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Original Article

CXCL14 regulates ovarian endometriosis progression by targeting PCNA

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Abstract: Objective: To explore the regulatory function and mechanism of CXCL14 in endometriosis. Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine the expression of CXCL14 in eutopic and ectopic endometrial stromal cells (ESCs) derived from patients with endometriosis and in situ endometrial stromal cells derived from healthy individuals. Alterations in cell proliferation and migration capabilities were assessed through Cell Counting Kit-8 (CCK8) and transwell assays following the silencing or overexpression of CXCL14. Mass spectrometry was employed to identify potential interacting proteins of CXCL14, and proliferating cell nuclear antigen (PCNA) was selected for further investigation. The regulatory mechanism of PCNA by CXCL14 was further examined using co-immunoprecipitation (co-IP), Western blotting, and cellular experiments. Results: CXCL14 was highly expressed in ovarian endometriosis. The proliferative and migratory abilities of ESCs were positively correlated with CXCL14 expression levels. Moreover, CXCL14 interacted with PCNA. Silencing CXCL14 expression increased PCNA ubiquitination and promoted its degradation. Conversely, overexpression of PCNA mitigated the inhibitory effects of CXCL14 silencing on ESCs. Conclusions: CXCL14 may regulate PCNA through the ubiquitination pathway, thereby promoting the development and progression of endometrial stromal cells. This study provides new insights into the pathogenesis of endometriosis, highlighting the potential of CXCL14 as a therapeutic target.

Keywords: Endometriosis, CXCL14, PCNA, ubiquitination

Introduction

Endometriosis is a prevalent gynecological disorder characterized by the presence of endometrial tissues outside the uterine cavity. It affects approximately 5-10% of women worldwide. Clinical manifestations often include lower abdominal pain, abnormal menstruation, and even infertility [1], with ovarian endometriosis being the most common type. Endometriosis significantly impacts patients' physical health, mental health, and quality of life [2]. Among the various pathophysiological mechanisms of endometriosis, the retrograde menstruation theory is widely recognized. However, a growing body of evidence indicates that endometriosis also has significant effects on systemic health [3]. For instance, women diagnosed with endometriosis via laparoscopy have a significantly higher risk of myocardial infarction compared to healthy women [4]. Additionally, women with endometriosis are more likely to suffer from depression and anxiety [5]. Although the pathogenesis of endometriosis remains unclear, numerous immune-related cells are associated with the occurrence and progression of the disease. For example, compared to the control group, patients with endometriosis exhibit a decreased phagocytic capacity of peritoneal macrophages [6]. Furthermore, the infiltration of neutrophils in the peritoneal fluid of patients with endometriosis is increased [7]. Co-culture with macrophages enhances the clonogenic and invasive capabilities of endometriotic stromal cells [8].

Ubiquitin-proteasome degradation is a common pathway for protein degradation, and it is a complex process. Ubiquitination requires the coordinated action of the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin-ligating enzyme E3 [9],

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which facilitates the sequential binding of ubiquitin to the lysine residues of the target protein. The E3 enzyme is responsible for specifically identifying the target protein [10], and is crucial for the process of ubiquitination. The ubiquitin-proteasome degradation pathway plays a significant role in various diseases. For example, it is involved in the occurrence and development of breast cancer [11]. Additionally, 2-deoxy-d-ribose (dRib) induces ferroptosis in renal tubular epithelial cells by degrading the xCT protein through the ubiquitin-proteasome system (UPS), thus reducing intracellular cysteine uptake [12]. Ubiquitination also contributes to the pathogenesis of endometriosis. For instance, Yunjun Sun et al. discovered that the carboxyl terminus of Hsc70-interacting protein (CHIP) interacts with high mobility group box-1 protein (HMGB1), promoting its ubiquitination-mediated degradation and suppressing aerobic glycolysis and the progression of endometriosis [13].

C-X-C motif chemokine ligand 14 (CXCL14), also known as breast and kidney-expressed chemokine (BRAK), is a member of the chemokine CXC family located on human chromosome 5q31. It induces the migration of immune cells and plays roles in immune surveillance, inflammation, and cancer [14, 15]. CXCL14's role in various diseases is context-dependent. In oral squamous cell carcinoma (OSCC), CXCL14 expression is significantly downregulated in lymph node metastatic carcinoma compared to the primary tumor, suggesting a potential involvement in preventing invasion and metastasis [16]. Moreover, obese patients, especially those with type 2 diabetes, have lower circulating levels of CXCL14 and CXCL14, which are negatively correlated with body mass index (BMI) and blood glucose/insulin stability impairment index [17]. CXCL14 expression was also markedly reduced in both HPV-positive cancers in the head, neck, and cervix [18]. However, a report indicated that CXCL14 was overexpressed in lung cancer, promoting epithelial-mesenchymal transition (EMT) and the metastasis of non-small cell lung cancer (NSCLC) cells through ACKR2 [19]. Additionally, Yanyang Xu et al. found that in osteosarcoma, high levels of CXCL14 stimulated fibroblasts to generate transforming growth factor-β (TGF-β) and enhanced the invasion and migration of osteosarcoma cells [20]. In pancreatic cancer, CXCL14 expression was upregulated, significantly increasing the invasiveness of pancreatic cancer cells [21].

The mechanism of CXCL14 in endometriosis remains underexplored. This study aimed to investigate the role of CXCL14 in the pathogenesis and progression of endometriosis. We examined CXCL14 expression in endometrial stromal cells (ESCs) from healthy individuals (in situ endometrium) and patients with endometriosis (eutopic and ectopic endometrium). Additionally, we assessed how CXCL14 influenced the proliferative and migratory capabilities of ESCs. Furthermore, we investigated the interaction between CXCL14 and proliferating cell nuclear antigen (PCNA) as well as its involvement in ubiquitination, a key protein degradation pathway.

Materials and methods

Tissue collection, as well as cell isolation and culture

We collected 20 eutopic endometrium samples from individuals without endometriosis, 22 eutopic endometrium samples from patients with endometriosis, and 24 ectopic endometrium samples from patients with ovarian endometriosis between February 2023 and July 2023. All patients had not received any special treatment for at least six months prior and they provided informed consent. This research was approved by the Ethics Committee of Suzhou Hospital affiliated with Nanjing Medical University (GSKY20210216). CXCL14 expression was determined through literature review. Endometrial stromal cells (ESCs) were derived from control endometrium (EuCo) of individuals without endometriosis, eutopic endometrium from endometriosis (EuEM) and ectopic endometrium of patients with endometrioma (OvEM). ESCs were isolated by mincing endometrial specimens into small pieces, which were then digested with type IV collagenase (Sigma, USA) in a thermostatic shaking incubator at 37°C and 150 rpm for approximately one hour. The digested tissue suspension was sequentially filtered through sterile 70 μm and 40 μm screens. The filtrate was rinsed repeatedly with DMEM/F12 medium containing 10% fetal bovine serum (FBS; ScienCell, USA). The rinsed filtrate was collected and centrifuged at 1,000 rpm for five minutes. The cell pellet was suspended in the culture medium and incubated in a 5% CO_2 atmosphere at 37°C [22].

