Knockdown of CCL28 inhibits endometriosis stromal cell proliferation and invasion via ERK signaling pathway inactivation

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Abstract. Endometriosis (EM), the presence of functional endometrial glands and stroma outside the uterine cavity, is a common gynecological disorder. At present, the pathogenesis of EM has not been fully elucidated, so there is still a lack of effective therapy. The present study aimed to explore the role of C-C motif chemokine ligand 28 (CCL28) and its underlying mechanism in endometrial stromal cells to propose a novel therapy for EM treatment. The expression of CCL28 and CC chemokine receptor 10 (CCR10) were examined. After CCL28 knockdown or overexpression by lentivirus infection, cell proliferation and invasion were measured. It was revealed that compared with normal, the expression levels of CCL28 and CCR10 were significantly elevated in endometrial tissues of patients with EM. Knockdown of CCL28 in endometrial stromal cells significantly suppressed cell proliferation and invasion, and this was accompanied by significantly reduced expression levels of CCR10, MMP2, MMP9, integrin β1 (ITGB1) and phosphorylated (p)-ERK/ERK ratio. The addition of the CCL28 recombinant protein had an opposite effect to CCL28 downregulation. Furthermore, the ERK inhibitor, PD98059, reduced CCL28-induced cell proliferation and invasion, as well as the expression levels of MMP2, MMP9, ITGB1 and p-ERK. Therefore, the present study indicated that CCL28 may contribute to the progression of EM by regulating MMP2, MMP9 and ITGB1 expression and function via the activation of the ERK signaling pathway.

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Introduction

Endometriosis (EM), the presence of functional endometrial glands and stroma outside the uterine cavity, is a common gynecological disorder characterized by dysmenorrhea, chronic pelvic pain, menstrual abnormalities and infertility (1-4). Previous study demonstrated that EM affects ~10% of individuals who have a uterus, and that the probability of symptomatic perimenopause increases to 30-50% (5). Currently, the most common primary diagnostic method for EM is laparoscopy, supplemented with the screening for cancer antigen 125 (CA125) and endometrium antibody, as well as B-ultrasounds, x-ray and magnetic resonance imaging (6). Although EM is a benign disease, it has certain characteristics of malignant tumors, including cell invasion, new blood vessel generation, unlimited growth, reduced numbers of apoptotic cells, infiltration and destruction of surrounding tissues and metastasis (7). Therefore, inhibition of the growth and invasion of EM may be a possible treatment strategy for EM.

Chemokines are selective mediators of leukocyte migration to inflammatory sites (8). It has been demonstrated that chemokines are key players in a variety of physiological and pathological events, including chemotaxis, cell proliferation, apoptosis, angiogenesis and inflammatory processes/diseases (9,10). C-C motif chemokine ligand (CCL)28 is a mucosa-associated epithelial chemokine that is selectively expressed in certain mucosal tissue, such as epithelial mucosal tissues (11,12). CCL28 is a functional ligand for CC chemokine receptor (CCR)10, a member of the chemokine receptor family, which belongs to the G protein-coupled receptor superfamily, and is normally expressed by melanocytes, plasma cells and skin-homing T cells (13). Upregulation of CCR10 can facilitate cell proliferation and invasion in glioma, contributing to gliomagenesis (14). Furthermore, CCR10 can stimulate breast cancer cell invasion and migration by increasing MMP7 expression via ERK1/2 activation (15,16). In ectopic endometrial stromal cells, depletion of CCL27 can suppress cell proliferation, metastasis and adhesion (17). CCL28 induces apoptosis of decidual stromal cells via binding of CCR3/CCR10 in human spontaneous abortion (18). Furthermore, estrogen may serve a crucial role in the protection against genital infection by regulating mucosa-associated epithelial chemokine (MEC)/CCL28 expression in the uterus (19). There are also several studies

demonstrating that the ERK1/2 signaling pathway is associated with migration and apoptosis of endometrial stromal cells (20-24). Another study has reported that CCL28 can promote cell proliferation and metastasis in breast cancer via the MAPK signaling pathway (25). However, the function of CCL28 in endometrial stromal cells and its underlying mechanisms are still unknown.

The present study aimed to explore the role of CCL28 and its underlying mechanism in endometrial stromal cells to propose a novel therapy for EM treatment. It provided important leads for designing studies in the future to understand the mechanism of EM and aid in the development of novel therapeutic strategies.

Materials and methods

Patient samples. Patients who met the EM diagnostic criteria (visual inspection/laparoscopy/laparotomy) participated in the study. EM is usually diagnosed by visual inspection of the pelvis during laparoscopy or laparotomy (6). After informed consent was obtained, the EM tissues from 15 patients (female, age range from 25 years to 55 years, mean age: 37 years, Shanghai, China) with deep-infiltrating EM who underwent laparoscopic treatment at the Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine (Shanghai, China) between February 2020 and December 2020 were removed via biopsy. Deep-infiltrating EM is located 5 mm below the surface of the peritoneum. Control endometrial samples were collected from 15 patients (female, age range from 23 years to 51 years, mean age: 35 years, Shanghai, China) without EM who underwent laparoscopy and hysteroscopy surgery for benign gynecological diseases. CCL28 and CCR10 expression was detected in these tissues by IHC. Furthermore, 80 serum samples (healthy individuals: 40; EM patients, 40) were collected to detect CCL28 levels by ELISA. Tissue samples were collected independent of menstrual cycle stage. The following inclusion criteria were used: i) EM was confirmed by two pathologists following laparoscopic biopsy; and ii) No preoperative chemotherapy or radiotherapy was received. Patients who had received hormonal treatment and birth control prior to enrollment were excluded from the study. The clinical characteristics of the patients and controls are shown in Table I. The Ethics Committee of Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine (Shanghai, China) approved all experiments involving patients.

Cell culture. Primary endometrial stromal cells derived from ectopic endometria of female patients with EM, or from normal endometria of female patients without EM, were isolated and cultured as previously described (26,27). Immunocytochemistry using anti-cytokeratin 19 and anti-Vimentin antibodies was performed to identify cell purity. The cells were cultured in DMEM/F12 (cat.no. SH30023.01B; Hyclone; Cytiva) containing 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.), 1% double antibiotics (penicillin and streptomycin mixture), 2 mM L-glutamine and 1 ng/ml fibroblast growth factor-2 at 37˚C in a 5% CO_2 incubator. 293T cells were cultured with DMEM containing 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.), 1% double antibiotics in a 37˚C, 5% CO_2 incubator.

