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Regulatory effects of CCDC3 on proliferation, migration, invasion and EMT of human cervical cancer cells

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Abstract. – OBJECTIVE: To elucidate the potential effects of Coiled coil domain-containing 3 (CCDC3) on proliferative, migratory, invasive potentials and epithelial-mesenchymal transition (EMT) of human cervical cancer cells.

MATERIALS AND METHODS: Protein and mRNA levels of CCDC3 in C33 and HeLa cells were determined by quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. Proliferative capacity and clonality of C33 and HeLa cells transfected with sh-CCDC3 were evaluated by cell counting kit-8 (CCK-8) and colony formation assay, respectively. Transwell assay and wound healing assay were conducted to determine the invasive and migratory potentials of cervical cancer cells with CCDC3 knockdown. Protein expressions of EMT-related genes in C33 and HeLa cells with CCDC3 knockdown were determined by Western blot.

RESULTS: Transfection of sh-CCDC3 in C33 and HeLa cells markedly inhibited CCDC3 expression compared with those transfected with sh-EGFP. CCDC3 knockdown remarkably attenuated proliferative, migratory and invasive capacities. Moreover, CCDC3 knockdown inhibited protein levels of EMT-related genes in C33 and HeLa cells.

CONCLUSIONS: Low expression of CCDC3 attenuated proliferative, migratory, invasive potentials and EMT of cervical cancer cells. Hence, CCDC3 may be utilized as a novel therapeutic target for cervical cancer.

Key Words:
Cervical cancer, Proliferation, Invasion, Migration, CCDC3, EMT.

Introduction

The incidence of cervical cancer (CC) ranks second among female malignancies worldwide. It is estimated that there are approximately 529,000 new cases of CC each year, and 275,000 women die from CC throughout the world[1]. CC is also the most common malignancy threatening the health of female reproductive system in China. Lymph node metastasis, organ metastasis and tumor recurrence, are the major reasons for CC-induced tumor death. It is urgent to reveal the metastatic mechanism of CC, so as to improve the clinical outcomes and prognosis of CC patients. Coiled coil domain containing (CCDC) proteins and their epigenetic changes are associated with many malignancies. In recent years, several studies have shown that CCDCs are directly related to invasive, migratory and metastatic phenotypes of tumor cells, including CCDC6[2,3], CCDC8[4], CCDC116[5], CCDC19[6], CCDC62[7], CCDC67[8], CCDC68[9], CCDC98[10,11], CCDC134[12] and CCDC152[13]. They participate in gene transcription, cell cycle progression, apoptosis, tumor invasion and many other biological processes. CCDC3 is a newly discovered gene encoding Favine/CCDC3 (NCBI: NP_083080). It serves as a cell-secreting factor, and is highly expressed in adipose tissues and aorta. Liao et al[14] found that CCDC3, as a downstream gene of p63 network, regulates hepatic lipid metabolism by inhibiting hepatic lipogenesis. Kobayashi et al[15] showed the promotive effect of Favine on adipogenesis. Azad et al[16] revealed that CCDC3 inhibits the pro-inflammatory response induced by TNF-α/NF-κB in ECs. Ugi et al[17] pointed out that CCDC3 is highly expressed in visceral adipose tissues of abdominal obesity population. Kobayashi et al[18] have shown that CCDC3 is secreted by adipose cells and endothelial cells, and its expression is influenced by hormone and nutrient levels. So far, it is unclear whether CCDC3 is involved in tumor diseases. We screened out CCDC3 gene through

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analyzing datasets downloaded from Gene Expression Omnibus (GEO) database. It is speculated that CCDC3 may have a potential relationship with the stemeness of tumor cells. Here, we analyzed the molecular mechanism of CCDC3 in the development of CC, and provided references for clinical diagnosis and treatment.

