required to determine the precise mechanisms through which CCDC7 and IL-6/VEGF interact.

Abbreviations

CCDC7, coiled coil domain containing 7; IL, interleukin; VEGF, vascular endothelial growth factor; JAK, Janus kinase, STAT, signal transducer and activator of transcription; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositol 3 kinase; MEK1/2, MAP kinase kinase; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide; DAB, diaminobenzidine; DMSO, dimethylsulfoxide; HRP, horseradish peroxidase; ANOVA, analysis of variance.

Ethical Statement

All animal experiments were carried out in accordance with the regulations of the People's Republic of China on the administration of experimental animals and performed according to protocols approved by the Animal Care and Use Committee of Sichuan University. The experiments on humans or human tissues were undertaken with the understanding and written informed consent from the patient donors. The study methodologies were approved by the Sichuan University ethics committee.

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Disclosure

We certify that regarding this paper, no actual or potential conflicts of interests exist; the authors declare that they have no conflicts of interest associated with the publication of the present work.

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