Plasmid construction and lentivirus packaging. Targeting different sites of CCL28, three interference sequences were synthesized (Table II). Short hairpin RNA (sh) constructs were created using double chain annealing and inserted into the pLKO.1-Puro vector (Addgene, Inc.) at *Age*I-*Eco*RI restriction sites, while a negative control shRNA (shNC) as a negative control. Subsequently, plasmids of pLKO.1-Puro-shCCL28-1, -2 and -3 (1,000 ng) were co-transfected with viral packaging plasmids psPAX2 (900 ng) and pMD2.G (100 ng; Addgene, Inc.; packaging vector:envelope vector, 1:9) into 3rd generation 293T cells (ATCC) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C in a 5% CO_2 incubator. Following 48 h of incubation, viral particles were collected via ultracentrifugation at 55,000 x g, 4˚C for 2.5 h, and then the viral supernatant (MOI, 10) was used to infect EM stromal cells ($1x10^6$). After 24 h infection, the cells were cultured for 24 h with serum-free transfer solution before further experiments were performed.

ELISA. An ELISA was employed to detect CCL28 levels in the serum of patients with EM or in the supernatant of endometrial stromal cells ($1x10^6$ cells/ml). The Human MEC/CCL28 ELISA Kit (cat. no. RAB0072; Sigma-Aldrich; Merck KGaA) was used according to the manufacturer's protocol.

Immunohistochemical (IHC) detection. Tissue sections (4 μm) were washed with 0.02 M PBS and fixed with 4% formaldehyde for 30 min at room temperature. After three washes with 0.02 M PBS, samples were incubated in 0.3% H_2O_2 in a wet-box for 10 min and then blocked in 1% BSA (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. Subsequently, samples were incubated with primary antibodies against CCL28 (1:200; cat. no. 18214-1-AP; ProteinTech Group, Inc.) and CCR10 (1:100; cat. no. 22071-1-AP; ProteinTech Group, Inc.) for 1 h at room temperature. The primary incubation was then followed by a 30-min incubation with HRP-labeled secondary antibodies (cat. no. D-3004; Shanghai Changdao Biological Technology Co., Ltd.) at room temperature. Samples were then subjected to 3, 3'-diaminobenzidine (DAB) staining (cat. no. FL-6001; Shanghai Changdao Biological Technology Co., Ltd.), 3 min of hematoxylin staining (cat. no. 714094; Zhuhai Besso Biotechnology Co., Ltd.) and alcohol differentiation with 1% hydrochloric acid at room temperature, followed by washing with tap water for 10 min and drying in a 65˚C oven for 15 min. Finally, samples were made transparent in xylene for 3 min and sealed with neutral gum (cat. no. G8590; Solarbio) at room temperature. After drying in a 65˚C oven for 15 min, samples were imaged using an upright fluorescence microscope (ECLIPSE Ni; Nikon Corporation). CCL28 and CCR10 expression was analyzed using an image analysis system version 11.0 (IMS; Beijing Changheng Rongchuang Technology).

Immunocytochemical detection. Endometrial cells were cultured on coverslips for 24 h. The cells were washed with 0.02 M PBS to remove the medium, fixed with 4% formaldehyde for 30 min at room temperature and washed with 0.02 M PBS. Cells were permeated with 0.5% Triton X-100 (cat. no. T8200; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min

Table I. Clinical characteristics of patients.

Characteristics	Patients with endometriosis (n=40)	Healthy patients (n=40)	P-value
Age, year	35.55±3.64	31.32±5.87	0.092
BMI, kg/m^2	19.10±2.53	21.32±2.12	0.061
CA125, IU/ml	23.74±2.56	14.57±2.04	0.013
EM stage, n (%)			
III	21 (52.5)	NA	
IV	19 (47.5)	NA	
Benign conditions, n (%)			
Uterine myoma	NA	8 (20.0)	
Endometrial hyperplasia	NA	13 (32.5)	
Others	NA	19 (47.5)	
Menstrual phase, n (%)			0.171
Proliferative	21 (52.5)	27 (67.5)	
Secretory	19 (47.5)	13 (32.5)	

Data are presented as the mean ± SD or n (%). CA125, cancer antigen 125; NA, not applicable.

Table II. CCL28 sequences for gene silencing.

Target site name	Sequence (5'-3')
CCL28-1 (site: 167-185)	GCACGGAGGTTTCACATCA
CCL28-2 (site: 260-278)	CTGTCATCCTTCATGTCAA
CCL28-3 (site: 321-339)	GCAGTGGATGAAAGTGCAA

CCL28, C-C motif chemokine ligand 28.

at room temperature and then blocked with 1% BSA for 1 h at room temperature. Subsequently, cells were incubated with primary antibodies against CK19 (1:200; cat. no. ab52625; Abcam) and vimentin (1:500; cat. no. ab92547; Abcam) at 4˚C overnight. Following primary incubation, cells were incubated for 30 min with HRP-labeled secondary antibodies (cat. no. D-3004; Shanghai Changdao Biological Technology Co., Ltd.). Cells were then subjected to DAB staining. Finally, cells were imaged using an upright fluorescence microscope and the expression levels of CK19 and vimentin were analyzed using an image analysis system version 11.0 (IMS; Beijing Changheng Rongchuang Technology).

Flow cytometry analysis. The EM markers of CD10 and CD90 have been detected to verify the purity of endometrial stromal cells. Endometrial stromal cells in the logarithmic growth phase were digested, resuspended and counted. Resuspended cells (5,000,000-10,000,000) were centrifuged at 1,000 x g for 5 min to obtain the cell precipitants, and then incubated with the following antibodies: FITC Mouse Anti-Human CD10 (1:50; cat. no. 340925; BD Biosciences); FITC Mouse Anti-Human CD90 (1:100; cat. no. 561969; BD Biosciences); and FITC Mouse IgG1 (1:100; cat. no. 555748; BD Biosciences). After 30 min of incubation at 4˚C in the dark, the cells were detected using a Flow cytometer (CytoFLEX; Beckman Coulter, Inc.) and analyzed using BD Accuri™ C6 Software (Version 1.0.264.21; BD Biosciences).

Cell proliferation assay. Endometrial stromal cells in the logarithmic growth phase were digested with trypsin and cultured overnight in 96-well plates (cat. no. TR4001; TrueLine) at a density of 3,000 cells/well in a 37˚C, 5% CO_2 incubator. At 0, 12, 24 and 48 h of treatment of shNC, shCCL28-1and shCCL28-2, or different concentrations of CCL28 recombinant protein (0, 5, 10, 20 and 40 ng/ml), or vehicle + DMSO, CCL28 + DMSO, vehicle + PD98059 and CCL28 + PD98059, Cell Counting Kit-8 (CCK-8; cat. no. CP002; SAB Biotherapeutics, Inc.) reagent and serum-free medium were mixed at a volume ratio of 1:10. Subsequently, 100 μl CCK-8 mixture was added to the aforementioned groups. After 1 h incubation at 37˚C, the optical density at 450 nm was measured using a microplate reader.