Materials and Methods

Cell Culture

Human cervical cancer cell lines HeLa, SiHa, Caski and C33 were purchased from the CellBank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a 100-mm culture dish, supplied with 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from CC cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Internal reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the target gene CCDC3 were amplified using their cDNAs as a template. QRT-PCR reaction conditions were: pre-denaturation at 95°C for 30 s, followed by 38 cycles at 95°C for 10 s, 56°C for 30 s and 72°C for 10 s. Relative expressions of CCDC3 and GAPDH were calculated as $2^{-\Delta\Delta Ct}$. Primers of CCDC3 and GAPDH were shown: CCDC3, F: 5′-TGACTGGGAAATCCAGGAAGA-3′, R: 5′-CGTGGTCCTCCTCCTCAAAC-3′; GAPDH, F: 5′-TGGGGAAGGTGAAGGTCGG-3′, R: 5′-CTGGAAGATGGTGATGGGA-3′.

Western Blot

Total protein was extracted using the cell lysate for determining protein expression. Protein sample was quantified by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked in 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Plasmid Construction

Plasmid sequences were: sh-CCDC3, F: 5′-CCGGCCTCCGAATCATGAGATATTACTCGAGTAATATCTCATGATTCGGAGGTTTTTG-3′, R: 5′-AATTCAAAAACCTCCGAATCATGAGATATTACTCGAGCCTCCGAATCATGAGATATTA-3′; sh-EGFP, F: 5′-CCGGTACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTATTTTTG-3′, R: 5′-AATTCAAAAATACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTA-3′. Double strands with cohesive terminus were formed by the annealing primers. The pLKO.1-puro vector was double digested with EcoR I and Age I, ligated with double-stranded DNA and transformed into competent cells. The monoclonal antibody was identified by PCR and sequenced. Constructed plasmids were preserved at -20°C.

Transfection

Cells were seeded in 6-well plates with 5×10^5 cells per well. One day prior to transfection, serum-free medium was supplied. 2 μg sh-EGFP or sh-CCDC3 was diluted in 200 μL of serum-free medium. Meanwhile, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was diluted in 200 μL of serum-free medium at a ratio of 1:2.5. After 5 min maintenance, they were mixed together, maintained for 20-30 min, and finally added in each well. At 4-6 h, complete medium was replaced.

Cell Proliferation Assay

At 12 h of transfection, cells were digested and inoculated into 96-well plates with 1000 cells per well. After culture for 1, 2, 3, 4 and 5 days, respectively, 10 μL of cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) was supplied in each well. Cells were incubated in dark for 2 h, and the absorbance at 490 nm was recorded by a microplate reader.

Colony Formation Assay

Cells were seeded in a 6-well plate with 1000 cells per well and incubated at 37°C for 10 days. Subsequently, cells were fixed with methanol for 15 min and stained with 0.1% crystal violet for another 20 min. The number of colonies containing over 50 cells in each well was counted and photographed.

Wound Healing Assay

Cells were seeded into 24-well plates and subjected to serum starvation for 24 h in serum-free medium. Until 90% of confluence, an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Images were taken

at 0 and 24 h using an inverted microscope, respectively.

Migration and Invasion Assays

Matrigel was diluted with serum-free medium at a ratio of 1:9. The inner side of the chamber was coated with 100 μL of Matrigel overnight. 100 μL of suspension (5×10^4 cells/mL) was added to the upper layer of the Transwell chamber. 600 μL of medium with 10% FBS was added to the bottom layer of the chamber. After incubation for 24 h, fixation with methanol, trypan blue staining and phosphate-buffered saline (PBS) wash for three times, penetrating cells were photographed under a microscope. Migration assay procedures were as same as the above indicated, except for Matrigel pre-coating.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. Differences among multiple groups were analyzed by one-way ANOVA, followed by post-hoc test. $p<0.05$ was considered statistically significant.

