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Original Article

Inhibition of CDC42 in adenomyosis reduces the proliferation of endometrial stromal cells and the migration of epithelial cells

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Abstract: Objectives: Adenomyosis severely impairs fertility and quality of life. This study investigated the pathophysiologic role of cell division cycle 42 (CDC42) in adenomyosis by profiling its expression, identifying its binding partners, and performing functional assays on endometrial cells. Methods: Immunofluorescence was used to evaluate CDC42 expression in endometrial cells from adenomyosis patients. RNA sequencing (RNA-seq) was used to analyze transcriptomic changes following CDC42 knockdown. The effects of CDC42 inhibition on endometrial stromal cell proliferation and migration were assessed using cell viability and migration assays. Furthermore, coimmunoprecipitation coupled with mass spectrometry (Co-IP/MS) was conducted to identify CDC42-interacting proteins. Finally, conditioned medium from CDC42-knockdown stromal cells was used to evaluate the effects of paracrine signaling on endometrial epithelial cell migration. Results: CDC42 expression was significantly upregulated in the endometrial cells of adenomyosis patients. Transcriptomic analysis revealed that CDC42 knockdown downregulated the expression of genes associated with the cell cycle and cytokine signaling. Functionally, CDC42 inhibition markedly reduced stromal cell proliferation and attenuated the migration of both stromal and epithelial cells. Notably, conditioned medium from CDC42-knockdown cells also suppressed epithelial cell migration. Co-IP/MS revealed 70 CDC42-interacting proteins, and subsequent Gene Ontology (GO) enrichment analysis indicated their primary involvement in mRNA metabolism, cell adhesion, and Rho GTPase signaling pathways. Conclusions: CDC42 contributes to the pathogenesis of adenomyosis by promoting endometrial stromal cell proliferation and epithelial cell migration, likely by mediating cell cycle regulation and Rho GTPase signaling networks. Therefore, targeting CDC42 is a promising therapeutic strategy for the management of adenomyosis.

Keywords: CDC42, adenomyosis, stromal cells, epithelial cells, migration

Introduction

Endometriosis (EMs) is characterized by the presence and proliferation of endometrial tissue at diverse ectopic sites, particularly within the uterine myometrium (adenomyosis, ADs) and ovaries (ovarian endometriomas or "chocolate cysts"). Although benign, this condition elicits severe pain, significantly impairs patients' quality of life, and is a leading cause of female infertility [1-3]. Notably, EMs shares several cancer-like characteristics, including excessive proliferation, tissue invasion, and metastasis, and has a high recurrence rate [4, 5]. However, the cyclical shedding of this ectopic endometrium during menstruation may explain why malignant transformation in EMs remains relatively rare.

Despite its prevalence, the underlying pathogenesis of EMs remains incompletely understood. Sampson's widely accepted theory of retrograde menstruation cannot adequately explain the development of adenomyosis or extrapelvic endometriosis. Our previous research demonstrated that inhibition of the formin-

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like 2 (FMNL2) protein promotes the epithelialization of endometrial stromal cells, thereby impairing their migration [6]. Cell division cycle 42 (CDC42), a pivotal member of the Rho GTPase family, acts as an upstream regulator of FMNL2 and plays a crucial role in orchestrating the actin cytoskeleton [7-9]. Furthermore, CDC42 is abundantly expressed in ectopic endometrial tissues [10, 11]. Structurally, CDC42 binds to FMNL2, forming heterodimers that expose specific membrane-binding motifs. This complex recruits FMNL2 to the cell membrane and drives actin filament elongation at the leading edge of filopodia. Consequently, the CDC42/FMNL2 axis is highly actively involved in cell invasion, migration, and epithelial-mesenchymal transition (EMT) [9, 12].

Nevertheless, the specific function and molecular mechanisms of CDC42 in the pathogenesis of adenomyosis (ADs) remain elusive. In this study, we observed that CDC42 promoted the proliferation and migration of endometrial stromal cells by regulating networks associated with the TNF-α signaling pathway, cell cycle progression, and cell adhesion. Furthermore, the suppression of CDC42 attenuated cytokine secretion by stromal cells, which consequently reduced the migration capacity of epithelial cells by paracrine effects. These findings provide novel insight into the pathophysiologic role of CDC42 in adenomyosis and contribute significantly to the understanding of the molecular mechanisms driving the initiation and progression of this disease.

Materials and methods

Ethics statement

Informed patient consent was obtained prior to sample collection, and the study was approved by the Research Ethics Review Committee of Jiangxi Maternal and Child Health Hospital (EC-KY-202007).

Human sample collection

All tissue samples were obtained from Jiangxi Maternal and Child Health Hospital in Nanchang, Jiangxi Province, China. Normal endometrial tissue samples from the control group were obtained from patients who underwent hysteroscopic surgery for benign gynecological diseases other than EMs. The eutopic endometrial tissue and ectopic endometrial tissue of EMs patients were obtained from EMs patients undergoing laparoscopic surgery. All patients were histologically confirmed.

Primary cell isolation and culture

Human primary EMs eutopic stromal cells (ZQ7 and ZQ19) were provided by the central laboratory of Jiangxi Maternal and Child Health Hospital, and HEK293T cells were donated by Professor Cheng Yan from Nanjing Medical University. 293T cells were cultured in Dulbecco's modified Eagle's medium (Boster, China), and EMs primary ZQ7 and ZQ19 cells were cultured in DMEM/F12 (Boster, China). All the cells were cultured under standard conditions (37°C and 5% CO_2) in medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Immunofluorescence

All procedures were conducted in accordance with the guidelines provided by the fluorescence staining kit. The sections were soaked in xylene for 1 h and 100% ethanol for 30 min, after which the residual liquid was removed by washing with PBS. The tissue sections were subsequently immersed in a sodium citrate solution, heated to boiling in a microwave oven, removed for 10 minutes, and subsequently reheated twice. After cooling at room temperature, the sections were incubated with a CDC42 antibody (Proteintech, 1:2,000, 10804-1-AP) at 4°C overnight. The next day, the unbound antibodies were removed by a 1× PBS wash, and then, the sections were incubated with goat anti-rabbit IgG coupled with Alexa Fluor 488 or 568 at room temperature for 90 minutes in the dark. After performing a second wash with 1× PBS, DAPI staining was conducted, and the resulting images were captured using a Zeiss Scope. A1 fluorescence microscope after the film was sealed. The fluorescence intensity was analyzed using ImagePro plus software, and the fluorescence values of the photos were quantitatively analyzed using GraphPad software.