Cell invasion assay. Endometrial stromal cells in the logarithmic growth phase were digested with trypsin and seeded into 6-well plates at a density of 300,000 cells/well. After 24 h of culture at 37˚C, the stromal cells were transduced with shCCL28 (shCCL28-1, shCCL28-2) lentivirus for 48 h, or pre-treated with PD98059 (an ERK inhibitor; 10 μmol/l; S1177; Selleck) for 30 min at 37˚C. Subsequently, cells were treated with CCL28 recombinant protein for 48 h at 37˚C and then collected for Transwell detection. For the cell invasion assay, a 24-well Transwell plate was used (pore size, 8 μm; MilliporeSigma; Merck KGaA). The upper chamber of the Transwell plate was coated with 30 μl Matrigel at 37˚C for 30 min and 2x10^5 cells in 200 μl DMEM/F12 were added. DMEM/F12 containing 10% FBS was added to the lower chamber. After a 48-h incubation at37˚C, the membrane was fixed with 4% formaldehyde and stained with 0.5% crystal violet (1 ml) for 30 min at room temperature. The number

Table III. Sequences of primers for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
CCL28	F: CTGATGGGGATTGTGACTTG
	R: TGGTGTTTCTTCCTGTGGC
CCR10	F: AGGGCTGGAGTCTGGGAAGTG
	R: CACGATGACGGAGACCAAGTGT
MMP2	F: GGGAGTACTGCAAGTTCCCCTTCTT
	R: TGGAAGCGGAATGGAAAC
MMP9	F: AGGACGGCAATGCTGATG
	R: TCGTAGTTGGCGGTGGTG
ITGB1	F: AATGTAACCAACCGTAGC
	R: GGTCAATGGGATAGTCTTC
GAPDH	F: AATCCCATCACCATCTTC
	R: AGGCTGTTGTCATACTTC

CCL28, C-C motif chemokine ligand 28; CCR10, C chemokine receptor 10; ITGB1, integrin β1; F, forward; R, reverse.

of invasive cells was counted at a magnification of x200 via a light microscope (XDS-500C; Shanghai Caikang Optical Instrument Co., Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from endometrial tissues or stromal cells using TRIzol® reagent (cat. no. 1596-026; Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was quantified and underwent RNA integrity confirmation. Total RNA (1 μg) was reverse transcribed into complementary DNA using a Reverse Transcription Kit (cat. no. K1622; Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions as follows: 37˚C for 60 min; 85˚C for 5 min; 4˚C for 5 min. qPCR was subsequently performed using an ABI-7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a SYBR-Green PCR Kit (cat. no. K0223; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation: 10 min at 95˚C; followed by 40 cycles of denaturation, elongation and annealing at 15 sec at 95˚C and 45 sec at 60˚C. CCL28, CCR10, MMP2, MMP9 and ITGB1 mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (28) and normalized to the internal reference gene GAPDH. The primers are listed in Table III.

Western blotting. Total protein was extracted from endometrial stromal cells using RIPA buffer (containing protease and phosphatase inhibitors; cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). Following total protein quantification using a BCA kit (cat. no. 23223; Thermo Fisher Scientific, Inc.), 25 μl of protein/lane was separated via SDS-PAGE using 10 and 12% gels before being transferred onto polyvinylidene fluoride membranes (cat. no. HATF00010; MilliporeSigma; Merck KGaA). Membranes were blocked in 5% skimmed milk (BD Biosciences) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4˚C, with gentle shaking, with primary antibodies against CCL28 (dilution, 1:500; cat. no. ab196567; Abcam), CCR10 (dilution, 1:250; cat. no. ab3904; Abcam), MMP2 (dilution, 1:5,000; cat. no. ab37150; Abcam), MMP9 (dilution, 1:1,000; cat. no. ab194316; Abcam), ITGB1 (1:1,000; cat. no. ab24693; Abcam), phosphorylated (p)-ERK (dilution, 1:1,000; cat. no. ab214362; Abcam), ERK (dilution, 1:10,000; cat. no. ab184699; Abcam) and GAPDH (dilution, 1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.). The membranes were washed three times with TBS-0.05% Tween-20 (TBST), followed by a 2-h incubation at room temperature with HRP-conjugated goat anti-rabbit (cat. no. A0208) and goat anti-mouse (catalog no. A0216) secondary antibody (dilution, 1:1,000; Beyotime Institute of Biotechnology). Membranes were washed with TBST and visualized using a chemiluminescent reagent (cat. no. WBKLS0100; MilliporeSigma; Merck KGaA) and ECL imaging system (Tanon-5200; Tanon Science and Technology Co., Ltd.). ImageJ version 1.47 (National Institutes of Health) was used to semi-quantify protein expression levels using GAPDH as a loading control.

Gelatinase zymography. Total protein from cells was isolated using RIPA buffer, quantified by a BCA kit (cat. no. 23223; Thermo Fisher Scientific, Inc.) and 25 μl protein per lane was separated via 10% SDS-PAGE containing 1% gelatin. The gels were then washed with eluent (2.5% Triton X-100, 50 mM Tris-HCl, 5 mM $CaCl_2$; pH 7.6) twice for 30 min, and rinsed with rinsing solution (eluent without Triton X-100) twice for 20 min. The gels were subsequently incubated in incubation solution (50 mM Tris-HCL, 5 mM $CaCl_2$, 0.02% brij-35; pH 7.6) for 20 h at 37˚C. The gels were stained using Coomassie brilliant blue staining solution for 3 h at room temperature in a low-speed shaker, and the staining solution was recovered. A decolorizing solution (30% methanol and 10% acetic acid) was added to highlight clear bands on a blue background for 30 min at room temperature. Images of the gels were then captured for observation using a gel imager.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 7.0 software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons among more than two groups, whereas unpaired Student's t-tests were used for statistical comparisons between two groups. For Table I, the t-test was used for age, BMI and cancer antigen-125 comparisons between the patient and control groups. The χ^2 test was used for comparisons between menstrual phases of the patient and control groups. Data are presented as the mean ± standard deviation of ≥3 independent experimental repeats. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CCL28 and CCR10 are highly expressed in the serum and endometrial tissues of patients with EM. CCL28 levels in the serum of patients with EM were significantly higher compared with those of the healthy controls (Fig. 1A). Furthermore, significantly higher mRNA (Fig. 1B and C) and protein (Fig. 1D) expression levels of CCL28 and CCR10 were observed in EM tissues compared with healthy tissues. IHC staining also demonstrated the high expression levels of CCL28 and CCR10