Results

Detection of CCDC3 Expression in Cervical Cancer Cell Lines and Construction of Stably Interfered CCDC3 Cell Line

QRT-PCR data showed higher mRNA level of CCDC3 in C33 and HeLa cells than SiHa and Caski cells ($p<0.05$, Figure 1A). Identically, Western blot results also indicated higher protein expression of CCDC3 in C33 and HeLa cells (Figure 1B). Hence, we selected C33 and HeLa cells with high expression of CCDC3 in this study for the next experiments. The constructed sh-EGFP and sh-CCDC3

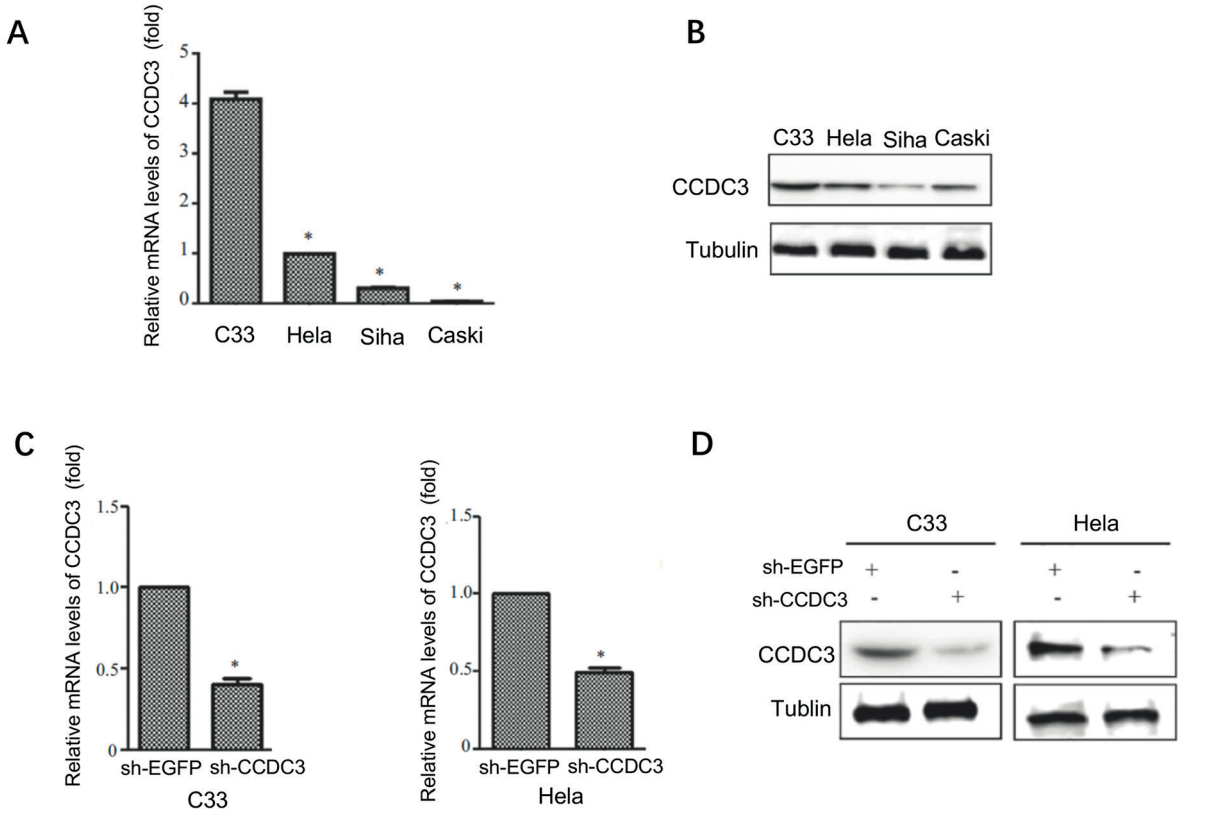

Figure 1. CCDC3 expression in cervical cancer cells and transfection efficacy of sh-CCDC3. ***A,*** The mRNA level of CCDC3 in C33, HeLa, SiHa and Caski cells. ***B,*** The protein level of CCDC3 in C33, HeLa, SiHa and Caski cells. ***C,*** The mRNA level of CCDC3 in C33 and HeLa cells transfected with sh-EGFP or sh-CCDC3. ***D,*** The protein level of CCDC3 in C33 and HeLa cells transfected with sh-EGFP or sh-CCDC3. *$p<0.05$.