Human telomerase reverse transcriptase (hTERT)-induced immortalized cells (iheESCs) and immortalized human endometriotic stromal cells (ihESCs) were purchased from IMMOCELL (China) and were authenticated by STR analysis. The cells were cultured in DMEM/F12 (MeilunBio, China) medium, supplemented with 10% FBS (ScienCell, USA) and 1% penicillin-streptomycin (NCM, China) at 37°C and 5% CO_2 in a culture incubator. Additionally, 293T cells were cultured in DMEM (MeilunBio, China) supplemented with 10% FBS (Excell Bio, New Zealand) and 1% antibiotics (NCM, China) under the same conditions as described above.

Cell transfection

When the cell density reached 60-80%, cells were transfected with the relevant siRNA (GenePharma, China) using lipofectamine RNAiMAX (Invitrogen, USA). The siRNA sequences were as follows: si-NC 5'-UUCUCCGAACGUGUCACGUTT-3', si-CXCL14-1# 5'-UGGACGGGUCCAAAUGCAATT-3', si-CXCL14-2# 5'-GCGAGGAGAAGAUGGUUAUTT-3', si-PCNA-1# 5'-GCGUGAACCUCACCAGUAUTT-3', si-PCNA-2# 5'-CCGGCAAUGAAGAUAUCAUTT-3'.

Following the provided protocol, the X-treme GENE HP DNA Transfection Reagent (Roche, Switzerland) was used to introduce overexpression plasmids into the cells. All overexpression plasmids were designed and constructed by Sangon Biotechnology Inc. (Shanghai, China). These plasmids included: pcDNA3.1-Flag-CXCL14, pEGFP-PCNA and pRK5-HA-Ub.

In the experiments involving MG132 (a proteasome inhibitor), cells were transfected for 48 hours and then treated with 20 μM MG132 for six hours. Afterward, further experiments were conducted.

RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from treated cells using Trizol reagent (Vazyme, China), then, cDNA was generated using reverse transcription-related reagents (Cat.R323-01, Vazyme, China). The expression levels of relative mRNA were assessed with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China) utilizing an Applied Biosystems 7500 Real-Time PCR System. The following provided the relevant primer information: 18s RNA-F: 5'-AAACGGCTACCACATCCAAG-3', 18s RNA-R: 5'-CCTCCAATGGATCCTCGTTA-3', CXCL14-F: 5'-TCCTGTGATGGCGAGACAAA-3', CXCL14-R: 5'-GTGTTGGGAACCTCACATGC-3', PCNA-F: 5'-ACTCGTCCCACGTCTCTTTG-3', PCNA-R: 5'-CATTGCCGGCGCATTTTAGT-3'.

Western blotting

Cells were washed twice with precooled PBS and lysed using RIPA buffer (RIPA, NCM) containing 1% PMSF (Beyotime) as a protease inhibitor. The supernatant was collected, and protein concentration was quantified using the BCA assay kit (BCA, NCM). Finally, proteins were denatured by heating at 100°C. Then, the samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, GenScript, China) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, USA). The membranes were blocked with skimmed milk for one hour and incubated with the corresponding primary antibody at 4°C overnight. The next day, the membranes were washed with TBST (Tris buffered saline with 0.1% Tween-20) three times and incubated with the corresponding secondary antibody at room temperature for one hour. Then, the membranes underwent three additional washes. Finally, images were acquired by the protein blot detection system and quantified with Image-Pro Plus 6.0 as previously described [23]. The antibodies that were utilized comprised anti-CXCL14 (1:1000; ab137541; Abcam), anti-PCNA (1:2000; 10205-2-AP; Proteintech), anti-Tubulin (1:3000, Cat No. AT819, Beyotime), anti-HA (1:500; sc-7392; Santa Cruz), anti-GFP (1:2000; ab290; Abcam) and anti-Flag (1:1000; F1804; Sigma).

Cell proliferation assays

After transfection, the cells were plated into 96-well plates at a density of 3,750 cells per well. Cell viability was evaluated using the Cell Counting Kit-8 (CCK8; APE×BIO) according to the standard protocol previously described [24, 25]. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad Model 680, USA) at 0, 24, 48, 72 and 96 hours.

Cell migration assays

Cell migration was assessed using a transwell assay in a 24-well plate with an 8 μm pore membrane (Corning, USA). Specifically, 300 μl of serum-free medium containing 50,000 cells

was added to the upper chamber, while 700 μl of complete medium was used as a chemotactic agent in the lower chamber. After an incubation period of 48 hours, migrating cells in the lower chamber were fixed and stained, as previously described [26-28]. Images were then captured using a microscope for quantification.

Co-immunoprecipitation (co-IP) and liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Primary human endometrial stromal cells were transfected with either the empty vector or pcDNA3.1-Flag-CXCL14 plasmid. After 48-72 hours, cells were collected and lysed using RIPA lysis buffer (NCM, China). The samples were centrifuged to remove cell debris, and the supernatants were carefully collected. To reduce non-specific protein binding, 20 μL of protein A/G magnetic beads (Vazyme, China) were added to each supernatant, followed by incubation at a low speed for 3 hours. Subsequently, the lysates were incubated overnight (12-16 hours) at 4°C with an appropriate amount of anti-FLAG nanobody agarose beads (AlpaLifeBio, China). The immunoprecipitates were washed thrice using RIPA lysis buffer to remove unbound proteins. The eluted samples were heated at 100°C for 10 minutes with 1× sodium dodecyl sulfate (SDS) buffer. The samples were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and LC-MS/MS was used to analyze the immunoprecipitation (IP) products, as previously described [28, 29].

Ubiquitination assay

Cells were transfected with specific plasmids and siRNA, followed by cultivation for 48 hours. Before sample collection, the cells were treated with 20 μM MG132 for 6 hours to inhibit proteasomal degradation. Subsequently, the cells were lysed using RIPA lysis buffer, and PMSF was added to inhibit protease activity. GFP-PCNA was purified using anti-GFP magnetic beads (AlpaLifeBio, China). Thereafter, the samples were incubated with anti-GFP antibodies to assess PCNA expression, and anti-HA antibodies to detect ubiquitin (Ub).