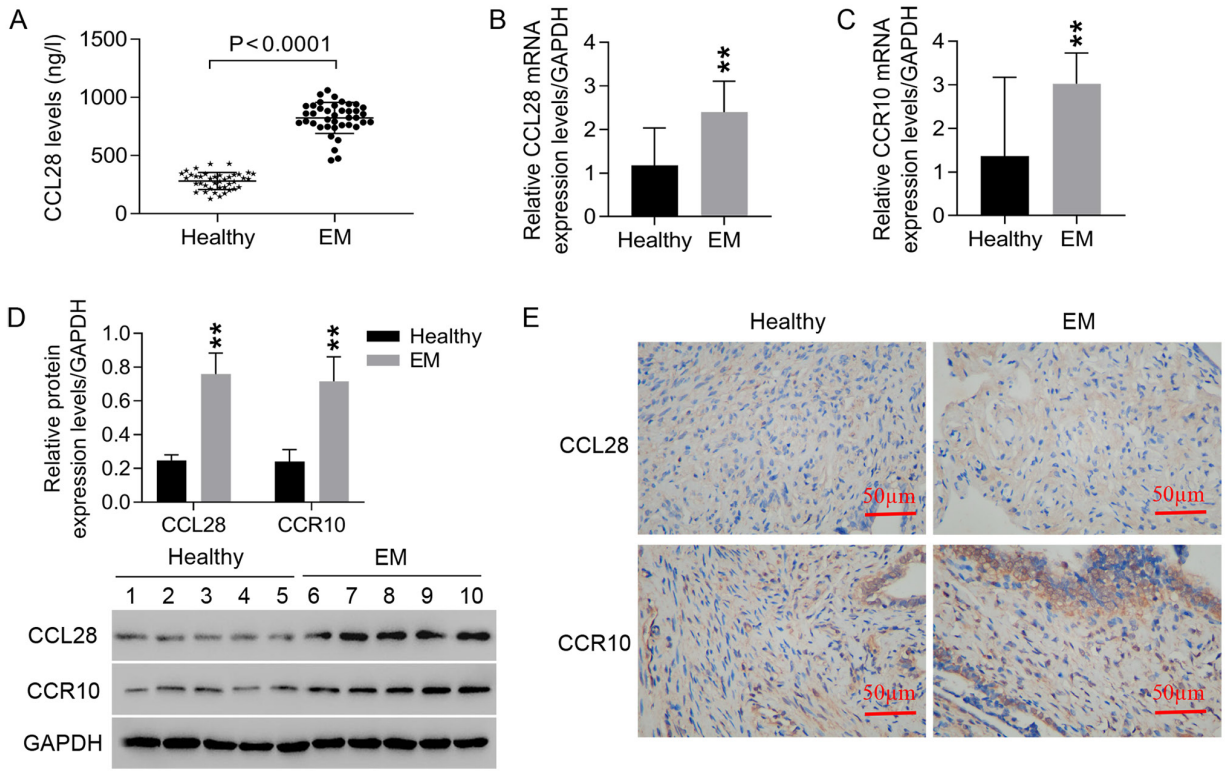

Figure 1. CCL28 and CCR10 are highly expressed in the serum and endometrial tissues of patients with EM. Serum samples of 40 patients with EM and 40 healthy patients were collected to detect CCL28 levels, and 15 endometrial tissue samples from patients with EM and 15 endometrial tissues from healthy patients were also collected. (A) CCL28 levels in the serum of patients with EM were detected using ELISA. mRNA expression levels of (B) CCL28 and (C) CCR10 in endometrial tissues of patients with EM were detected by reverse transcription-quantitative PCR. (D) Protein expression levels of CCL28 and CCR10 in endometrial tissues of patients with EM were detected using western blotting. (E) Protein expression levels of CCL28 and CCR10 in endometrial tissues of patients with EM were detected by immunohistochemistry (x200, 50 μm). **P<0.01 vs. normal. CCL28, C-C motif chemokine ligand 28; CCR10, CC chemokine receptor 10; EM, endometriosis.

in EM tissues compared with healthy tissues (Fig. 1E). These results indicated that increased expression levels of CCL28 and CCR10 may contribute to the progression of EM.

Knockdown of CCL28 expression in endometrial stromal cells via lentiviral transduction. Positive vimentin expression was observed in ectopic endometrial stromal cells, whereas CK19 expression was negative (Fig. 2A). Vimentin is a marker of endometrial stromal cells and CK19 is a marker of epithelial cells (29,30). CD10 and CD90 detection using flow cytometry verified that >95% of the isolated cells were EM stromal cells (Fig. 2B). To understand the function of CCL28 in EM, lentiviral transduction was used to downregulate CCL28 expression in ectopic endometrial stromal cells. Both mRNA and protein expression levels of CCL28 were significantly downregulated by shCCL28-1, -2 and -3 compared with negative control (shNC; Fig. 2C and D). Among the three, shCCL28-1 and shCCL28-2 were more efficient and selected for use in subsequent experiments.

Knockdown of CCL28 in endometrial stromal cells significantly suppresses cell proliferation and invasion. Following CCL28 knockdown, cell proliferation and invasion were evaluated. Cell proliferation (0-48 h) and cell invasion in shCCL28 endometrial stromal cells were significantly decreased compared with those in the shNC group (Fig. 3A and B). Compared with the shNC group, a significant decrease in CCL28 levels in the supernatant of shCCL28 endometrial stromal cells was observed (Fig. 3C). Both mRNA (Fig. 3D) and protein (Fig. 3F) expression levels of CCL28, CCR10, MMP2, MMP9 and ITGB1 in shCCL28 endometrial stromal cells were significantly reduced compared with those of shNC endometrial stromal cells. Knockdown of CCL28 also decreased the activities of MMP2 and MMP9 compared with the shNC group (Fig. 3E). Furthermore, CCL28 knockdown significantly decreased the protein expression levels of p-ERK/ERK ratio, compared with shNC (Fig. 3G). These results suggested that knockdown of CCL28 attenuated EM progression by inhibiting cell proliferation and invasion via the regulation of CCR10, MMP2, MMP9 and ITGB1 expression, and this may involve the ERK signaling pathway.