plasmids were confirmed by sequencing, followed by transfection efficacy verification. Both mRNA and protein levels of CCDC3 decreased in C33 and HeLa cells transfected with sh-CCDC3 than those transfected with sh-EGFP ($p<0.05$, Figure 1C and 1D). Hence, the qualified sh-CCDC3 could be utilized to effectively downregulate CCDC3 expression in cervical cancer cells.

Effects of CCDC3 Knockdown on Proliferative Potential of Cervical Cancer Cells

To analyze the effect of CCDC3 knockdown on the proliferative potential of HeLa and C33 cells, we examined the proliferative rate at the appointed time points (day 1, 2, 3, 4, and 5) after plasmid transfection. Compared with controls, the relative proliferative rate of cells transfected with sh-CCDC3 markedly decreased. The inhibited proliferative phenomenon was obvious as early as at day 2, and became more pronounced with the prolongation of cell culture until day 5 ($p<0.05$, Figure 2A and Figure 2B). Meanwhile, colony formation assay identically suggested that the number of colonies containing over 50 cells remarkably decreased after CCDC3 knockdown ($p<0.05$, Figure 2C and 2D). The above results indicated that downregulation of CCDC3 could inhibit the proliferative rate and colony formation ability of HeLa and C33 cells.

Effects of CCDC3 Knockdown on Migratory and Invasive Potentials of Cervical Cancer Cells

Wound healing assay showed longer migratory distance in C33 cells transfected with sh-EGFP (64±4%) than those transfected with