Western blotting

The protein was extracted using RIPA lysis buffer (Beyotime, Beijing, China), and the protein concentration was determined using a BCA

(Beyotime, Beijing, China) kit. The protein was separated by SDS-PAGE and transferred to a PVDF membrane, which was blocked in 5% milk and incubated overnight with anti-CDC42 rabbit monoclonal antibody (1:500; Cat No. 10155-1-AP; Proteintech, China), anti-β-actin (1:1,000; Cat No. sc-47778; Santa Cruz, USA) and anti-GAPDH (1:1,000; Cat No. sc-47724; Santa Cruz, USA). After incubation with the secondary antibody of the corresponding species (1:3,000, Proteintech, China), an ECL kit (Absin, Beijing, China) was used to expose the protein substrate present on the membrane. ImageJ and Prism 6 were used to quantify grayscale values in a linear range, and the image adjustments were consistent across the entire image.

Immunoprecipitation-mass spectrometry (IP-MS)

293T cells were inoculated at the appropriate density and cultured in 6-well plates for 18 hours. The CDC42 plasmid was transfected into cells (70%-80% percentage) by GBFETtene-ELITE (GB1001-250, Genebank Biosciences Inc., China). The cells were collected 24 hours after transfection and lysed using Pierce IP Lysis Buffer (Thermo, USA). Following ultrasonic lysis, the mixture was centrifuged at a constant temperature of 4°C and 14,000 × g for 15 minutes to remove impurities. Anti-CDC42 (Proteintech, China) was coupled with protein G agarose beads (Millipore, USA). The isolated protein was subjected to 8% SDS-PAGE and western blotting to obtain gel strips containing protein, which were packed into tubes and sealed. The samples were sent to Jingjie Biological Laboratory in Hangzhou, China, for protein profiling.

Knockdown of CDC42

293T, ZQ7, and ZQ19 cells were transfected with CDC42 siRNA using the GBFETTENe-Elite method (GB1001-250; Genebank Biosciences Inc.) according to the manufacturer's protocol. The negative control siRNA (si-NC) and three CDC42 siRNAs (si-CDC42 123 #1, #2, and #3) were purchased from Invitrogen. First, when the cell density was approximately 70%, 7.0 μl of GBI reagent was mixed with 50 pmol of siRNA double-stranded at room temperature to form a complex, and the mixture was added to the medium and transfected for 48 hours. Total protein and RNA were extracted from the transfected cells for western blotting and RT-qPCR.

Real-time PCR and RNA-seq

Real-time fluorescent quantitative PCR (RT-qPCR) was used to quantify the expression of CDC42 mRNA in endometrial cells. RNA-seq was performed by the High Throughput Laboratory of the University of China, Wuhan, China. In accordance with the manufacturer's protocol, total RNA was extracted from siControl and siRNA-transfected endometrial cells using TRIzol reagent (TIANGEN, DP424, China), and cDNA was synthesized using SYBR Premix Ex Taq II for qPCR. Quantitative PCR analysis was conducted using an ABI 7500 instrument (Applied Biosystems, USA). The $2^{-\Delta\Delta Ct}$ method was used, with adjustments made for the cycle phase. RNA-seq analysis was performed using the application Metascape, with custom analyses including "GO molecular function", "GO biological process" and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, as well as default parameters.

Cell migration

The expression of CDC42 in ZQ7 primary EMs cells was knocked down to observe changes in migration ability. A total of 1×10^4 cells from serum-free culture medium were inoculated into transwells (Bosch, Shanghai, China), and culture medium containing 20% fetal bovine serum was added to the outside of the transwells. After 48 h of culture, the cells were washed three times in 1× PBS. The migrating cells at the bottom of the well were fixed with 4% polymethanol solution for 20 min, the residual liquid was removed, and the cells were washed and stained with crystal violet (1%). The cells on the side of the well were wiped with a cotton swab and air dried, and pictures were taken with an Olympus X71. Six separate fields of view were counted for each transwell, and the average number of cells passing through the transwell is indicated in the plot.

Cell proliferation

EdU proliferation assays were performed according to the instructions of a 5-acetylidene-2-deoxyuridine (EdU) labeling/detection kit (RiboBio, Guangzhou, China). ZQ7/ZQ19 cells (density of 1.0×10^5 cells/well) were inoculated in 24-well plates and cultured for 16 hours. Forty-eight hours after the CDC42-siRNA was transfected, the EdU-labeled solution was diluted in the cell culture medium at a ratio of

1:3,000, and after the cells were incubated in the cell incubator for 18 hours, they were treated with 4% paraformaldehyde and 0.5% Triton X-100 and stained with anti-EdU working solution. DAPI staining was performed, and images were obtained using an Olympus X71 fluorescence microscope.

Statistical analysis

The data were analyzed and visualized using GraphPad Prism 8.0.1 software (GraphPad, USA) and are expressed as the mean ± SD. One-way analysis of variance was used to compare differences between immunofluorescence groups. Unpaired two-tailed Student's t tests were used to compare the differences between the migration and proliferation groups. All experiments were independently repeated at least three times. $P < 0.05$ was considered to indicate statistical significance.

Results

CDC42 is highly expressed in adenomyosis and endometrial carcinoma

To evaluate CDC42 protein expression, we performed immunofluorescence staining on control eutopic endometrium (n=17) and paired eutopic (n=16) and ectopic (n=16) tissues from patients with adenomyosis (ADs). Quantitative analysis of fluorescence intensity revealed that CDC42 expression was significantly upregulated in both the stromal and glandular epithelial cells of ADs tissues (both eutopic and ectopic) compared to that of the control group (**Figure 1A-C**). However, no significant difference in CDC42 expression was observed between the eutopic and ectopic lesions within the ADs cohort. Consistent with the tissue staining results, the results of the western blot analysis revealed markedly higher CDC42 protein levels in the ADs eutopic stromal cell line (12Z), primary EMs eutopic stromal cells (ZQ), and an endometrial carcinoma cell line (HEC-1B) than in the normal control stromal cell line (ThESCs) (**Figure 1D**). Furthermore, using software available online, we found that the CDC42 protein (Ualcan, http://ualcan.path.uab.edu/analysis-prot.html) and mRNA (TNMplot, https://tnmplot.com/analysis/) levels were greater in endometrial carcinoma tissue than in normal tissue (**Figure 1E** and **1F**) [13, 14]. Collectively, these findings indicate that CDC42 is abnormally overexpressed in both ADs lesions and endometrial carcinoma. This shared upregulation suggests that CDC42 overexpression may serve as a molecular marker associated with the malignant transformation of benign endometriotic lesions.

