Original Article

SMURF1-mediated ubiquitylation of SHP-1 promotes cell proliferation and invasion of endometrial stromal cells in endometriosis

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Background: Endometriosis is a widespread benign gynecological disorder. The signal transducer and activator of transcription 3 (STAT3) signaling pathway plays an important role in the pathogenesis of endometriosis through regulating proliferation and invasion of endometrial stromal cells. Furthermore, the protein tyrosine phosphatase (PTP), SH2 domain-containing phosphatase 1 (SHP-1), negatively regulates STAT3 activation. However, regulation of the SHP-1-STAT3 pathway in the pathogenesis of endometriosis remains unclear.

Methods: Cell proliferation and invasion were assessed by Cell Counting Kit-8 (CCK-8) assay and Transwell analysis, respectively, to investigate the role and regulation of the SHP-1-STAT3 pathway in the proliferation and invasion of endometrial stromal cells. Expression of Smad ubiquitin regulatory factor 1 (SMURF1), SHP-1, matrix metalloproteinase 2 (MMP2), MMP9, STAT3, and phospho-STAT3 (p-STAT3) level in patients with endometriosis were measured by Western blotting and/or immunohistochemical staining. The interaction between SMURF1 and SHP-1 was investigated by co-immunoprecipitation and ubiquitylation analysis.

Results: The present study demonstrated that downregulation of SHP-1 expression in patients with endometriosis was negatively correlated with SMURF1 expression. SMURF1, an E3 ubiquitin ligase, activated the STAT3 pathway via ubiquitylation and degradation of SHP-1. Furthermore, SMURF1 promoted cell proliferation and invasion of endometrial stromal cells by activating STAT3 signaling and expression of its downstream targets, MMP2 and MMP9, whereas SHP-1 demonstrated an inverse effect. Additionally, SHP-1 inhibited SMURF1-mediated cell invasion and proliferation of endometrial stromal cells.

Conclusions: Our findings indicate that SMURF1-mediated ubiquitylation of SHP-1 regulates endometrial stromal cell proliferation and invasion during endometriosis.

Keywords: Endometriosis; invasion; Smad ubiquitin regulatory factor 1 (SMURF1); SH2 domain-containing phosphatase 1 (SHP-1); ubiquitylation

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Introduction

Endometriosis is a common, chronic, gynecologic disease that affects 6–10% of women of child-bearing age, 35–50% of infertile women, and 40–60% of women with dysmenorrhea (1,2). It is characterized by the presence and growth of endometrial tissue at extrauterine sites, mainly on the pelvic peritoneum and ovaries (3). Symptoms include pelvic mass, pelvic pain, abnormal menstruation, dyspareunia, dysmenorrhea, and infertility (4), which are associated with some properties of malignant tissues, such as hyperplasia, cell invasion, and induction of metastasis (5), and can severely affect quality of life and pose a serious health burden to society (6,7). Endometriosis etiology is believed to be multifactorial, ranging from genetic causes to immunologic dysfunction due to environmental exposure to endocrine disrupting chemicals (8). There are several treatments for endometriosis, including conservative treatment (painkillers and/or hormones) and surgery (9). However, none of these treatment methods offer a complete cure, but aim to reduce pain, improve quality of life, and may increase the chance of pregnancy (10). The phenomenon of retrograde menstruation represents a classic theory relating to the pathogenesis of endometriosis (11). Although the majority of women experience retrograde menstruation, only 10% of women develop endometriosis (12), indicating that other factors may mediate the formation of endometriotic lesions. Ectopic endometrial stromal cells from endometriosis demonstrated increased proliferation, invasion, and migration compared with normal endometrial stromal cells from nonendometriotic controls (13,14). Furthermore, attachment of the endometrium to host peritoneum was shown to be important for endometriosis progression (15,16). This attachment is associated with the invasion and migration of endometrial stromal cells, as well as several regulatory factors related to cell invasion and migration, such as matrix metalloproteinases (MMPs) (17). Therefore, identifying mechanisms that potentially alter the activity of endometrial stromal cells is important to understanding the pathogenesis of endometriosis.