CCL28 recombinant proteins significantly increase CCL28 and CCR10 expression in healthy endometrial stromal cells. The results demonstrated that positive vimentin expression was observed in endometrial stromal cells from healthy controls, whereas CK19 expression was negative (Fig. 4A). Detection of CD10 and CD90 by flow cytometry verified that >95% of the isolated cells were healthy endometrial stromal cells (Fig. 4B). A series of CCL28 recombinant protein concentrations (0, 5, 10, 20 and 40 ng/ml) were used to treat healthy endometrial stromal cells. At 48 h, CCL28 could significantly promote healthy endometrial stromal cell proliferation in a dose-dependent manner compared with 0 ng/ml, whereas at 0, 12 and 24 h

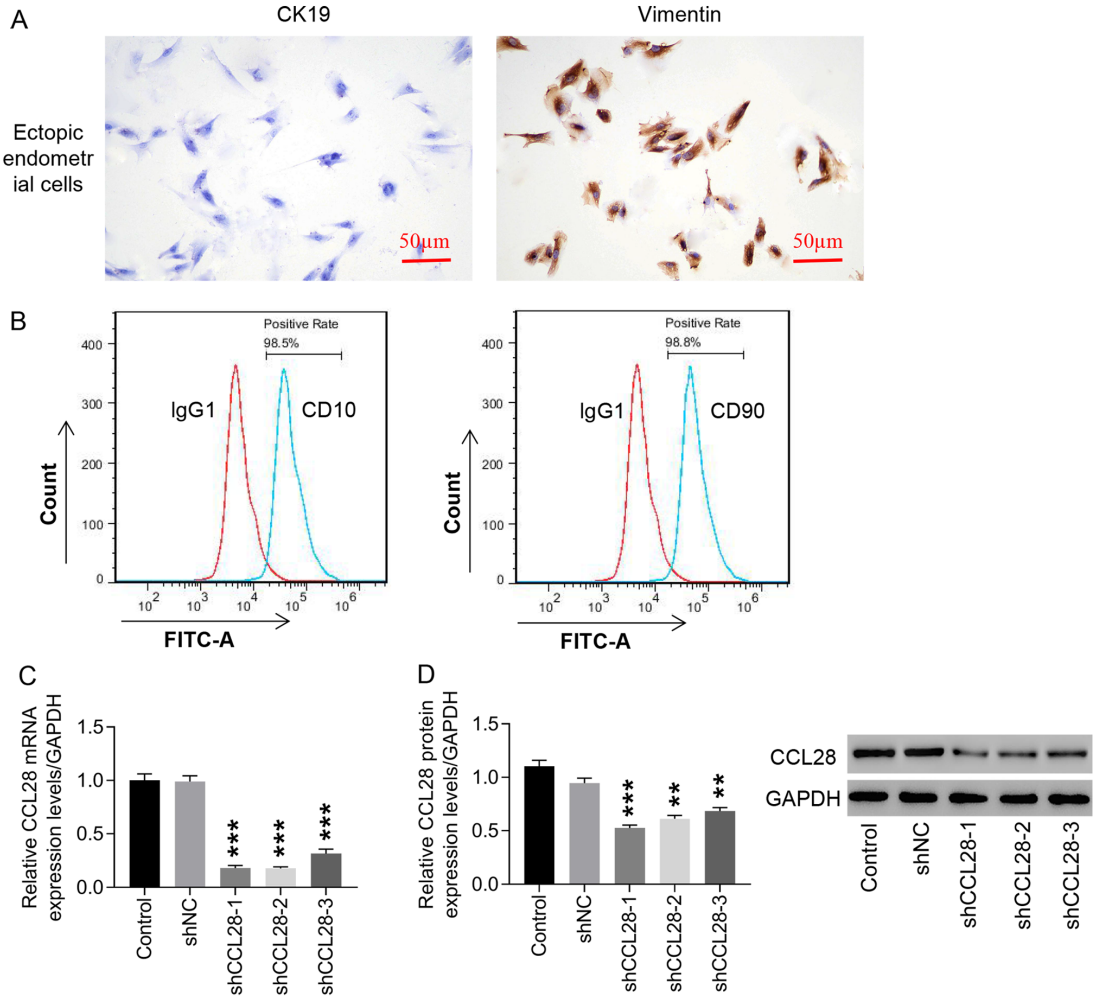

Figure 2. Knockdown of CCL28 expression in endometrial stromal cells by lentiviral transduction. (A) endometrial stromal cells were identified via immunocytochemistry, which was used to analyze CK19 and vimentin expression (x200, 50 μm). (B) CD10 and CD90 were detected using flow cytometry to identify the percentage of EM stromal cells. shCCL28-1, -2 and -3 were constructed to transduce endometrial stromal cells, CCL28 (C) mRNA and (D) protein expression levels were detected to determine the knockdown efficiency of the constructs. **P<0.01 and ***P<0.001 vs. shNC. Control, cells cultured with medium; shNC, cells infected with negative control lentivirus; shCCL28-1, -2 and -3, cells transduced with shCCL28 lentivirus-1, -2 and -3; CCL28, C-C motif chemokine ligand 28; sh, short hairpin RNA; CK19, cytokeratin-19.

no significant change was observed (Fig. 4C). In most cases there is still a small increase at 40 ng/ml and in general the increase in cell proliferation at different concentrations is small. Furthermore, after CCL28 recombinant protein treatment, both mRNA and protein expression levels of CCL28 and CCR10 were significantly increased in a dose-dependent manner compared with 0 ng/ml (Fig. 4D-F). Although there is still a small increase at 40 ng/ml and in general the increase of 40 ng/ml in cell proliferation and expression of CCL28 and CCR10 is smaller than that of 20 ng/ml. Based on these results, 20 ng/ml CCL28 recombinant protein was selected for the subsequent experiments.

CCL28 may contribute to EM progression by regulating MMP2, MMP9 and ITGB1 expression via activation of the ERK signaling pathway. The mechanism of the ERK signaling pathway in EM progression was explored. The results demonstrated that CCL28-induced proliferation and invasion of healthy endometrial stromal cells were markedly attenuated by PD98059 (ERK inhibitor; Fig. 5A and B). Furthermore, compared with vehicle + DMSO, CCL28 induced the activities of MMP2 and MMP9, which were markedly inhibited by PD98059 (Fig. 5C). CCL28-induced MMP2, MMP9 and ITGB1 protein expression levels, as well as p-ERK/ERK ratio, were significantly decreased by PD98059 (Fig. 5D and E). These results indicated that CCL28 may contribute to EM progression by regulating MMP2, MMP9 and ITGB1 expression via the ERK signaling pathway.