CDC42 knockdown alters the expression of cytokine- and cell cycle-related genes in endometrial stromal cells

To elucidate the downstream molecular mechanisms of CDC42 in endometriosis, we performed siRNA-mediated knockdown in primary stromal cells derived from two EMs patients. Quantitative PCR (qPCR) and western blot analyses confirmed that the knockdown efficiency of siRNA #1 was the highest (**Figure 2A** and **2B**). Consequently, we used siRNA #1 to transfect ZQ19 primary stromal cells and extracted RNA from biological triplicates for RNA sequencing (RNA-seq) analysis. Transcriptomic profiling revealed 611 differentially expressed genes (DEGs) (fold change ≥ 2) between the CDC42-knockdown and siControl groups, including 235 upregulated and 376 downregulated genes (**Figure 2C**). The volcano plot highlighted CDC42 as one of the most significantly downregulated genes, further validating the efficacy of our knockdown model (**Figure 2D**). Subsequent Gene Ontology (GO) enrichment analysis revealed that these DEGs were primarily localized to the extracellular space, cell surface, and plasma membrane (**Figure 2E**). In terms of molecular function, the DEGs were highly enriched in cytokine activity and chemokine receptor binding (**Figure 2F**). Furthermore, the predominant biological processes involved cytokine-mediated signaling pathways, inflammatory responses, and cellular responses to lipopolysaccharides. Notably, the cell cycle pathway contained the greatest number of altered genes (**Figure 2G**). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis further demonstrated that these DEGs were most significantly enriched in the TNF-α signaling pathway, cytokine-cytokine receptor interactions, and the NF-κB signaling pathway (**Figure 2H**). It is well known that TNF-α drives inflammation and cytokine production by the NF-κB axis and plays a critical role in cell cycle regulation [15]. On the basis of these transcriptomic profiles, we hypothesized that CDC42 inhibition suppresses TNF-α signaling, thereby attenuating