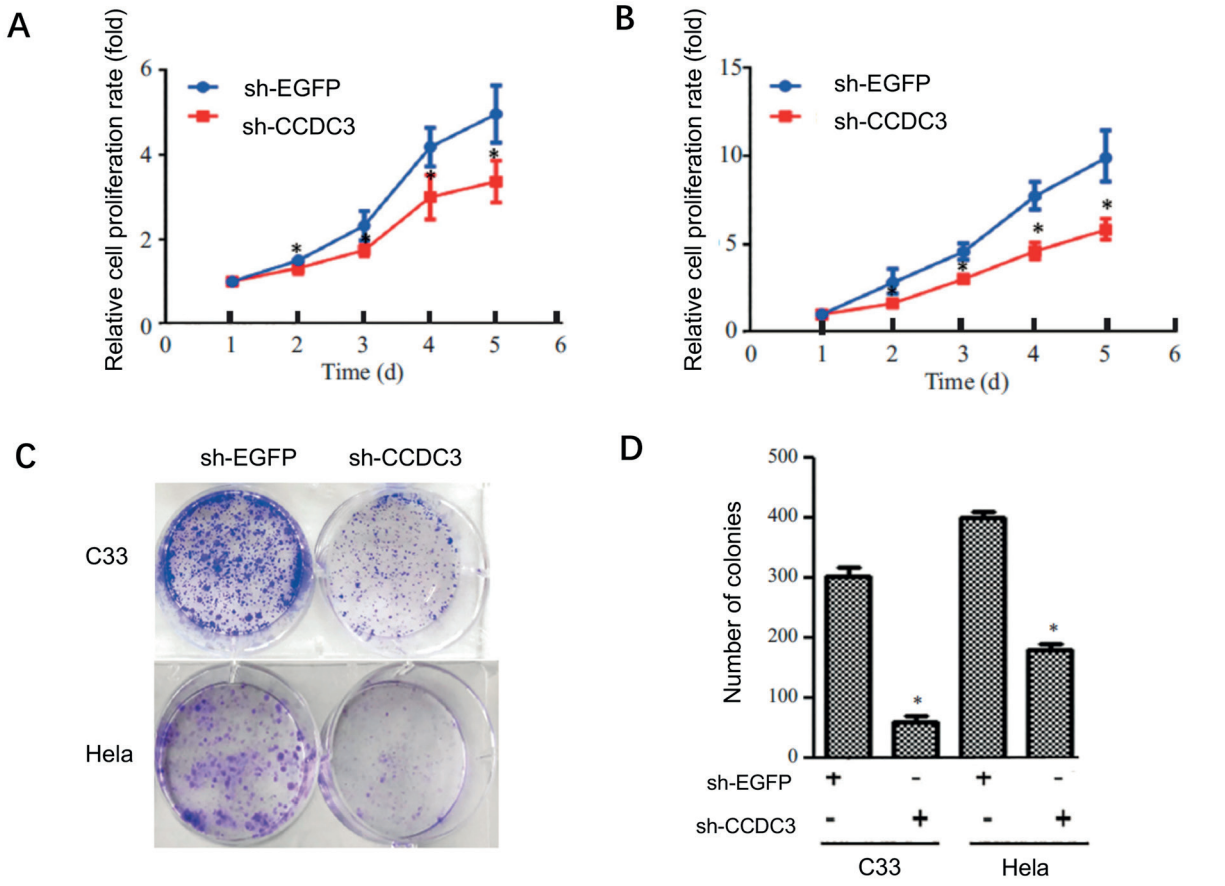

Figure 2. CCDC3 knockdown inhibited proliferation of cervical cancer cells. ***A,*** Proliferative rate of C33 cells transfected with sh-EGFP or sh-CCDC3. ***B,*** Proliferative rate of HeLa cells transfected with sh-EGFP or sh-CCDC3. ***C,*** Colony formation of C33 and HeLa cells transfected with sh-EGFP or sh-CCDC3. ***D,*** Number of colonies in C33 and HeLa cells transfected with sh-EGFP or sh-CCDC3. *$p<0.05$.