Signal transducer and activator of transcription 3 (STAT3) signaling plays an important role in cell proliferation, apoptosis, invasion, and immune response (18). A recent study showed that abnormal activation of STAT3 signaling plays an important role in the pathogenesis of endometriosis (19). SH2 domain-containing phosphatase 1 (SHP-1), which is a member of the protein tyrosine phosphatase (PTP) family and a negative regulator of phospho-STAT3 (p-STAT3), is involved in regulating various cellular processes including cell proliferation, cell cycle, migration, and invasion; however, little is known about its role in endometriosis. Crocin, which is an active component of saffron and is used in traditional Chinese medicine, has been shown to suppress STAT3 activation via induction of SHP-1 and improve endometriosis (20,21).

Ubiquitylation is a post-translational modification that plays a key role in the degradation of short-life regulatory proteins. The specificity of ubiquitin signaling for different biological processes and pathways is determined by a wide variety of monoubiquitination and polyubiquitination events, including many possible chain structures (22). Smad ubiquitin regulatory factor 1 (SMURF1) is a well-known Nedd4 family E3 ubiquitin ligase that regulates the cellular localization, mediates various processes including tumorigenesis, axon development, embryogenesis, and homeostasis by targeting substrate proteins for ubiquitination and proteasomal degradation (23-26), and activates STAT3 signaling (27). Recent studies have shown that SHP - 1 could be ubiquitinated and degraded into the ubiquitin-proteasome pathway (28). However, the ubiquitination mechanism of SHP - 1 in endometriosis remains unknown. Since bioinformatics analysis demonstrated that SHP-1 interacts with SMURF1, we hypothesized that SMURF1 and SHP-1 were involved in endometriosis via STAT3 signaling.

The present study investigated SMURF1, SHP-1, p-STAT3, and STAT3 levels in the ectopic endometrium or endometrial stromal cells and the effect of SMURF1 and SHP-1 on the biological behavior of endometrial stromal cells from women with and without endometriosis. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-2897).

Methods

Tissue collection and cell culture

Twelve active peritoneal endometriotic red, chocolate, and blue lesions were obtained from 12 women aged 23–45 (mean 34.6) years with endometriosis who underwent laparoscopic treatment at the Shanghai First Maternity and Infant Hospital, Tongji University from February 2019 to December 2019. Control endometrial samples were collected from 12 women aged 23–51 (mean 36.0) years without endometriosis who underwent laparoscopy and

Table 1 Patient clinical characteristics

Characteristics	Endometriosis group (n=12)	Control group (n=12)	P value
Age	35.75±3.84	31.45±5.97	0.094
BMI (kg/m^2)	19.30±2.63	21.52±2.22	0.065
CA-125 (IU/mL)	23.84±2.86	14.87±2.24	0.014
rASRM stage, n (%)			
III	5 (41.7)	NA	
IV	7 (58.3)	NA	
Benign conditions, n (%)			
Uterine myoma	NA	4 (33.3)	
Endometrial hyperplasia	NA	6 (50.0)	
Others	NA	2 (16.7)	
Menstrual phase, n (%)			0.6820
Proliferative	5 (41.7)	6 (50.0)	
Secretory	7 (58.3)	6 (50.0)	

Data are expressed as mean ± SD. BMI, body mass index; rASRM, revised American Society for Reproductive Medicine.

hysteroscopy surgery for benign gynecological diseases. Exclusion criteria for this study were patients who had received hormonal treatment and birth control prior to enrollment. The clinical characteristics of the patients and controls are shown in *Table 1*. Primary endometrial stromal cells derived from ectopic endometria of women with endometriosis or from normal endometria of women without endometriosis were isolated and cultured as previously described (15,16). Immunocytochemistry using anti-CK19 (Abcam, Cambridge, MA, USA; ab52625) and anti-vimentin (Abcam; ab92547) was performed to identify cell purity. After two to three passages, the purity of the stromal cells was >95%. Immunohistochemistry (IHC) was performed using anti-SMURF1 (Proteintech, Group, Inc., Chicago, IL, USA; 55175-1-AP), anti-SHP-1 (Abcam; ab32559) and anti-p-STAT3 (Abcam; ab76315) antibodies. Endometrial tissues (1.5 cm × 1.5 cm × 0.3 cm) were fixed with 10% formalin for 48 h. IHC staining was performed on 5-μm sections of paraffin-embedded tissues. The positive area within 10 successive fields (×400 magnification) was counted per section using a microscope (DS-Ri2, Nikon, Tokyo, Japan) as previously described (29). After fixing, permeation, and blocking, cells were collected for the detection of SMURF1 and SHP-1 protein expression by immunofluorescence staining using anti-SMURF1 antibody (Abcam; ab57573), anti-SHP-1 antibody (Abcam; ab124942), and Alexa Fluor-labeled IgG (H + L) antibody (Beyotime Biotechnology, Shanghai, China; A0428 and A0453) as previously described (30). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital, Tongji University (No. 2019tjdx351). Informed consent was obtained from all the patients.