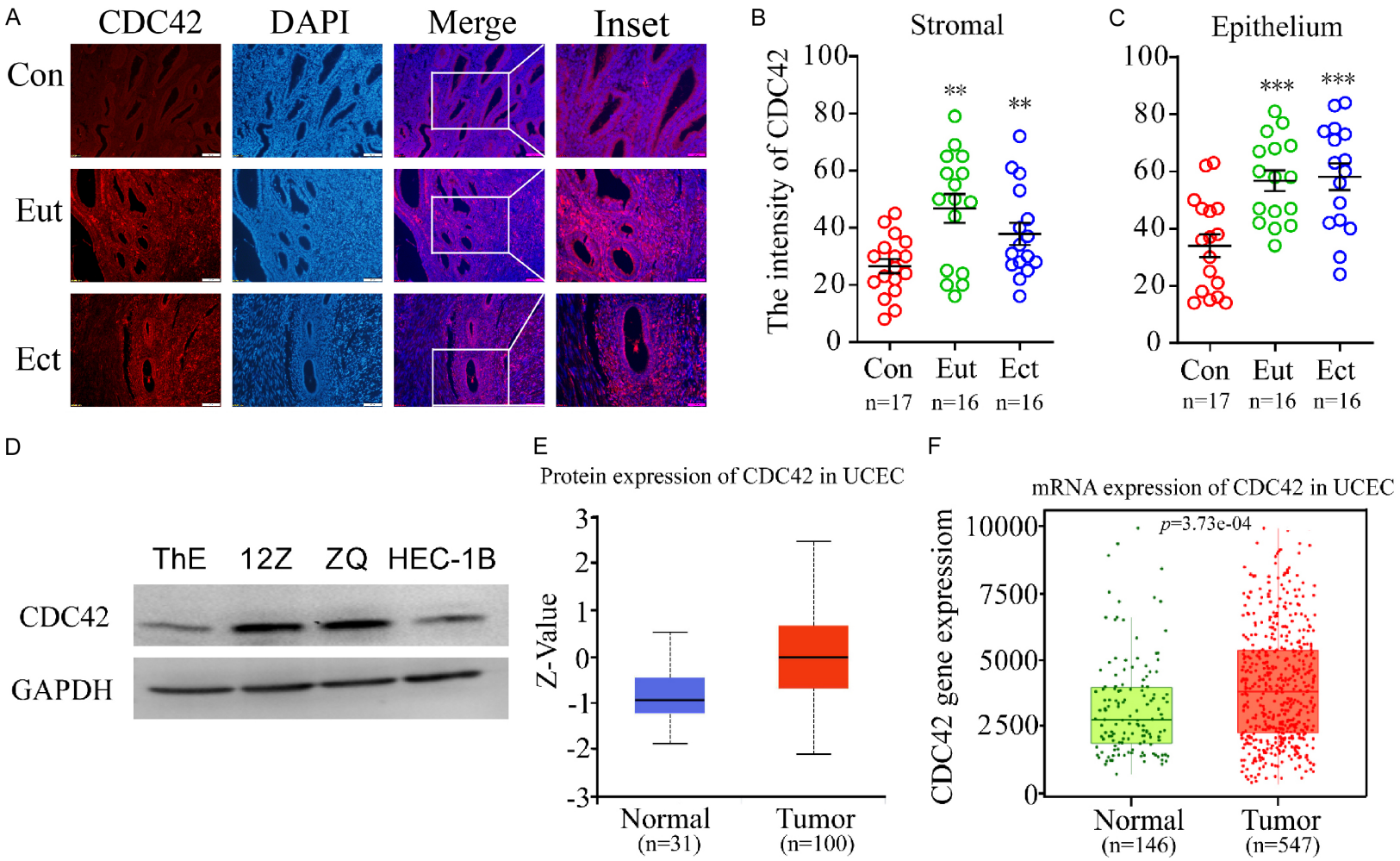

Figure 1. CDC42 is upregulated in EMs. A. Representative image showing the expression of CDC42 in the normal endometrium (Con), eutopic endometrium (Eut) and ectopic endometrium (Ect) of ADs by immunofluorescence (IF) staining; scale bar=200 μm. B, C. Statistical analysis of IF staining of CDC42 from normal endometrium (Con, n=17), eutopic endometrium (Eut, n=16) and ectopic endometrium (Ect, n=16) of ADs stromal and epithelial cells. D. Western blotting was used to verify the CDC42 protein level in ThESCs, 12Z, ZQ, and HEC-1B cells. E. The protein level of CDC42 between normal tissues (n=31) and primary tumor tissues (n=100) from the UALCAN online database. Z values represent standard deviations from the median across samples for the given cancer type. The $\log_2$ spectral count ratio values from CPTAC were first normalized within each sample profile and then normalized across samples. F. CDC42 gene expression levels in normal tissue (n=146) and UCEC tissue (n=547) (TNMPLOT online database). *p* values were determined by Student's t test; $^{**}P < 0.01$, $^{***}P < 0.001$.

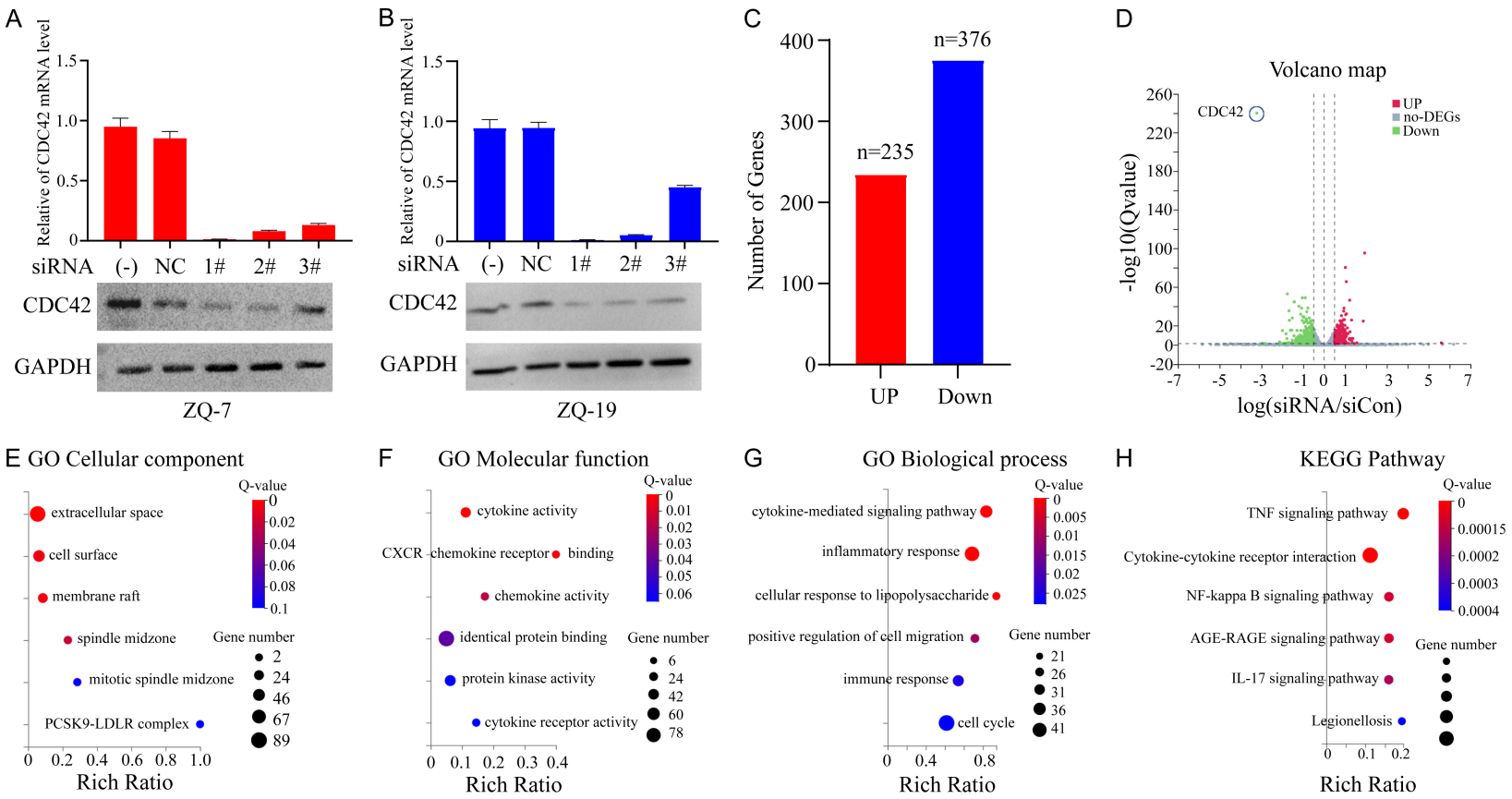

Figure 2. siCDC42 influences the expression of genes involved in cytokine-mediated, inflammatory response, and cell migration signaling pathways. A, B. ZQ7 and ZQ19 cells were transfected with CDC42 siRNA (#1, #2 and #3) for 24 hours or 48 hours. The expression level of CDC42 mRNA or protein after the knockdown of CDC42 was detected by qPCR or western blot. C. The number of differentially expressed genes between the NC group and the siCDC42 group. D. The volcano plot shows the differentially expressed genes after knockdown of CDC42. The green dots represent the down-regulated genes, the red dots represent the up-regulated genes, and CDC42 is circled. E-H. Differentially expressed genes in GO enrichment analysis (cellular component, molecular function, and biological process terms) and KEGG pathway analysis.

cytokine/chemokine expression and impeding cell cycle progression at the transcriptional level.