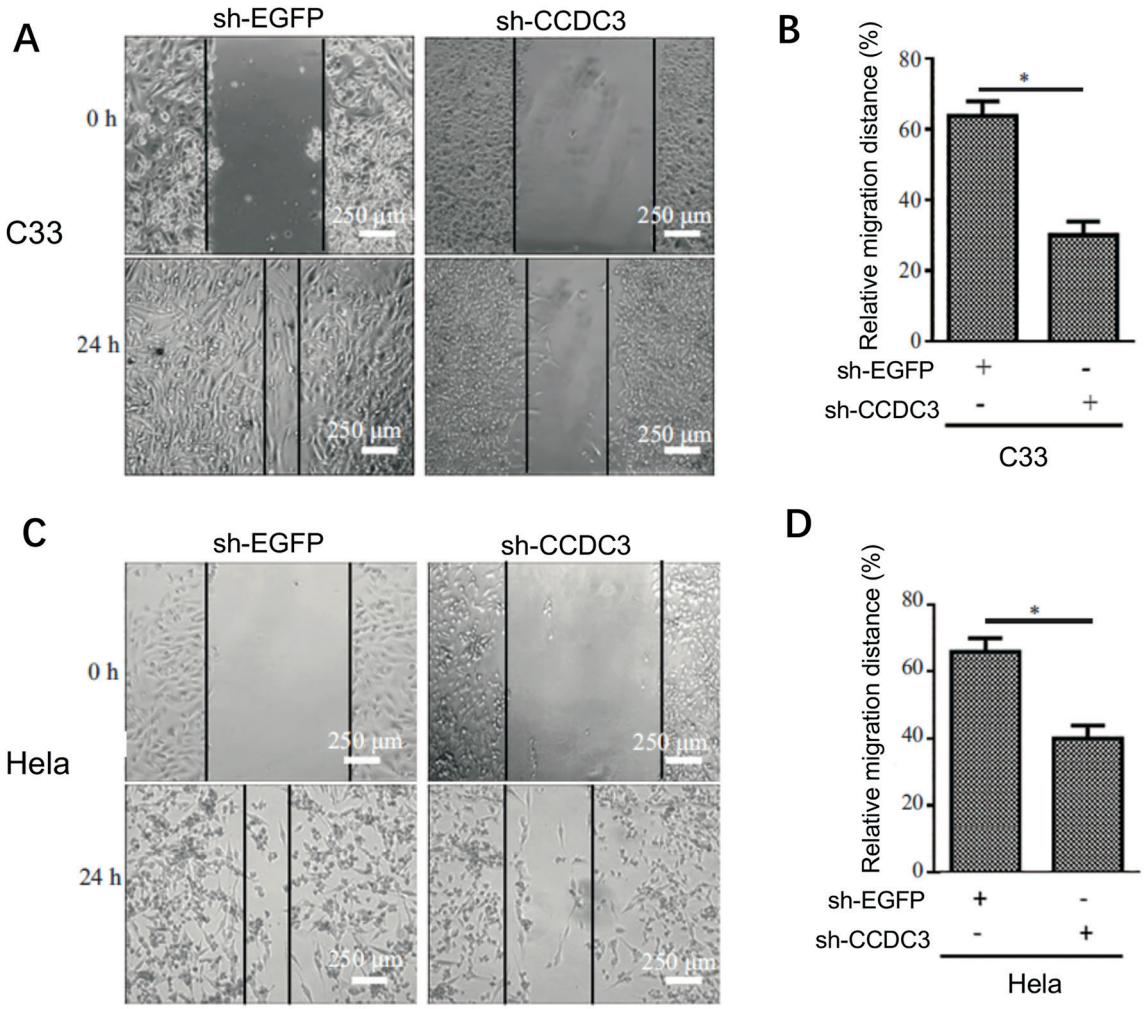

Figure 3. CCDC3 knockdown inhibited migration of cervical cancer cells. ***A,*** Migration of C33 cells transfected with sh-EGFP or sh-CCDC3. ***B,*** Relative migratory distance of C33 cells transfected with sh-EGFP or sh-CCDC3. ***C,*** Migration of HeLa cells transfected with sh-EGFP or sh-CCDC3. ***D,*** Relative migratory distance of HeLa cells transfected with sh-EGFP or sh-CCDC3. Bar = 250 μm, *p<0.05.

sh-CCDC3 (30±4%), and the difference was statistically significant (p<0.05, Figure 3A and 3B). Similar trends were also observed in HeLa cells. CCDC3 knockdown decreased migratory distance in comparison with those controls (40±4% *vs.* 66±4, p<0.05, Figure 3C and 3D). Transwell assay revealed that the number of invasive C33 cells transfected with sh-EGFP or sh-CCDC3 was 287±5 and 188±5, respectively (p<0.05, Figure 4A and 4B). We also observed the similar trends in HeLa cells, with 80±5 and 20±5 invasive cells in sh-EGFP group and sh-CCDC3 group, respectively (p<0.05, Figure 4C and 4D). We may conclude that CCDC3 down-regulation attenuated migratory and invasive potentials of cervical cancer cells.

CCDC3 Knockdown Reversed Epithelial-Mesenchymal Transition (EMT) of Cervical Cancer Cells

Further exploration found that downregulation of CCDC3 in C33 and HeLa cells downregulated protein expressions of interstitial phenotypic markers (N-cadherin, vimentin), CC-specific interstitial phenotypic marker YKL-40 and fibronectin, but upregulated epithelial marker E-cadherin (Figure 5). It is suggested that EMT of cervical cancer cells was reversed by CCDC3 knockdown.