Construction of the lentivirus

pLKO.1 lentiviral vectors containing shRNA targeting human SMURF1 (NM_001199847.1) or human SHP-1 (NM_002831.6) were synthesized by Sangon Biotech (Shanghai, China). shRNA sequences as well as scrambled sequences are shown in *Table 2*. A nonspecific scrambled shRNA was used as negative control (shNC). Full-length human SMURF1 or SHP-1 cDNA sequence was inserted into the pLVX-puro lentiviral vector and an empty pLVX-puro vector was used as negative control (blank vector). The production, purification, and titration of lentivirus were performed as previously described (31). Lipofectamine 2000 (Invitrogen, New York, CA, USA) was used to transfect pLKO.1 or pLVX-puro lentiviral vectors along with packaging plasmids psPAX2 and pMD2G into 293T cells according to the manufacturer's instructions. Recombined lentiviral vectors were collected 48 h after transfection and used to infect endometrial stromal cells.

Flow cytometric analysis of gene expression

Flow cytometric analysis of gene expression was performed as previously described (32) using anti-CK19 (Abcam; ab52625) and anti-vimentin (Abcam, ab92547) antibodies.

Cell Counting Kit-8 (CCK-8) assay

Cells were trypsinized and counted under a microscope and cells were seeded into wells at a density of 3×10^3 cell/well. Each 100 μL of suspension was seeded in 96-well plates and cultured overnight. Endometrial stromal cells were infected with pLKO.1-SMURF1-shRNA, pLKO.1-SHP-1-shRNA, pLVX-Puro-SMURF1, or pLVX-Puro-SHP-1 lentivirus. After incubation for 12, 24, and 48 h, 100 μL of CCK-8 (Dojindo, Kumamoto, Japan) solution [CCK-8, serum-free medium (1:10)] was added to each well and incubated at

Table 2 shRNA sequences as well as scrambled sequences used in this study

shRNA targeting position	Sequence (5'-3')
SMURF1-shRNA-1 [529–547]	CCAGCACTATGATCTATAT
SMURF1-shRNA-2 [682–700]	GCGTTTGGATCTATGCAAA
SMURF1-shRNA-3 [1,048–1,066]	GCCCGAAGGCTACGAACAA
SHP-1-shRNA-1 [156–174]	GGATGGTGAGGTGGTTTCA
SHP-1-shRNA-2 [331–349]	GGATGGTGAGGTGGTTTCA
SHP-1-shRNA-3 [429–447]	GCACCATCATCCACCTCAA

Table 3 Primer sequences used in this study

Gene	Sequences
SMURF1	F: 5'-TGAAGATGCGACCGAAAG-3'
	R: 5'-CTGGAAGAGCCCGTAATAAG-3'
SHP-1	F: 5'-AGGCTGTTGTCATACTTC-3'
	R: 5'-TGGCGATGTAGGTCTTAG-3'
GAPDH	F: 5'-AATCCCATCACCATCTTC-3'
	R: 5'-AGGCTGTTGTCATACTTC-3'

37 ℃ for 1 h. Thereafter, the absorbance value (optical density) at 450 nm, which indicates cell proliferation, was measured using a microplate reader (Wellscan MK3, Thermo/Labsystems, Finland).

Transwell assay

Cell invasion was measured after endometrial stromal cells were infected with pLKO.1-SMURF1-shRNA, pLKO.1-SHP-1-shRNA, pLVX-Puro-SMURF1, or pLVX-Puro-SHP-1 as previously described (17).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from endometriotic tissue samples or endometrial stromal cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) was used to reverse transcribe mRNA into cDNA and qPCR was performed by SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany) using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as a reference gene, and the primer sequences used in this study are shown in *Table 3*.