CDC42 knockdown inhibits endometrial stromal cell proliferation

To validate the RNA-seq findings, we first generated heatmaps to visualize the differential expression of genes enriched in the cell cycle and TNF-α signaling pathways between the control and CDC42-knockdown groups (**Figure 3A**, **3B**). We subsequently performed quantitative PCR (qPCR) to assess the mRNA levels of representative cell cycle-related genes identified in the heatmap. As expected, CDC42 knockdown significantly downregulated the expression of PCNA, MCM7, CDK4, and c-MYC (**Figure 3C**). Similarly, qPCR analysis of key TNF-α pathway targets revealed significant decreases in the expression of CSF3, CXCL10, and CXCL11 following CDC42 depletion, whereas CSF2 expression remained relatively unchanged (**Figure 3D**).

Furthermore, bioinformatics analysis using the TCGA and TNM plot databases for endometrial carcinoma cohorts demonstrated a strong positive correlation between CDC42 expression and both MCM7 and CXCL11 expression ($P < 0.001$; **Figure 3E**, **3G**). To assess the functional effect of these transcriptomic changes, we performed an EdU incorporation assay in endometrial stromal cells. Consistent with the gene expression data, CDC42 knockdown significantly reduced the proportion of EdU-positive cells, indicating impaired DNA synthesis (**Figure 3F**, **3H**). Collectively, these data demonstrate that CDC42 inhibition suppresses endometrial stromal cell proliferation, which is likely driven by the downregulation of critical genes in the cell cycle and TNF-α signaling networks.

CDC42 interacts with focal adhesion and extracellular matrix proteins

While the aforementioned RNA-seq profiling provided insight into the signaling pathways and cellular functions modulated by CDC42, its specific protein-protein interactome was unclear. Therefore, we performed coimmunoprecipitation coupled with mass spectrometry (Co-IP/MS) using HEK-293T cells transfected with either EGFP-tagged or MYC-tagged CDC42 plasmids. Western blot analysis initially confirmed the successful immunoprecipitation of the tagged CDC42 proteins (**Figure 4A**).

After the nonspecific background from the control vectors was filtered out, 24 and 36 unique proteins that interact exclusively with EGFP-CDC42 and MYC-CDC42, respectively, were identified by mass spectrometry (**Figure 4B**). Crucially, 11 proteins, including CDC42 itself, were identified in both experimental groups, thereby validating the reliability of our IP-MS approach (**Figure 4C**). Among these common interactors, two proteins are of particular interest. The first is junction plakoglobin (JUP), a key structural component crucial for cell-cell junctions and cell adhesion [16, 17], strongly suggesting that CDC42 directly participates in cell adhesion and migration. The second is Immunoglobulin lambda variable 4-60 (IGLV4-60), a secreted membrane-associated glycoprotein involved in immune responses [18]. We next performed enrichment analysis on the 71 specific CDC42-interacting proteins. Gene Ontology (GO) analysis revealed that these interactors were significantly enriched in RNA metabolic processes (biological processes) and were localized primarily to focal adhesions and extracellular regions (cellular components) (**Figure 4D**). Furthermore, Reactome pathway analysis revealed enrichment of Rho GTPase cycle signaling, perfectly aligning with the intrinsic function of CDC42 as a Rho family small GTPase (**Figure 4E**). Collectively, these interactome data demonstrate that CDC42 directly binds to extracellular adhesion-related proteins, further corroborating its capacity to drive cell migration through physical molecular interactions.

Knockdown of CDC42 in stromal cells inhibits epithelial cell migration

To validate the functional implications of CDC-42, we first knocked down CDC42 in ZQ7 endometrial stromal cells and assessed their migratory capacity using a transwell assay. As anticipated, CDC42 depletion significantly impaired the autonomous migration of stromal cells (**Figure 5A** and **5B**). Furthermore, our earlier omics data indicated that CDC42 transcriptionally regulates cytokines and chemokines and physically interacts with extracellular matrix proteins. In ectopic endometriotic lesions, endometrial stromal and epithelial cells coexist intimately, with the stroma encapsulating the