Protein half-life assay

293T cells were transfected with either si-NC or si-CXCL14 for 48-72 hours. Then, the cells were treated with cycloheximide (CHX) at a concentration of, 100 μg/mL to block protein synthesis. Finally, total proteins were collected from the cells following treatment. The expression levels of PCNA were quantified through Western blotting.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 9. All experiments were performed at least three times independently. Results were expressed as mean ± SD. Student's t-test and one-way ANOVA were employed for comparisons. A *p*-value of less than 0.05 was deemed statistically significant.

Results

CXCL14 exhibits high expression levels in ectopic endometrial stromal cells (ecESCs) from endometrioma

We used RT-qPCR to evaluate the expression levels of CXCL14 in ESCs derived from the control endometrium (EuCo), eutopic endometrium from endometriosis (EuEM), and ectopic endometrium of patients with endometrioma (OvEM). The mRNA levels of CXCL14 were significantly elevated in the OvEM group compared to ESCs from EuCo and EuEM. However, no significant difference in CXCL14 expression was observed between EuCo and EuEM (**Figure 1A**).

Silencing of CXCL14 suppresses ecESCs proliferation and migration

To investigate the effect of CXCL14 on the proliferation and migration of *ecESCs*, we knocked down CXCL14 in ecESCs by transfecting them with siRNA. The efficiency of siRNA knockdown was confirmed using RT-qPCR analysis (**Figure 1B**). CCK-8 proliferation assays and transwell assays revealed that, compared to the negative control (si-NC), both proliferation and migration capabilities were significantly reduced following the inhibition of CXCL14 expression in ecESCs (**Figure 1C-E**).

Overexpression of CXCL14 enhances the proliferation and migration of eutopic endometrial stromal cells (euESCs)

We transfected Flag-CXCL14 into euESCs for overexpressing CXCL14, which was verified by Western blotting (**Figure 2A**). The results of

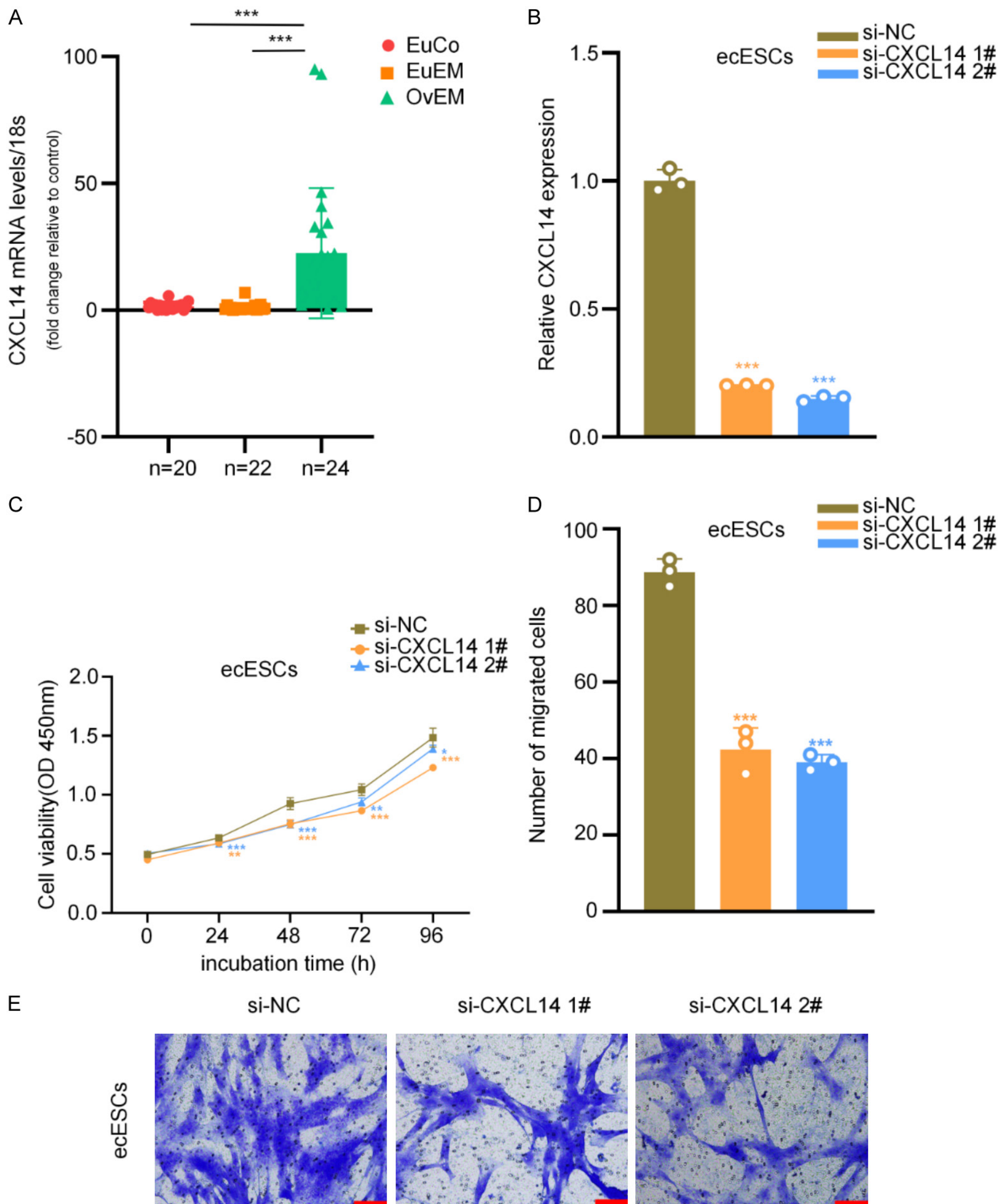

Figure 1. C-X-C motif chemokine ligand 14 (CXCL14) exhibits high expression levels in ectopic endometrial stromal cells (ecESCs) from endometrioma. Silencing of CXCL14 suppresses ectopic endometrial stromal cells proliferation and migration. A. The mRNA levels of CXCL14 in EuCo, EuEM and OvEM were examined by RT-qPCR. B. CXCL14 mRNA level in ecESCs transfected with si-NC and si-CXCL14. C. Cell Counting Kit-8 (CCK8) assays showed the proliferation of ecESCs after CXCL14 knockdown. D, E. Transwell experiments showed that the migration of ecESCs was reduced after CXCL14 knockdown. Scale bar = 100 μm. Each experiment was independently repeated three times. *P<0.05, **P<0.01, ***P<0.001 compared with si-NC.