Discussion

Local recurrence and distant metastasis of tumors are mainly caused by malignant behaviors of tumor invasion and metastasis, which are the key factors leading to treatment failure and poor prognosis in CC patients. Tumor invasion and metastasis are complex progresses involving multiple factors and pathways, which are achieved by direct diffusion, transvascular diffusion and implantation. In this study, CCDC3 was screened out by analyzing the downloaded datasets from GEO. We presumed that CCDC3 was associated with stem cells and tumor progression. Subsequently, we analyzed co-expressions of CCDC3 and known intestinal cancer-related genes (data not shown). It is found that CCDC3 was negatively correlated with tumor-suppressor genes APC, PROC, ATOH1 and C21ORF3. Besides, CCDC3 was highly expressed in small intestinal stem cells and intestinal cancer in a similar pattern with SOX9, OLFM4, ASCL2 and LGR5. Therefore, we believed that CCDC3 may exert a close relationship with the stemness of tumor cells, and is expected to become a candidate gene for cancer stem cells. Tumor cells could spread to the surroundings and invade to normal tissues at a certain stage of growth. The acquisition of infiltration ability is a crucial part in the malignant process of tumor cells. EMT allows tumor cells to present stronger ability to invade. In this work, wound-healing assay revealed that cervical cancer cells undergoing EMT could migrate faster and heal the artificial wound in a shorter period of time. It is well known that EMT is of great significance in tumor cell infiltration. Matrigel is similar to components of the basement membrane, such as collagen, laminin, proteoglycans, cytokines and enzymes. Cells undergoing EMT can normally grow on Matrigel and penetrate