Western blotting

RIPA cell lysate containing protease and phosphatase inhibitors (Sigma, USA) was used to lyse the endometriotic tissues or endometrial stromal cells. Total protein was fractionated using 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA, USA) for 30 min at 4 ℃, and then incubated at 4 ℃ for 12 h with anti-SMURF1 (Abcam; ab57573), anti-SHP-1 (Abcam; ab32559), anti-MMP2 (Abcam; ab97779), anti-MMP9 (Abcam; ab76003), anti-p-STAT3 (Abcam; ab76315), anti-STAT3 (Abcam; ab68153), or anti-GAPDH antibody (Abcam; ab9485) antibody followed by incubation for 1 h at 37 ℃ with secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China; A0208 and A0216). An enhanced chemiluminescence substrate kit (Amersham Biosciences, Piscataway, NJ, USA) was used to quantify protein expression. Target protein expression was quantified relative to GAPDH by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each experiment was performed three times.

Co-immunoprecipitation and ubiquitylation analysis

Cell lysates were homogenized using RIPA lysis buffer (Beyotime Biotechnology, China) then incubated with or without anti-SMURF1 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA; sc-100616), anti-SHP-1 antibody (Abcam; ab227503), or control IgG (Santa Cruz Biotech.; sc-2027) overnight at 4 ℃ followed by an incubation with protein A/G Plus agarose beads (Santa Cruz Biotech.; sc-2003) for 2 h at 4 ℃. The immunoprecipitated complexes were washed three times in lysis buffer and analyzed by Western blotting. The membrane was then incubated with the secondary antibody, IPKine HRP, mouse anti-rabbit IgG LCS (Abbkine, California, CA, USA; A25022), to eliminate heavy chain interference.

Protein stability assay

Cycloheximide (CHX; 0.1 mg/mL) was added to cell culture medium and cells were harvested at the indicated time points to examine SHP-1 protein turnover. Cells

were then lysed, and cell lysates were subjected to Western blotting with anti-SHP-1 and anti-GAPDH as indicated. SHP-1 protein levels were quantified relative to GAPDH using ImageJ software.

Statistical analysis

Results were presented as mean ± SD of at least three samples in triplicate. Statistical analysis was performed using SPSS software (version 18.0, IBM Corp., Armonk, NY, USA). Where appropriate, data were log-transformed prior to statistical analysis to meet normality assumptions. If normality assumptions were met, means comparisons were performed using unpaired *t*-test for two groups or one- or two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons among groups. P values <0.05 were considered statistically significant.

Results

Downregulation of SHP-1 expression in ectopic endometrium of patients

STAT3 signaling was activated in the eutopic endometrium from women with endometriosis (19), and dysregulation of STAT3 signaling was shown to play an important role in the pathogenesis of endometriosis via regulation of endometriotic stromal cell proliferation, apoptosis, invasion, and migration (33,34). SHP-1 is a PTP that reduces the level of p-STAT3 and subsequently blocks the dimerization of p-STAT3. Therefore, we proposed that SHP-1 may be associated with endometriosis through the STAT3 signaling. SHP-1 expression in endometrium from women with or without endometriosis was measured to investigate the role of SHP-1 in the pathogenesis of endometriosis. SHP-1 protein levels were significantly downregulated in ectopic endometrial tissue compared with normal controls (*Figure 1A*,*B*).

Correlation analyses between predicated E3 ubiquitin ligases and SHP-1 in ectopic endometrium of patients

Bioinformatics analysis was performed using UbiBrowser (http://ubibrowser.ncpsb.org/) (*Figure 2A*) to further study the regulation of SHP-1 in endometriosis. Expression levels of four predicted E3 ubiquitin ligases with higher confidence levels, including SMURF1, CBL, MDM2, and SYVN1, except for BMI1 whose expression was not different between patients with or without endometriosis (35), were examined in ectopic endometrium from women with endometriosis or in normal endometrium from women without endometriosis. SMURF1, CBL, MDM2, and SYVN1 protein levels were significantly upregulated in ectopic endometrium compared with normal controls (*Figure 2B*,*C*,*D*,*E*,*F*). Moreover, there was a negative correlation between SMURF1 and SHP-1 expression in patients with endometriosis (*Figure 2G*), and no significant correlation was detected between other ubiquitin ligases and SHP-1 in patients with endometriosis (data not shown). Therefore, this protein was used for further experiments.