CCK-8 proliferation assays and transwell assays indicated that the overexpression of CXCL14 promoted the proliferation and migration of euESCs (**Figure 2B-D**).

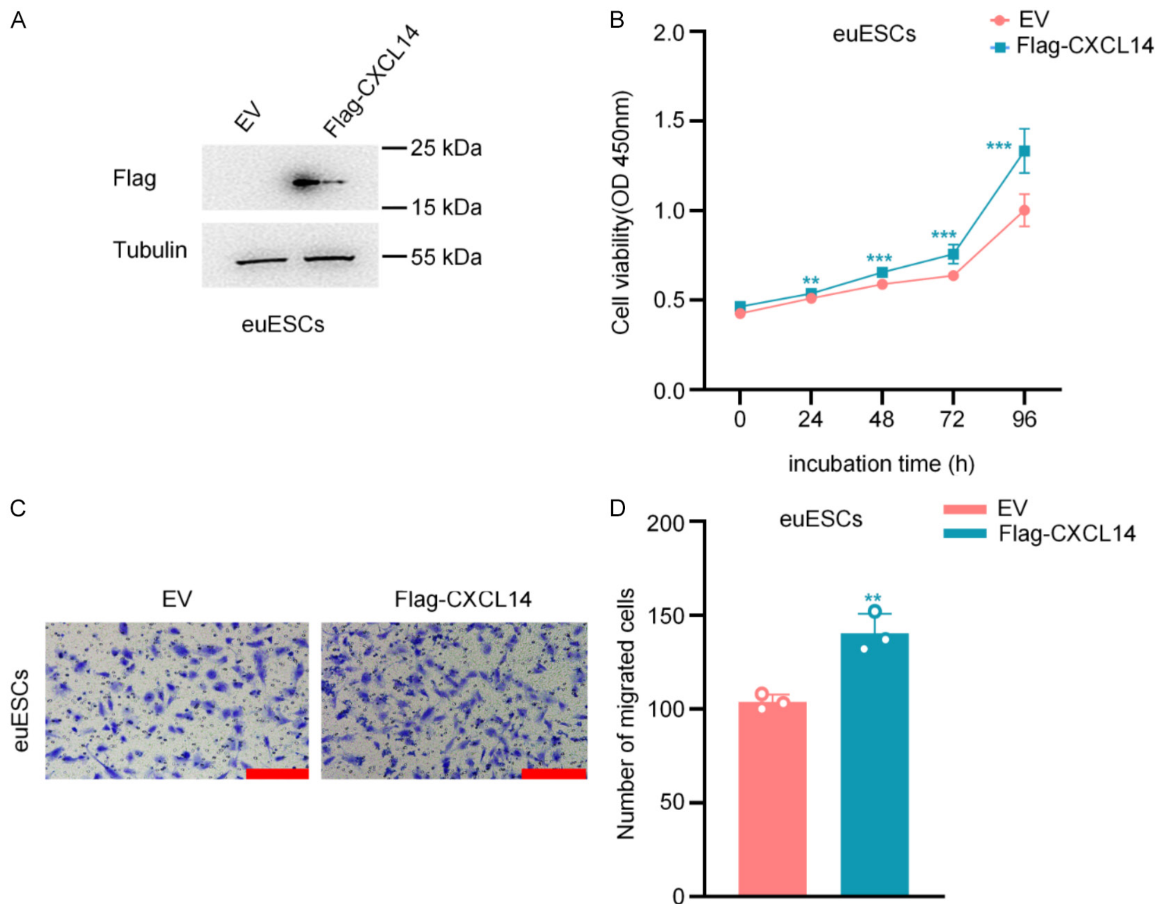

Figure 2. Overexpression of CXCL14 enhances the proliferation and migration of euESCs. A. Western blotting analysis of CXCL14 expression in euESCs transfected with the Flag-CXCL14 plasmid and empty vector. B. CCK8 assays showed the proliferation of euESCs after CXCL14 overexpression. C, D. Transwell experiments showed that the migration of euESCs was enhanced after CXCL14 overexpression. Scale bar = 200 μm. Each experiment was independently repeated three times. *P<0.05, **P<0.01, ***P<0.001 compared with empty vector.

CXCL14 interacts with PCNA

To further investigate the molecular mechanism of CXCL14 in ESCs, we purified and enriched CXCL14 through co-immunoprecipitation (co-IP), followed by mass spectrometry analysis of the resulting product. This approach led to the identification of 31 interacting proteins with unique peptide ≥3 (Supplementary Table 1). Proliferating cell nuclear antigen (PCNA), a well-known molecular marker of proliferation, was among the identified interactors [30]. In the context of a mouse model of endometriosis, PCNA staining has been used to assess ESC proliferation [31]. Furthermore, it was reported that after 6 months of treatment with the levonorgestrel-releasing intrauterine system (LNG-IUS), PCNA expression was reduced in both eutopic and ectopic endometrial tissues [32]. Based on these findings, we focused on further investigating the interaction between CXCL14 and PCNA. Given the limitations in the passage of primary ESCs and the challenges associated with protein collection, we conducted an immunoprecipitation (IP) assay in 293T cells co-overexpressing both CXCL14 and PCNA to validate their interaction (**Figure 3A-C**).

PCNA promotes the proliferation and migration of ESCs

Due to the limited passage number of primary ESCs, we chose to knock down PCNA in immortalized human endometriotic stromal cells (ihESCs) using siRNA to investigate the role of PCNA in the proliferation and migration of ESCs. The knockdown efficiency of si-PCNA was confirmed via RT-qPCR analysis (**Figure 4A**). CCK-8 proliferation assays and transwell assays showed that, compared to the negative