Discussion

Previous research has suggested that chemokine ligands serve important roles in the development and progression of EM. For example, the levels of CCL2 are elevated in the peritoneal fluid of female patients with EM (31,32), and can enhance endometrial stromal cell survival and invasion (23). A previous study also demonstrated that proinflammatory cytokines contribute to the development of EM by upregulating the secretion of CCL20 in endometrial stromal cells (33). Furthermore, it has been reported that certain chemokines, such as CCL2 and CCL5, have the potential to be biomarkers for EM (34,35). As for chemokine CCL28, a study has revealed that it is elevated

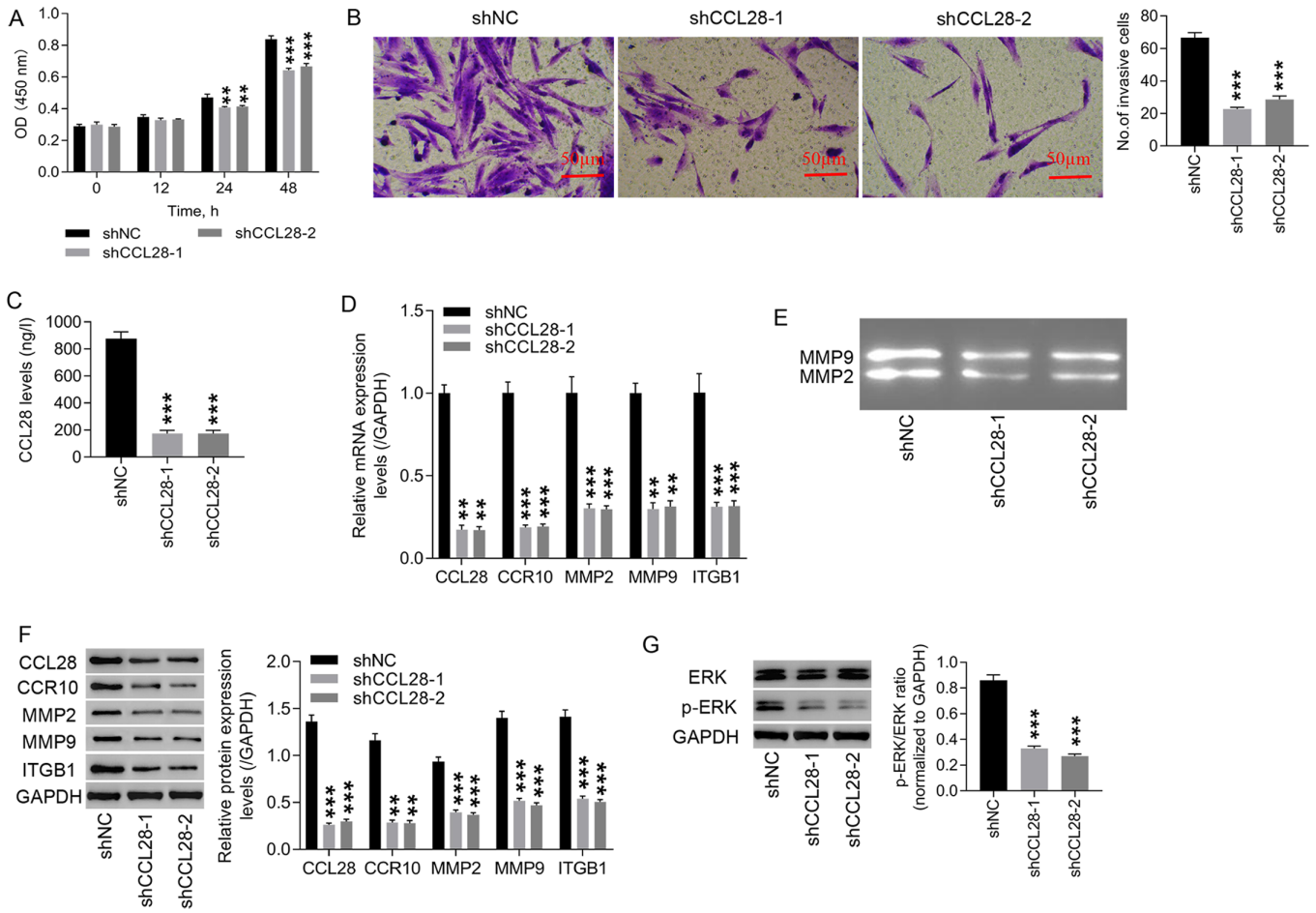

Figure 3. Knockdown of CCL28 in endometrial stromal cells significantly suppresses cell proliferation and invasion. (A) Following CCL28 knockdown in endometrial stromal cells, cell proliferation was detected using a Cell Counting Kit-8 assay at 0, 12, 24 and 48 h. (B) Cell invasion at 48 h was detected using a Transwell invasion assay (x200, 50 μm). (C) CCL28 levels in endometrial stromal cell supernatants were detected using an ELISA. (D) mRNA expression levels of CCL28, CCR10, MMP2, MMP9 and ITGB1 were examined using reverse transcription-quantitative PCR. (E) Activities of MMP2 and MMP9 were detected using gelatinase zymography. (F) Protein expression levels of CCL28, CCR10, MMP2, MMP9 and ITGB1 were detected via western blotting. (G) Protein expression levels of p-ERK/ERK ratio were detected via western blotting. **P<0.01 and ***P<0.001 vs. shNC. shNC, cells infected with negative control lentivirus; shCCL28-1 and -2, cells transduced with shCCL28 lentivirus-1 and -2; CCL28, C-C motif chemokine ligand 28; CCR10, CC chemokine receptor 10; ITGB1, integrin β1; sh, short hairpin RNA; p, phosphorylated; OD, optical density.

in the serum of patients with celiac disease and decreases following treatment (36). In *Helicobacter pylori* infection, upregulated CCL28 expression is associated with a risk of gastritis and peptic ulcer disease (37). In the present study, significantly elevated CCL28 expression was observed in the serum and endometrial tissues of patients with EM, alongside significantly increased CCR10 expression. Knockdown of CCL28 in endometrial stromal cells significantly suppressed cell proliferation and invasion. These results are consistent with a report that depletion of CCL27 can suppress cell proliferation and metastasis in ectopic endometrial stromal cells (17). Therefore, these results suggested that CCL28 may serve a critical role in the progression of EM, and knockdown of CCL28 may attenuate EM by inhibiting cell proliferation and invasion.