SMURF1, SHP-1, and STAT3 expression and p-STAT3 levels in ectopic endometrial stromal cells of patients

Expression of SMURF1, SHP-1, and STAT3 in ectopic endometrium and control endometrium was measured by IHC to further examine the role of SMURF1, SHP-1, and STAT3 signaling in endometriosis pathogenesis. Protein levels of SMURF1 and p-STAT3, but not SHP-1, were significantly upregulated in ectopic endometrium compared with normal endometrium from women without endometriosis (*Figure 3A*,*B*). Immunocytochemical staining was used to confirm endometrial stromal cells isolated from ectopic or normal endometrium, and SMURF1, SHP-1 and STAT3 expression, and p-STAT3 levels were also measured. IHC analysis revealed that isolated cells were negative for CK19 and positive for vimentin (*Figure 3C*) and most of these cells expressed vimentin, as determined by flow cytometric analysis (*Figure 3D*), suggesting that these cells were endometrial stromal cells. Moreover, SMURF1 expression and p-STAT3 levels were upregulated, whereas SHP-1 expression was downregulated in ectopic endometrial stromal cells compared with normal endometrial stromal cells (*Figure 3E*,*F*,*G*). However, STAT3 expression did not change between the two groups.

SMURF1 binds to and promotes ubiquitylation of SHP-1

SMURF1 coimmunoprecipitated with SHP-1 (*Figure 4A*), and reciprocal immunoprecipitation with SHP-1 antibodies also reduced SMURF1. Immunofluorescence microscopy revealed the overall distribution of SMURF1 and SHP-1 were similar (*Figure 4B*), suggesting an interaction between SMURF1 and SHP-1 in ectopic endometrial stromal cells. Since SMURF1 is an E3 ubiquitin ligase, we hypothesized

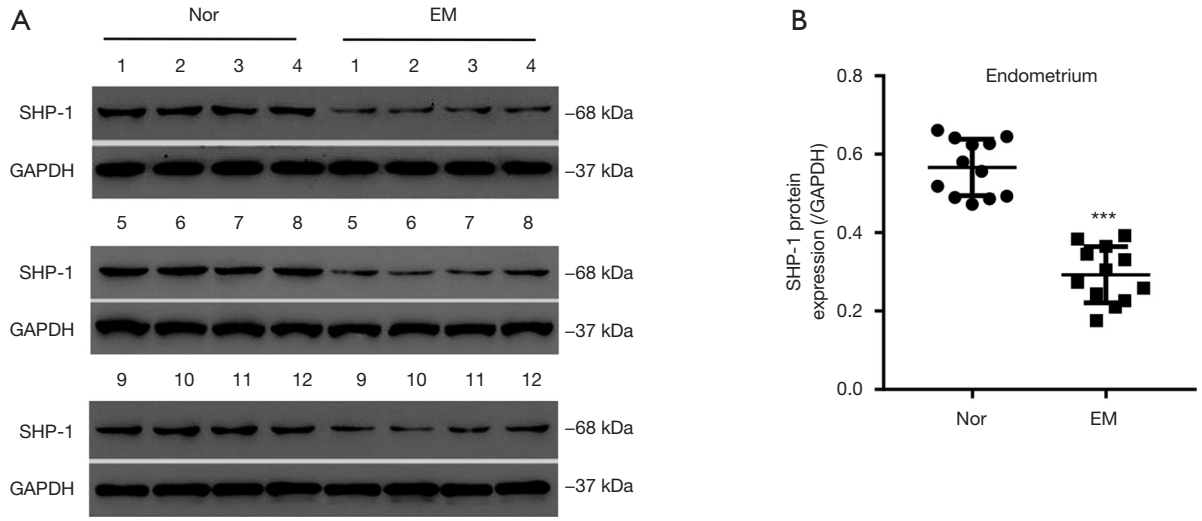
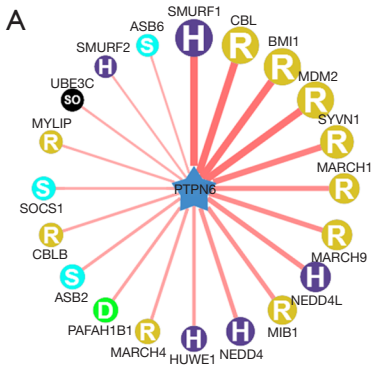