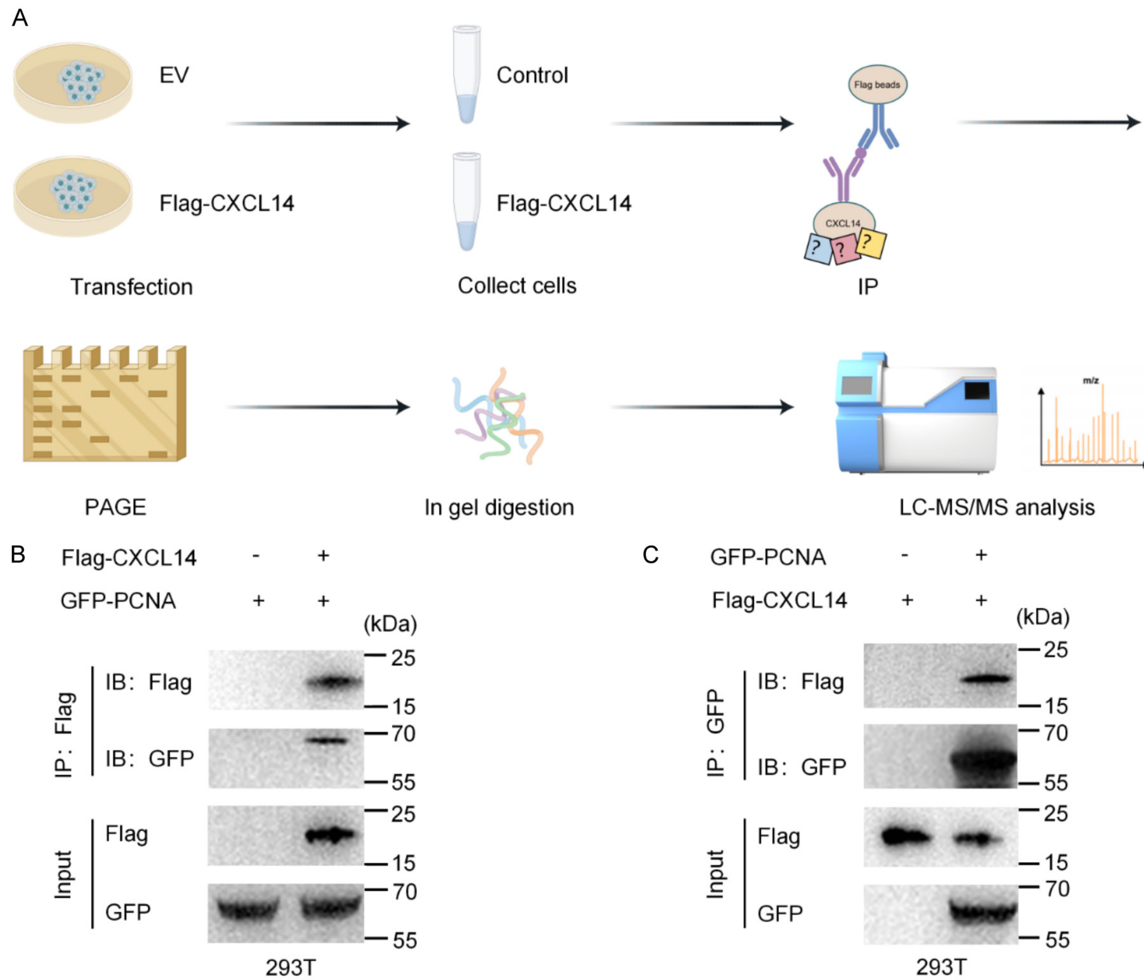

Figure 3. CXCL14 interacts with PCNA. A. The diagram identifying proteins that interact with CXCL14 in ecESCs. Flag-CXCL14 plasmid and empty vector was transfected into ecESCs. Total protein was harvested and subjected to immunoprecipitation (IP), with anti-Flag beads, SDS-PAGE of the immunoprecipitated products, in-gel digestion, and LC-MS/MS (By Figdraw). B, C. Reciprocal IP assays of 293T cells lysated with anti-Flag and anti-GFP beads, followed by Western blotting analysis with anti-Flag or anti-GFP antibodies.

control si-NC, both proliferation and migration were significantly impaired in ihESCs with reduced PCNA expression (**Figure 4B-D**). Additionally, we transfected GFP-PCNA into human telomerase reverse transcriptase (hTERT)-induced immortalized cells (iheESCs) to overexpress PCNA, with Western blotting confirming successful overexpression (**Figure 4E**). The results from CCK-8 proliferation and transwell assays demonstrated that overexpression of PCNA enhanced both the proliferation and migration of iheESCs (**Figure 4F-H**).

CXCL14 destabilizes PCNA by promoting ubiquitination

To further investigate how CXCL14 regulates PCNA, we examined changes in PCNA expression by modulating CXCL14 levels in ESCs. We found that knocking down CXCL14 in ihESCs led to a decrease in PCNA expression, whereas overexpressing CXCL14 resulted in a corresponding increase in PCNA expression (**Figure 5A-D**). To confirm these findings, we used 293T cells to assess the effect of CXCL14 on PCNA protein stability through a cycloheximide (CHX) experiment, due to the limitations of working with ESCs. Western blot analysis revealed that the protein level of endogenous PCNA in 293T cells was also reduced when CXCL14 was knocked down (**Figure 5E**, **5F**). These results suggest that, upon CXCL14 knockdown, PCNA undergoes degradation, with a reduced half-life, indicating that CXCL14 may play a role in preventing PCNA degradation and maintaining its stability.