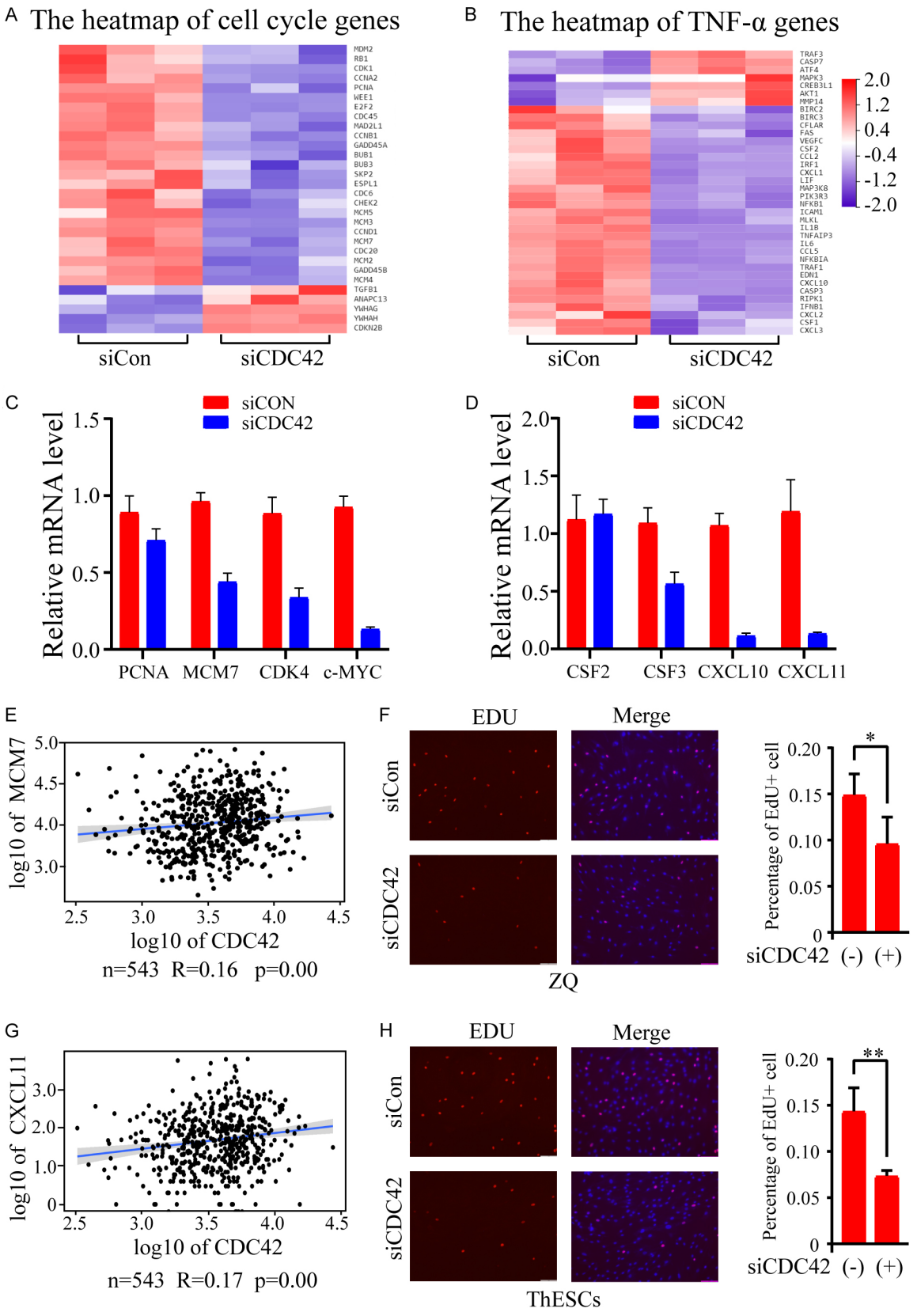

Figure 3. Knockdown of CDC42 expression inhibits the proliferation of stromal cells. A, B. Heatmap of differentially expressed genes in the cell cycle and TNF-α pathway after knockdown of CDC42 expression. C, D. show the mRNA expression of genes in the cell cycle and TNF-α pathway, respectively, detected by qPCR after knocking down CDC42

expression. E. The mRNA expression level of c-MYC was positively correlated with that of CDC42 in GEPIA. F. Results of the EdU incorporation assays of ZQ7 cells transiently transfected with siCon or siCDC42. The percentage of EdU-positive cells was blindly calculated by counting nine nonoverlapping fields. G. The mRNA expression level of CXCL11 was positively correlated with that of CDC42 in GEPIA. H. EdU incorporation assays of ZQ19 cells transiently transfected with siCon or siCDC42. The percentage of EdU-positive cells was blindly calculated by counting nine nonoverlapping fields. *p* values were determined by Student's t test; $*P < 0.05$, $**P < 0.01$.

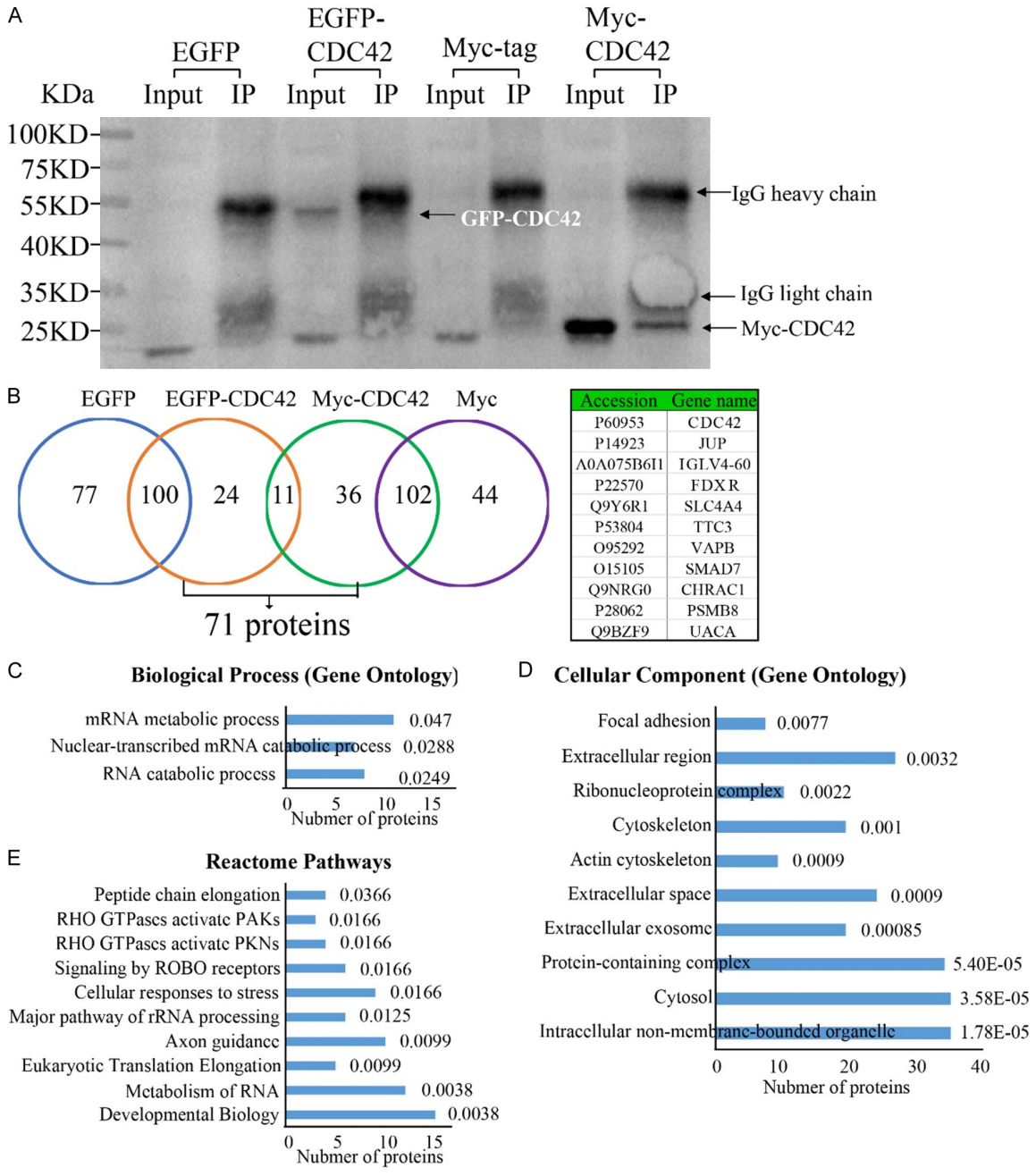

Figure 4. The proteins Interacting with CDC42. A. EGFP, EGFP-CDC42, Myc tag, and Myc-CDC42 plasmids were transfected into 293T cells, respectively. After 24 hours, the proteins were collected for CO-IP and Western blot was performed by using CDC42 antibodies. B. Perform mass spectrometry analysis on the four IP groups mentioned above, and the final intersection contains 11 proteins. C-E. GO enrichment analysis (Biological Process and Cellular Component) and Reactome pathways of the mass spectrometry data of two sets of CO-IP samples transfected with CDC42.