Figure 1 SHP-1 expression levels in ectopic endometrium from patients with endometriosis. (A,B) Representative SHP-1 protein expression in ectopic endometrium from women with endometriosis (EM1–4) or in normal endometrium from women without endometriosis (Nor1–4) was measured by Western blotting. Results are presented as mean ± SD of 12 samples in triplicate and means comparisons were performed using unpaired *t*-test. ***, P<0.001 *vs.* Nor groups.

that SMURF1 could regulate the protein level of SHP-1. SMURF1 expression vector significantly increased the *SMURF1* mRNA expression levels by 4.89-fold and protein levels by 2.40-fold compared with the blank vector in ectopic endometrial stromal cells (*Figure 4C*). SMURF1-shRNA-1, SMURF1-shRNA-2, and SMURF1-shRNA-3 significantly decreased *SMURF1* mRNA expression levels by 80.8%, 83.1%, and 77.0%, respectively, and protein expression levels by 82.2%, 84.4%, and 75.1%, respectively, compared with shNC in ectopic endometrial stromal cells (*Figure 4D*). Moreover, downregulation or upregulation of SMURF1 significantly increased or decreased SHP-1 protein levels, respectively, with no effect on *SHP-1* mRNA levels (*Figure 4E*). Moreover, proteasome inhibitor MG132 significantly reversed the decreased SHP-1 protein expression, indicating that SMURF1 decreased SHP-1 levels in a proteasome-dependent manner (*Figure 4F*). To further establish whether SMURF1 inhibits SHP-1 stability, cells were treated with CHX and the half-life of SHP-1 was determined. SHP-1 stability was dramatically decreased in SMURF1-overexpressing ectopic endometrial stromal cells (*Figure 4G*). These results demonstrate that SMURF1 destabilizes SHP-1. We next examined whether SMURF1 regulates the ubiquitylation level of SHP-1 in ectopic endometrial stromal cells. SMURF1 silencing decreased polyubiquitylation level of SHP-1 (*Figure 4H*).

Silencing of SMURF1 and/or SHP-1 regulates the proliferation and invasion of ectopic endometrial stromal cells

Ectopic endometrial stromal cells were infected with SMURF1-shRNA and/or SHP-1-shRNA and the cell proliferation and invasion were measured in order to examine the effect of SMURF1 and SHP-1 on regulating the development of endometriosis. SHP-1-shRNA infection significantly decreased the expression of SHP-1 compared with that of shNC (*Figure 5A,B*). Furthermore, SMURF1-shRNA infection significantly decreased cell proliferation and cell invasion compared with shNC at 48 h, whereas SHP-1-shRNA infection demonstrated an inverse effect (*Figure 5C,D*). Interestingly, SHP-1-shRNA also significantly inhibited the SMURF1-shRNA-mediated decrease in cell proliferation and invasion. The number of invading cells in the shNC, SMURF1-shRNA, SHP-1-shRNA, and SMURF1-SHP-1-shRNA groups were 67±7.21, 19.3±3.05, 129.3±3.06, and 76.7±1.53, respectively. STAT3 signaling and its downstream targets, MMP2 and MMP9, were also analyzed by Western blotting. SMURF1-shRNA infection significantly decreased the expression of MMP2 and MMP9 as well as p-STAT3 levels compared with shNC, whereas SHP-1-shRNA infection demonstrated an inverse effect (*Figure 5E,F,G*). Interestingly, SHP-1-shRNA also significantly inhibited a SMURF1-shRNA-



Predicated E3 ligases	RecName	Score
SMURF1	E3 ubiquitin-protein ligase SMURF1	0.792
CBL	E3 ubiquitin-protein ligase CBL	0.789
BMI1	Polycomb complex protein BMI-1	0.788
MDM2	E3 ubiquitin-protein ligase Mdm2	0.787
SYVN1	E3 ubiquitin-protein ligase synoviolin	0.762