Furthermore, the potential mechanisms by which CCL28 regulated EM stromal cell proliferation and invasion were investigated. A significant decrease in mRNA and protein expression levels of CCR10, MMP2, MMP9 and ITGB1, as well as decreased ERK1/2 phosphorylation, were observed in shCCL28 endometrial stromal cells. Subsequently, various concentrations of CCL28 recombinant proteins (between 5 and 40 ng/ml) were tested on healthy endometrial stromal cells. Although there is still a small increase at 40 ng/ml and in general the increase of 40 ng/ml in cell proliferation and expression of CCL28 and CCR10 is smaller than that of 20 ng/ml. Thus, the concentration of 20 ng/ml was chosen for subsequent experiments. Treatment with 20 ng/ml CCL28 recombinant proteins significantly induced cell proliferation and invasion at 48 h, and relative protein levels of MMP2, MMP9, ITGB1 and p-ERK were significantly attenuated by the ERK inhibitor, PD98059. MMPs are important in tumor metastasis as a result of their degradation capacity of extracellular matrix (38). Gelatinases MMP2 and MMP9 are used as prognostic factors in numerous types of solid tumors (39,40). A previous study reported that compared with that in the normal endometrium, expression of MMPs is much greater in ectopic endometrium (41). MMP9 has been reported to be associated with the grade and stage of endometrial cancer, whereas MMP2 expression is related to CA125 expression and clinical progression in endometrial carcinoma (42,43). MMP9 overexpression enhances the invasion of the endometrium

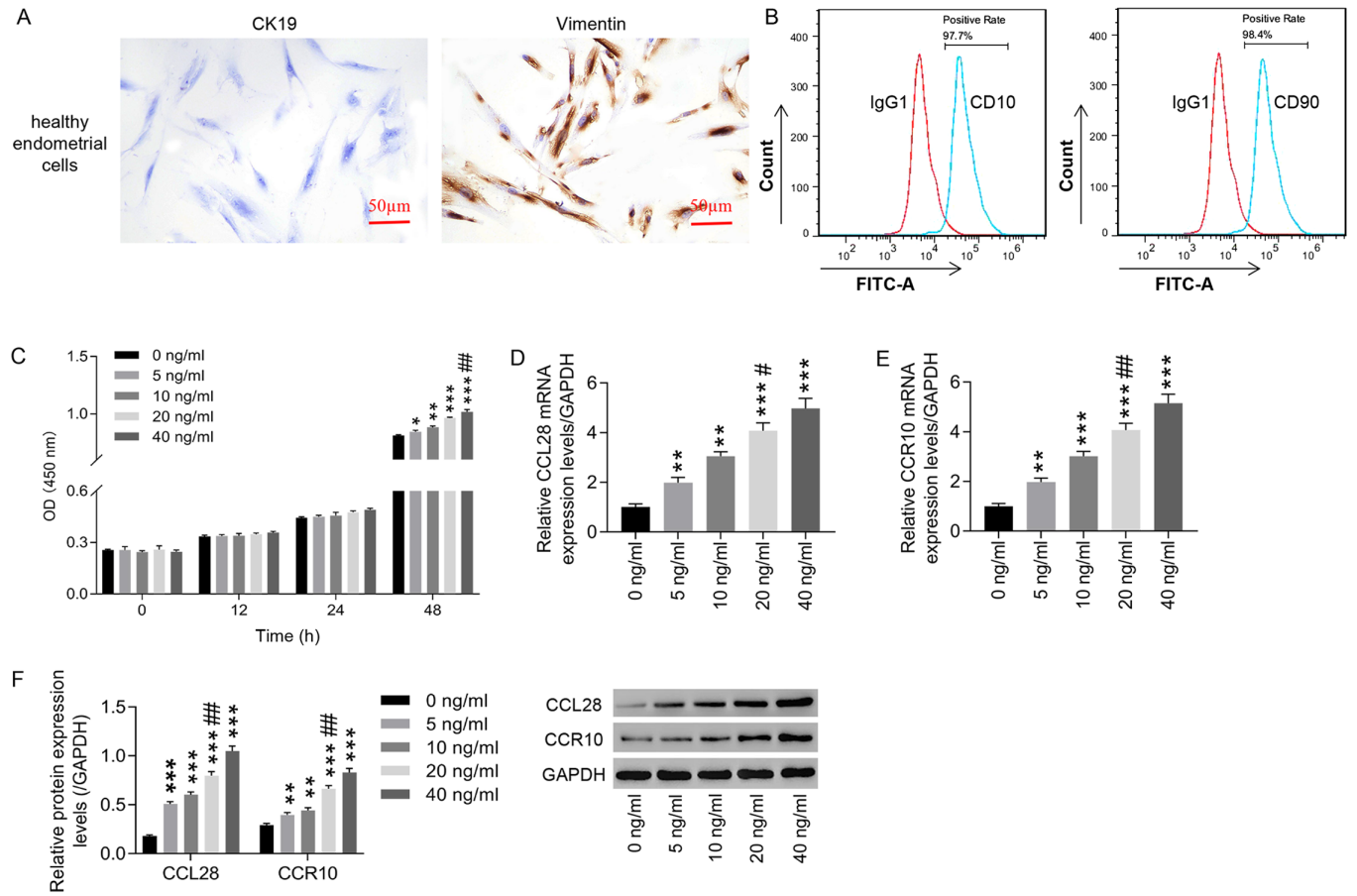

Figure 4. Treatment with CCL28 recombinant protein increases CCL28 and CCR10 expression in healthy human endometrial stromal cells. (A) Healthy human endometrial stromal cells were identified using immunocytochemistry to analyze CK19 and vimentin expression (x200, 50 μm). (B) CD10 and CD90 were detected via flow cytometry to identify the percentage of healthy endometrial stromal cells. Healthy human endometrial stromal cells were then treated with CCL28 recombinant protein at concentrations of 0, 5, 10, 20 and 40 ng/ml. (C) Cell proliferation was detected using a Cell Counting Kit-8 assay to determine the effect of CCL28 recombinant protein. Subsequently, at 48 h after CCL28 recombinant protein treatment, the mRNA expression levels of (D) CCL28 and (E) CCR10 were detected using reverse transcription-quantitative PCR. (F) Relative protein expression levels of CCL28 and CCR10 were analyzed via western blotting at 48 h after CCL28 recombinant protein treatment. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 ng/ml; and $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. 10 ng/ml. CCL28, C-C motif chemokine ligand 28; CCR10, CC chemokine receptor 10; CK19, cytokeratin-19; OD, optical density.

and the ability to degrade the extracellular matrix, loosening the connection between cells, thereby providing allowing ectopic endometrial tissue to enter the myometrium (44). MMP9 may also be involved in the formation of lesion blood vessels, providing nutrition for ectopic endometrium (45-47). Furthermore, previous studies have reported that the ERK1/2 signaling pathway is linked to EM progression, such as in endometriotic cell migration and apoptosis (20,22). It has also been demonstrated that estrogen can upregulate the expression levels of MMP2/MMP9 in endometrial epithelial cells via the VEGF-ERK1/2 signaling pathway (48). Furthermore, long non-coding RNA BRAF-activated non-protein coding RNA, can promote cell proliferation and invasion in endometrial cancer cells by regulating MMP2 and MMP1 via the ERK/MAPK signaling pathway (49). The present study demonstrated that CCL28 knockdown markedly inhibited the activities of MMP2 and MMP9, whereas CCL28 induced MMP2 and MMP9 activity, which was counteracted by the ERK inhibitor PD98059. Therefore, it can be inferred that CCL28 may promote endometrial stromal cell proliferation and invasion by regulating MMP2 and MMP9 via the ERK signaling pathway.