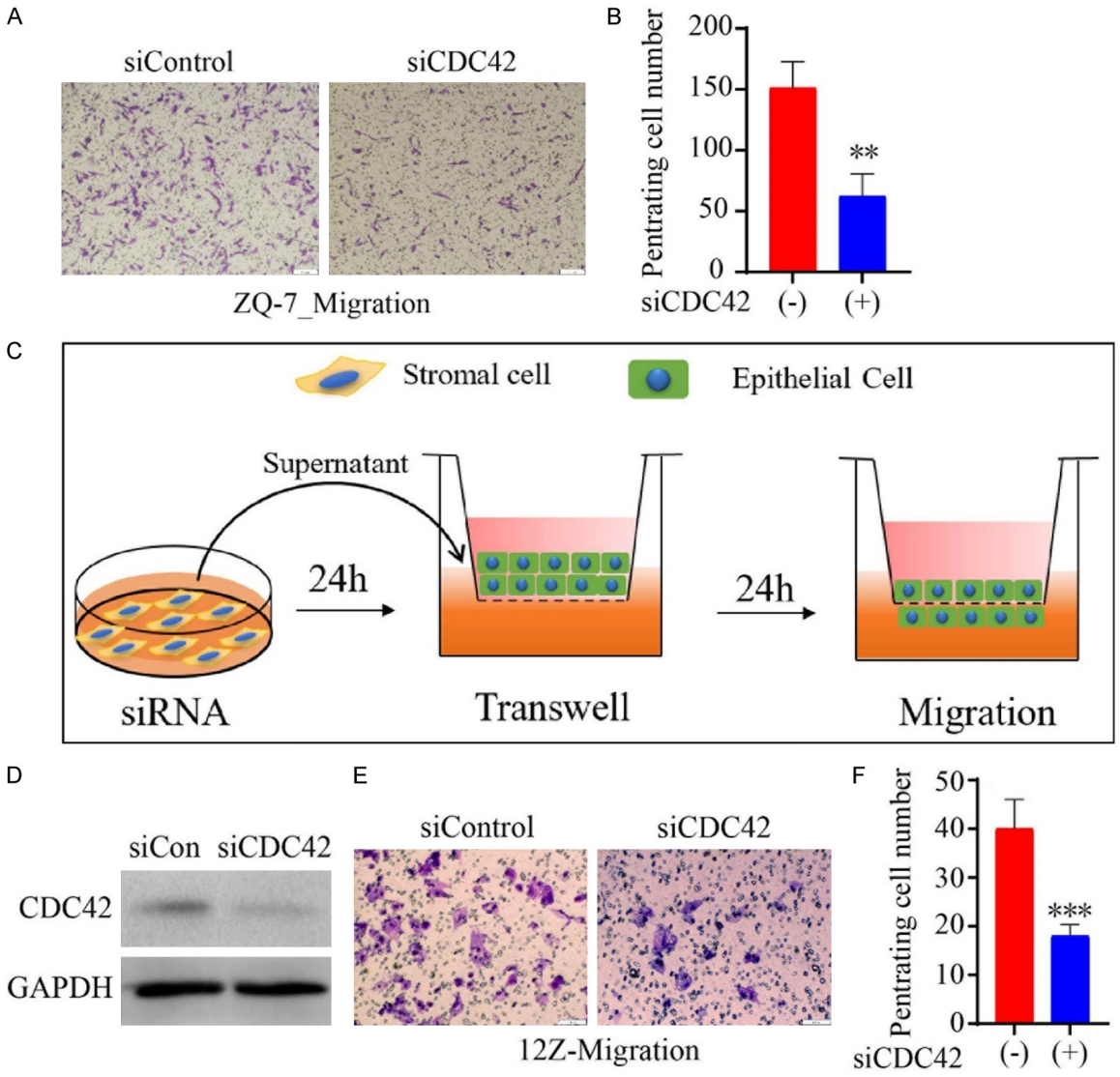

Figure 5. CDC42 inhibits the migration of stromal and epithelial cells. A. After transfection with siCon and siCDC42 for 24 hours, the migration of ZQ7 stromal cells was detected using a transwell assay. B. Statistical analysis of cell migration. C. Flowchart of the 12Z cell migration experiment. Stromal cells transfected with siRNA were added to the lower layer of the cell culture medium, and the migration of 12Z cells was analyzed using a transwell assay. D. After CDC42 was knocked down in stromal cells, the expression of CDC42 was detected using western blotting. E. After the steps shown in the flow chart, the migration of epithelial cells was analyzed. F. Statistical analysis of 12Z cell migration; *p* values were determined by Student's t test; $^{**}P < 0.01$, $^{***}P < 0.001$.

glandular epithelium to orchestrate a supportive microenvironment. This result strongly suggests that CDC42 may modulate the tissue microenvironment by secretory pathways.

To investigate whether secreted factors from stromal cells influence epithelial cell behavior, we performed a conditioned medium (CM) coculture assay. Briefly, culture supernatants from stromal cells transfected with either control or CDC42 siRNA were collected after 24 hours, filtered, and placed in the lower chambers of transwell plates. Epithelial cells were subsequently seeded into the upper chambers, and their migration was evaluated 24 hours later (**Figure 5C**). The results demonstrated that compared with control CM, CM derived from CDC42-knockdown stromal cells significantly attenuated the migration of epithelial cells (**Figure 5D-F**). Taken together, these findings indicate that CDC42 not only drives the intrinsic migration of endometrial stromal cells

but also promotes the migration of adjacent glandular epithelial cells through the paracrine secretion of extracellular components.