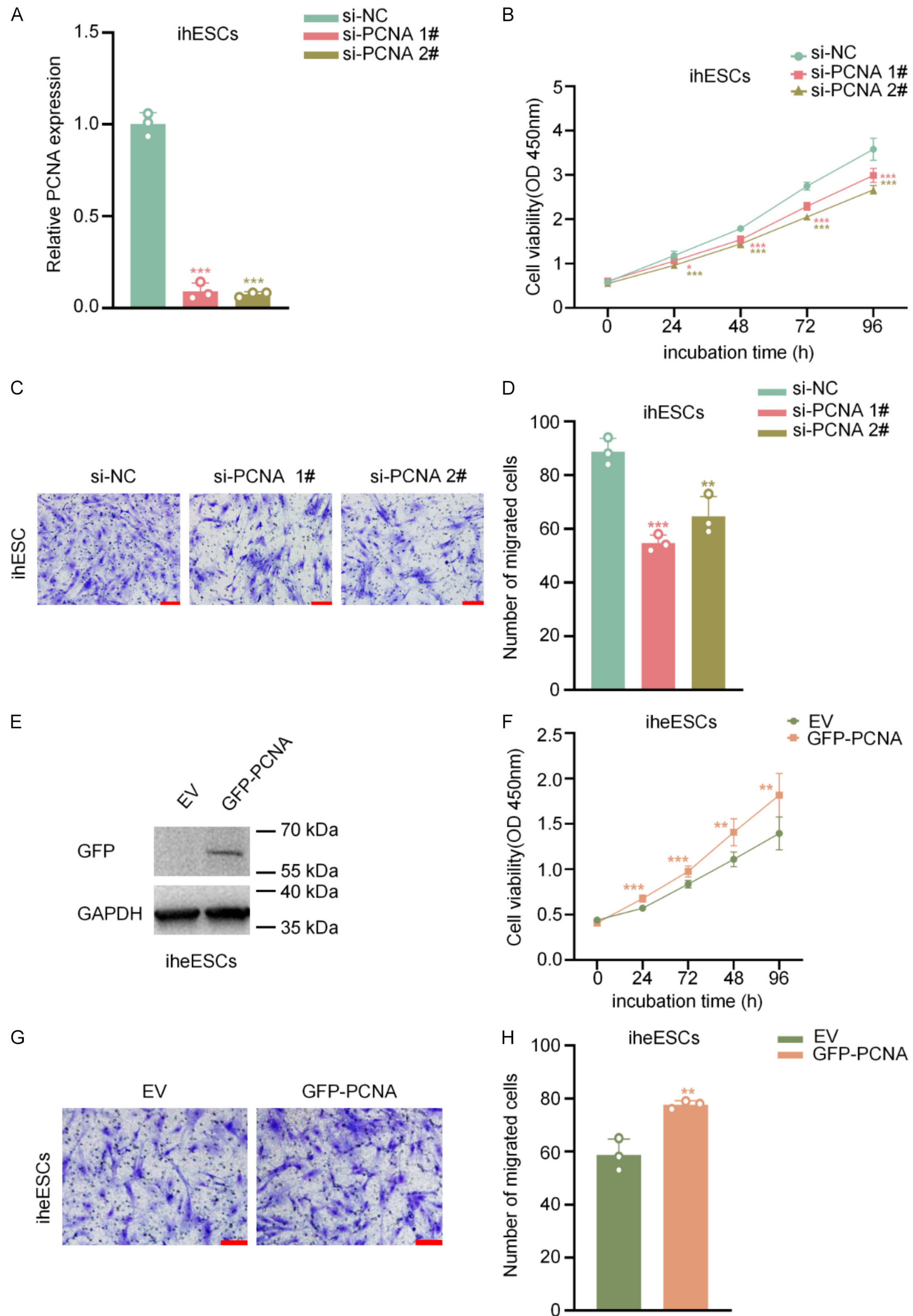

Figure 4. PCNA promotes the proliferation and migration of ESCs. A. PCNA mRNA levels were measured by qRT-PCR in immortalized human endometriotic stromal cells (ihESCs) transfected with si-NC and si-PCNA. B. CCK8 assays

showed the proliferation of ihESCs after PCNA knockdown. C, D. Transwell experiments showed that the migration of ihESCs was reduced after PCNA knockdown. Scale bar = 100 μm. E. Western blotting analysis of PCNA expression in human telomerase reverse transcriptase (hTERT)-induced immortalized cells (iheESCs) transfected with the pEGFP-PCNA plasmid and empty vector. F. CCK8 assays showed the proliferation of iheESCs after PCNA overexpression. G, H. Transwell experiments showed that the migration of iheESCs was enhanced after PCNA overexpression. Scale bar = 100 μm. Each experiment was independently repeated three times. *P<0.05, **P<0.01, ***P<0.001 compared with si-NC or empty vector.

Given that ubiquitination is a primary pathway for protein degradation, we hypothesized that CXCL14 might regulate PCNA through this mechanism. To test this, we knocked down CXCL14 in 293T cells and co-transfected them with PCNA and Ub plasmids. Co-immunoprecipitation (Co-IP) assays revealed a significant increase in the ubiquitination of PCNA following CXCL14 knockdown in 293T cells treated with MG132 for 6 hours (**Figure 5G**). These findings suggest that CXCL14 may regulate PCNA stability through the ubiquitin-proteasome pathway.

PCNA overexpression mitigates the phenotype of ihESCs induced by the silencing of CXCL14

To investigate the potential role of CXCL14 in ESC development through PCNA, we transfected induced human ESCs (ihESCs) with a PCNA plasmid while knocking down CXCL14. We found that, compared to the negative control group, the CXCL14 knockdown group exhibited significantly reduced PCNA expression. In contrast, the expression of PCNA was elevated in the si-CXCL14+OE-PCNA group compared to the CXCL14 knockdown group (**Figure 6A**, **6B**). CCK8 and transwell assays showed that silencing CXCL14 inhibited the proliferation and migration of ihESCs, whereas overexpression of PCNA in CXCL14-silenced cells alleviated these effects (**Figure 6C-E**). These results suggested that PCNA mitigate the inhibitory effects of si-CXCL14 on ESCs, highlighting the CXCL14's role in promoting ESCs development through the regulation of PCNA.

Discussion

Although endometriosis is a benign disorder, it shares similar characteristics with cancer, such as lesion dissemination and tissue invasion [33]. CXCL14 is a highly conserved homeostatic chemokine that extensively modulates chemotaxis, differentiation, and activation of various immune cell types [34]. CXCL14 is highly expressed in patients with advanced ovarian cancer, promoting the proliferation, migration, and invasion of ovarian cancer cells, and correlates with an unfavorable prognosis [35, 36]. Additionally, CXCL14 expression is upregulated in endometrial carcinoma, and patients with higher CXCL14 expression tend to have better overall survival [37]. It has also been reported that the expression of CXCL14 in the endometriosis group is 12.5 times higher than in the control group [38]. This finding aligns with our research, which also demonstrated that CXCL14 was highly expressed in endometrial stromal cells (ESCs) from endometriosis endometrial stromal cells (ESCs) from endometriosis, enhancing their proliferation and migration.

The ubiquitin-proteasome system (UPS) is mediated by a cascade of ubiquitin (Ub) enzymes. Ub is initially activated by the E1 enzyme and then transferred to the E2 conjugating enzyme. The E2 Ub-conjugating enzyme subsequently transfers Ub to the E3 Ub ligase via transferase activity. The transferase binds Ub to the lysine (Lys) residues of the target protein, forming an isopeptide bond with the C-terminal glycine (Gly), This process is repeated multiple times using the lysine of Ub as the substrate, resulting in the formation of a Ub chain [11, 39]. Ubiquitination is involved in various cellular processes, such as DNA damage response, cell metabolism, and lipid metabolism. For example, a high cholesterol load promotes the ubiquitination of proteins involved in cholesterol biosynthesis and uptake, while concurrently reducing the ubiquitination of proteins associated with cholesterol efflux [40]. Several studies have investigated the role of ubiquitination in endometriosis. For instance, tripartite motif (TRIM) 59 inhibits PPM1A through ubiquitination, activating the TGF-β/Smad pathway and facilitating the invasion of endometrial stromal cells in endometriosis [41].