Integrins are important adhesion molecules on the surface of endometrial cells, are closely related to the pathogenesis of EM and serve important roles in signal transduction (50). ITGB1 is a member of the integrins. ITGB1 expression has been demonstrated to be upregulated in the endometrium of patients with EM, with microRNA-183 impacting EM progression by regulating stromal cell ITGB1 expression and function (51).

In the present study, ITGB1 expression was positively regulated by CCL28, and this was counteracted by the ERK inhibitor PD98059. Therefore, these results indicated that CCL28 may also regulate ITGB1 expression and function in endometrial stromal cells via the ERK signaling pathway. However, the effect of CCL28 on the development of EM was only investigated at the cellular level. Therefore, *in vivo* experiments are required to further confirm the therapeutic effect of CCL28 on EM and further explore underlying molecular mechanisms to expand the clinical application of CCL28. Although additional studies are required to further verify the function and mechanisms of CCL28 in the development of EM, the present study implied that CCL28 could be considered as a potential target for EM treatment.

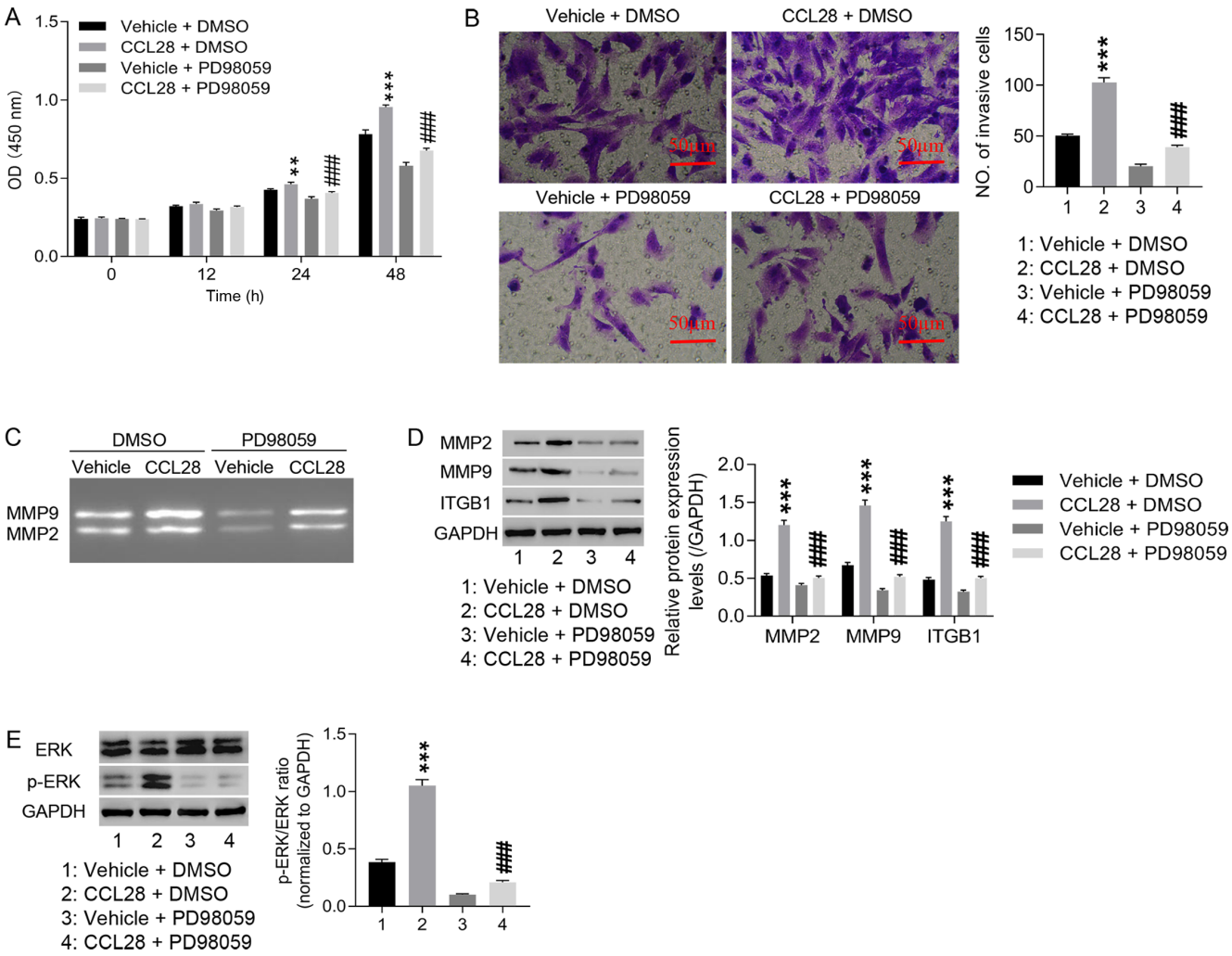

Figure 5. CCL28 may contribute to endometriosis progression by regulating MMP2, MMP9 and ITGB1 expression via activating the ERK signaling pathway. Healthy endometrial stromal cells were pre-treated with 10 μmol/l PD98059 (ERK inhibitor) for 30 min, and then treated with 20 ng/ml CCL28 recombinant protein for 48 h. (A) Cell proliferation was detected using the Cell Counting Kit-8 assay at 0, 12, 24 and 48 h. (B) Cell invasion was detected using the Transwell invasion assay at 48 h (x200, 50 μm). (C) MMP2 and MMP9 activity was detected via gelatinase zymography. Relative protein expression levels of (D) MMP2, MMP9 and ITGB1 and (E) the p-ERK/ERK ratio were analyzed via western blotting. **P<0.01, ***P<0.001 vs. vehicle + DMSO; and $^{\#\#\#}$P<0.001 vs. CCL28 + vehicle. Vehicle, solvent of CCL28 recombinant proteins; DMSO, solvent of PD98059; CCL28, C-C motif chemokine ligand 28; ITGB1, integrin β1; p, phosphorylated; OD, optical density.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HC and QZ conceived and designed the study. YW, FZ, WSu and WSh performed the experiments and collected and analyzed the data. HC and QZ wrote and revised the manuscript. HC and QZ have confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments conducted in the present study were approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital, School of Medicine, Tongji University (approval no. KS2154; Shanghai, China), and written informed consent was obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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