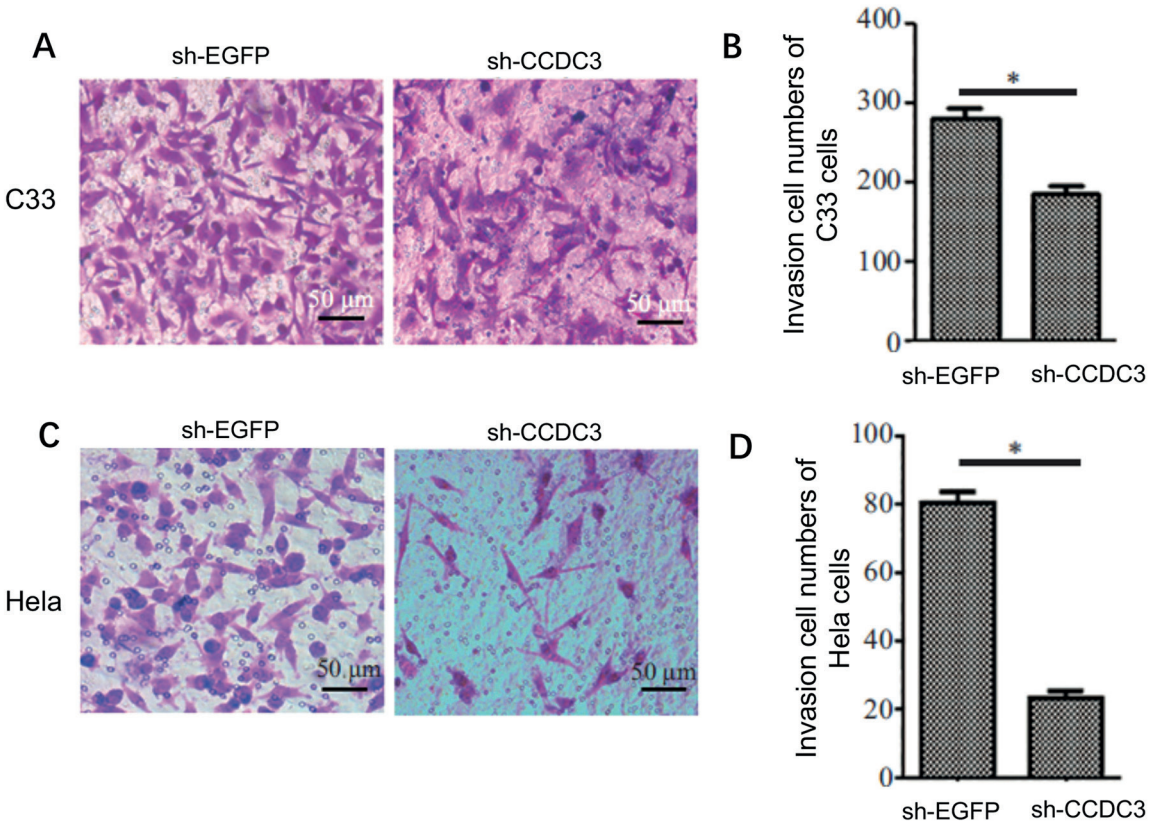

Figure 4. CCDC3 knockdown inhibited invasion of cervical cancer cells. ***A,*** Transwell image of invasive C33 cells transfected with sh-EGFP or sh-CCDC3. ***B,*** Invasion cell number of C33 cells transfected with sh-EGFP or sh-CCDC3. ***C,*** Transwell image of invasive HeLa cells transfected with sh-EGFP or sh-CCDC3. ***D,*** Invasion cell number of HeLa cells transfected with sh-EGFP or sh-CCDC3. Bar = 50 μm, *$p<0.05$.

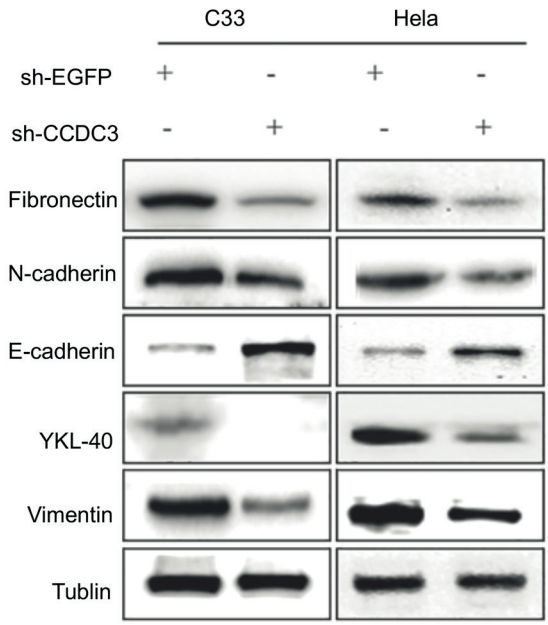

Figure 5. CCDC3 knockdown reversed EMT of cervical cancer cells. Western blot analyses of Fibronectin, N-cadherin, E-cadherin, YKL-40 and Vimentin in C33 and HeLa cells transfected with sh-EGFP or sh-CCDC3.

it, suggesting that EMT may be an important mechanism for tumor cells to invade through the basement membrane. Invasive ability of cells is an important indicator reflecting the malignant degree of tumors. Cells with a higher degree of malignancy tend to present stronger invasive ability to infiltrate and further impair adjacent tissues[19]. Highly invasive cells often exhibit active cell motility, which is another basic and key factor for tumor invasion. These two malignant phenotypes allow tumor cells to metastasize to distant organs. Our experimental results clarified that CCDC3 knockdown remarkably inhibited the invasive ability of cervical cancer cells. In addition, Western blot analyses confirmed that CCDC3 knockdown markedly inhibited protein expressions of EMT-related genes, showing the potential effect of CCDC3 on regulating EMT in CC. The promotive effects of CCDC3 on proliferative, migratory and invasive potentials of cervical cancer cells have been proved in this study. Further in-depth explorations are needed to elucidate the specific molecular mechanism of CCDC3 in the regulation of cervical cancer cell behaviors. This study provides a theoretical basis for developing CCDC3 as a novel therapeutic target for CC.

Conclusions

We detected that low expression of CCDC3 attenuated proliferative, migratory, invasive and EMT potentials of cervical cancer cells. Hence, CCDC3 may be utilized as a novel therapeutic target for cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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