Mass spectrometry identified PCNA as a CXCL14-interacting protein, which was confirmed by co-immunoprecipitation and Western blotting. We found that knockdown of CXCL14 increased the ubiquitination of PCNA, leading to

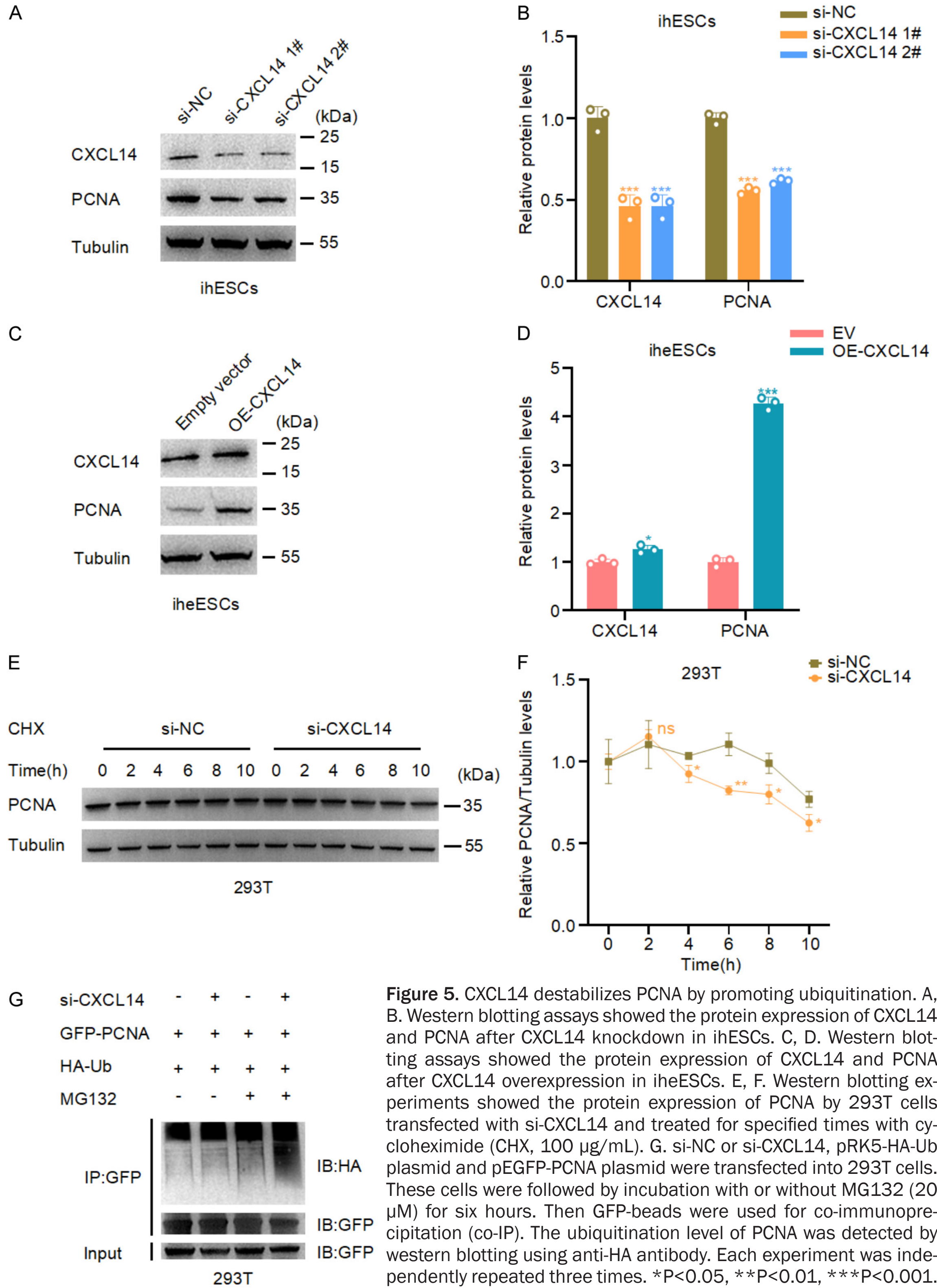

Figure 5. CXCL14 destabilizes PCNA by promoting ubiquitination. A, B. Western blotting assays showed the protein expression of CXCL14 and PCNA after CXCL14 knockdown in ihESCs. C, D. Western blotting assays showed the protein expression of CXCL14 and PCNA after CXCL14 overexpression in iheESCs. E, F. Western blotting experiments showed the protein expression of PCNA by 293T cells transfected with si-CXCL14 and treated for specified times with cycloheximide (CHX, 100 μg/mL). G. si-NC or si-CXCL14, pRK5-HA-Ub plasmid and pEGFP-PCNA plasmid were transfected into 293T cells. These cells were followed by incubation with or without MG132 (20 μM) for six hours. Then GFP-beads were used for co-immunoprecipitation (co-IP). The ubiquitination level of PCNA was detected by western blotting using anti-HA antibody. Each experiment was independently repeated three times. *P<0.05, **P<0.01, ***P<0.001.

its degradation. However, these results were only verified in 293T cells and have not yet been confirmed in endometrial stromal cells (ESCs). Therefore, future research should identify the ubiquitination sites and validate these findings in ESCs from endometriosis.