Discussion

The etiology of endometriosis (EMs) is a multifaceted process influenced by various factors, leading to the acquisition and deterioration of cellular function, and is characterized by notable heterogeneity. Epigenetic mechanisms play pivotal roles in regulating the emergence and persistence of immunologic, histologic, and biologic irregularities observed in both eutopic and ectopic endometria among EMs patients [19]. In terms of treatment, surgical intervention for advanced-stage EMs (stage III or IV) has more advantages [20]. However, in early-stage EMs patients who wish to maintain their fertility, hormone therapy may be a more advantageous option [21]. Additionally, the use of assisted reproductive technology following surgical intervention for EMs substantially increases the likelihood of conception [22]. Nevertheless, despite the use of pharmaceutical interventions for pain management, the recurrence rate of surgical procedures remains elevated, and the implementation of assisted reproductive technology imposes a protracted timeline and financial strain on families. As the understanding of endometriosis as a systemic disease expands, research endeavors are presently focused on exploring innovative molecular targets. These targets have the potential to improve the precision of endometriosis diagnosis and may present opportunities for therapeutic intervention [23].

In recent years, through genome-wide association studies (GWASs) and high-throughput genotyping techniques, the annotation of CDC42 on chromosome 1 as a susceptibility gene for endometriosis has been proposed [11, 24]. CDC42 is a widely recognized therapeutic target for cancer treatment and is frequently linked to mutations or amplifications in tumor cells [25]. Additionally, it plays a pivotal role in directing the development of ducts in vascular endothelial cells and promoting the migration and infiltration of tumor cells [26, 27]. Moreover, CDC42 is instrumental in the polarization morphogenesis of epithelial cells and exerts a non-localized effect by orchestrating diverse processes, such as apical membrane morphogenesis, luminal formation, and junction maturation [10]. Activation of CDC42 disrupts cell-cell contact, which is a prerequisite for the dissemination of EMs cells and the loss of the epithelial phenotype [28]. Further investigation has provided evidence that macrophages positive for CDC42 can impede the malignant progression of ovarian endometriosis [29]. The findings of this study revealed a substantial presence of CDC42 in both the eutopic and ectopic tissues of individuals with endometriosis, as demonstrated by immunofluorescence analysis. Notably, this expression pattern was predominantly observed in the cell membrane surrounding the gland, which is consistent with the established function of CDC42 in regulating actin and its subsequent influence on cytoskeletal composition [30]. Consequently, we proceeded to validate the expression of CDC42 in diverse endometrial cell types. Compared to that of stromal cells, the expression of CDC42 in endometrial epithelial cells was significantly greater, suggesting a potential association between CDC42 and the formation and oscillation of cilia [31]. Additionally, upregulated CDC42 expression was observed in primary endometriotic cells, providing initial evidence for the specific expression of CDC42 in endometrial cells and its increased expression in EMs cells.

Moreover, the use of high-throughput transcriptome RNA analysis allows for a more detailed examination of intricate protein interactions. We performed siRNA transfection to downregulate CDC42 expression in EMs cells, aiming to investigate alterations in cellular regulatory pathways and potential changes in associated protein expression. RNA-seq data, as illustrated by differential expression heatmaps and KEGG pathway analysis, revealed the regulatory effects of CDC42 on diverse cellular processes, including cell adhesion, cell cycle, migration, and endocytosis. Subsequent hierarchical analysis and quantitative polymerase chain reaction (qPCR) results further substantiated the noteworthy influence of CDC42 on the expression of proteins linked to cell adhesion and the cell cycle. Notably, the modification pattern of specific cell cycle proteins aligned with the observed alterations in the expression of CDC42.

In future experiments, we will use various techniques, including co-IP, to validate the precision

of protein expression in relevant pathways and elucidate the upstream and downstream regulatory mechanisms involved. The purported interactions between CDC42 and this specific subset of proteins likely represent a combination of genuine physiological regulatory mechanisms and experimental cell-lysis artifacts. Physiologically, CDC42-driven actin remodeling may anchor the membrane channel SLC4A4, coordinate with the ER protein VAPB to supply lipids for membrane protrusions, interact with UACA for cytoskeletal scaffolding, and bridge TGF-\betaβ signaling through SMAD7 to drive cell motility. Furthermore, the E3 ligase TTC3 and the proteasome subunit PSMB8 likely cooperate to regulate CDC42 turnover through ubiquitin-mediated degradation to maintain strict signaling homeostasis. While the interaction with the chromatin remodeling factor CHRAC1 suggests a noncanonical nuclear function for CDC42, its detection alongside the inner mitochondrial enzyme FDXR and the secreted antibody IGLV4-60 are almost certainly false-positive *in vitro* artifacts resulting from the disruption of physical cellular compartments during protein extraction.

Within the framework of the menstrual reflux hypothesis, endometrial cells can retrograde into the pelvic cavity through the fallopian tubes to form ectopic foci [32]. This phenomenon can be attributed to the increase in the movement of endometrial cells, wherein the abnormal expression of CDC42 facilitates cell movement by promoting the formation of actin polymers and elongation of filopodia [33]. Experiments on cell movement confirmed that inhibiting CDC42 resulted in a decrease in its expression in primary endometriotic cells, thereby reducing the number of cells transferred to the posterior region of the migration chamber. Importantly, this reduction was significantly different from that of the control group. This study highlights that the overexpression of CDC42 can increase the migratory capability of endometriosis-associated mesenchymal cells, thereby facilitating their metastatic propensity and the development of EMs lesions. Consequently, this discovery serves as supplementary empirical evidence supporting the retrograde menstruation hypothesis. Nevertheless, our investigation remains unfinished, as the exact mechanism through which CDC42 regulates EMs cell migration has yet to be comprehensively elucidated. Additionally, the precise mechanisms regulating cell adhesion and the interaction between cyclins and CDC42 have not been fully elucidated. These areas are promising avenues for future investigation.

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Informed patient consent was obtained prior to sample collection.

Disclosure of conflict of interest

None.

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