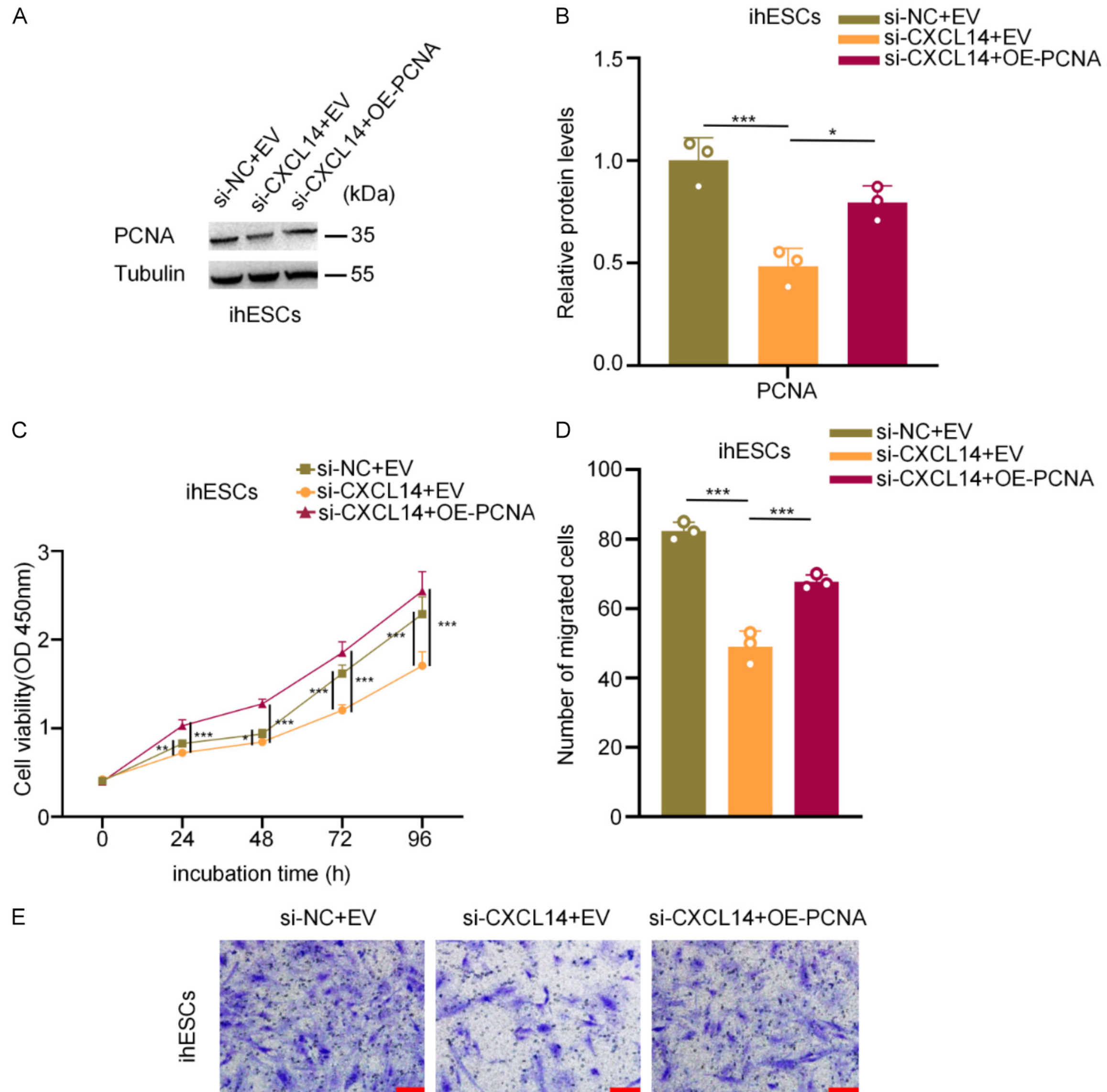

Figure 6. PCNA overexpression mitigates the phenotype of ihESCs induced by the silencing of CXCL14. A, B. Western blotting analysis of PCNA expression in ihESCs transfected with si-NC+EV, si-CXCL14+EV or si-CXCL14+OE-PCNA. C. CCK8 assays of cell viability. D, E. Transwell assays of cell migration. Scale bar = 100 μm. Each experiment was independently repeated three times. *P<0.05, **P<0.01, ***P<0.001.

Proliferating cell nuclear antigen (PCNA), located on the negative strand of chromosome 20p12.2, is a member of the DNA sliding clamp family and forms a homologous trimeric ring with three hydrophobic pockets [42]. PCNA plays a crucial role in DNA replication and repair, and contributes to processes such as the cell cycle, apoptosis, and chromosome metabolism [43]. Several studies have investigated the role of PCNA in endometriosis. Hyun Jin Kim et al. established an in vivo model using dienogest treatment and found that the size of the implanted endometrial tissue in mice was significantly reduced, accompanied by decreased PCNA expression [44]. Wei Liu et al. demonstrated that the expression of GSTM4 was significantly increased in the endometrium of patients with endometriosis. After treatment with NBDHEX (a GST inhibitor), the proliferation, migration, and invasion abilities of ESCs decreased, apoptosis increased, and PCNA expression was simultaneously reduced [45]. Previous studies have also shown that si-CXCL14 treatment can inhibit PCNA expression in L929

cells [46]. Our research confirmed the regulatory effect of CXCL14 on PCNA in ESCs. Additionally, our findings showed that the knockdown of PCNA inhibited ESC proliferation and migration, while overexpression of PCNA had a promoting effect. Furthermore, rescue experiments demonstrated that PCNA could counteract the inhibitory effect of CXCL14 knockdown on ESCs. However, the specific mechanism underlying the interaction between CXCL14 and PCNA still requires further investigation.

Our study has some limitations. For example, the collection of endometriosis samples was relatively limited, focusing solely on ovarian endometriosis and not including other types, such as peritoneal endometriosis. Future studies should aim to collect a broader range of samples to provide a more comprehensive understanding of the role of CXCL14 in different forms of endometriosis. Additionally, in vivo experiments, such as animal models, are necessary to confirm the regulatory mechanism of CXCL14 and PCNA.

In conclusion, our research provides new insights into the regulatory function and mechanism of CXCL14 in ovarian endometriosis. The positive correlation between CXCL14 expression and the proliferation and migration abilities of ESCs, as well as the involvement of PCNA in this process, suggests that CXCL14 may play a significant role in the pathogenesis of endometriosis. These findings, which have not been previously reported in endometriosis studies, may contribute to a better understanding of the disease and offer new diagnostic targets.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Estimation of protein expression levels using the LFQ algorithm with unique peptide ≥3. LC-MC/MS identified 31 proteins in ectopic endometrial stromal cells (ecESCs)

Gene names	Unique peptides		LFQ intensity	
	Flag-EV	Flag-CXCL14	Flag-EV	Flag-CXCL14
OASL	0	7	0	14970000
ACTN4	0	5	0	9368100
SNRNP40; DKFZp434D199	0	5	0	12933000
GNAS	0	4	0	6364500
PPP2R1A	0	4	0	8512400
TXN	0	4	0	20794000
FDPS	0	4	0	11117000
REEP5	0	4	0	13547000
PSMD5	0	4	0	6166100
RUVBL2	0	4	0	5260000
LPP	0	3	0	14166000
IDH3B	0	3	0	4057400
ACAA2	0	3	0	6170200
CXCL14	0	3	0	18632000
ASCC1	0	3	0	11188000
HARS	0	3	0	5950400
XPO1	0	3	0	3543000
NAP1L1	0	3	0	14952000
PICALM; SNAP91	0	3	0	4792200
YLPM1	0	3	0	6137400
TRIP11	0	3	0	14243000
SAP18	0	3	0	20289000
LONP1	0	3	0	5599700
PCNA	0	3	0	6317900
PCMT1	0	3	0	9509000
SUCLG1	0	3	0	6424400
CKAP5	0	3	0	4455800
CACTIN	0	3	0	4768400
RPL36AL; RPL36A; RPL36A-HNRNPH2	0	3	0	22464000
BUD13	0	3	0	6163100
GOPC	0	3	0	4412900