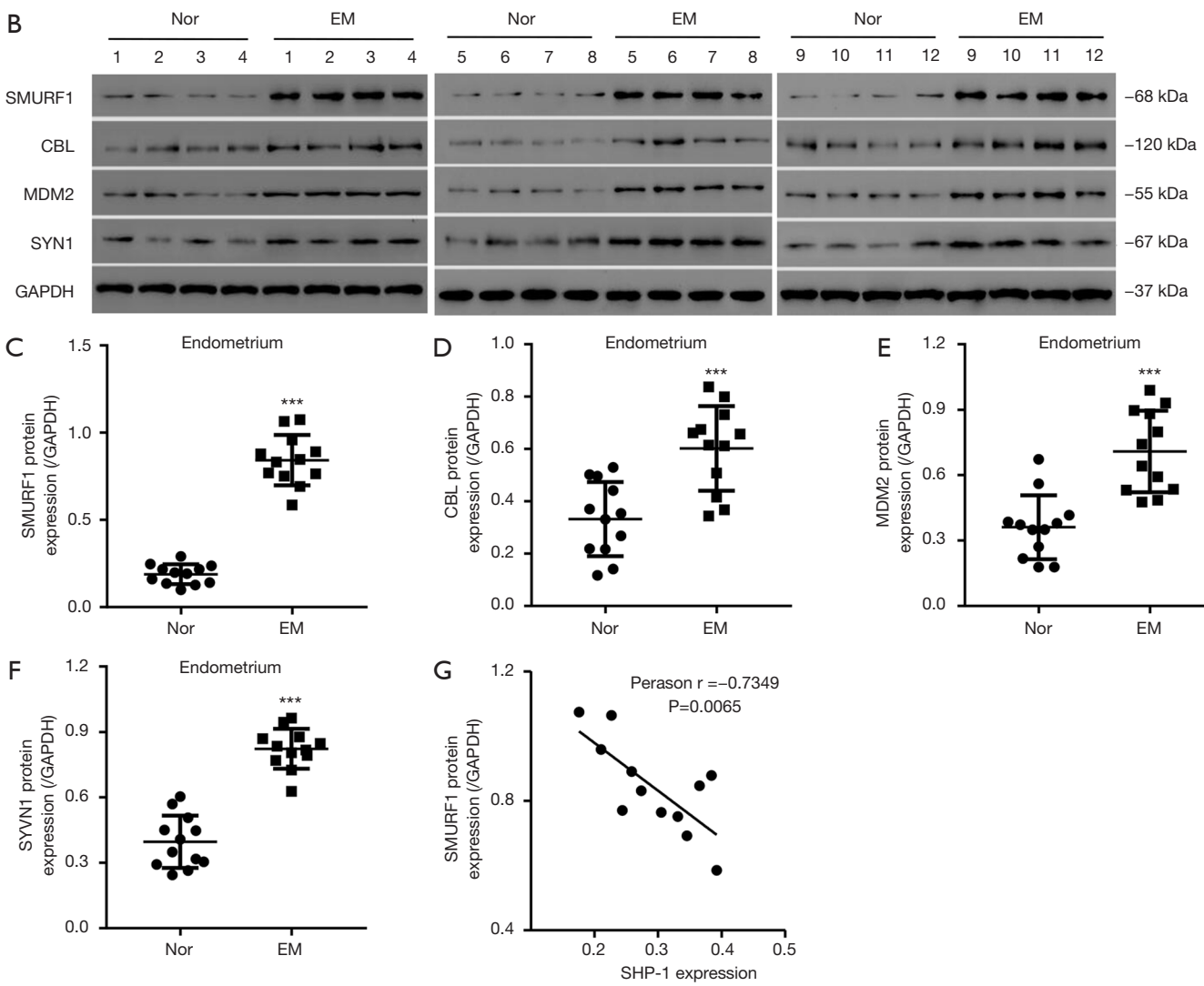


Figure 2 Expression of predicated E3 ligases and correlation analysis in ectopic endometrium from patients with endometriosis. (A) Top 20 predicated human E3 ubiquitin ligases interact with SHP-1. Five high confidence interactions between SHP-1 and human E3 ubiquitin ligases are shown. Representative SMURF1 (B,C), CBL (B,D), MDM2 (B,E), and SYVN1 (B,F) expression levels in ectopic endometrium from women with endometriosis (EM1–4) or in normal endometrium from women without endometriosis (Nor1–4) were measured using Western blotting. Results are presented as mean ± SD of 12 samples in triplicate and means comparisons were performed using unpaired *t*-test. (G) Pearson correlation scatter plot in ectopic endometrium from patients with endometriosis (n=12). ***, P<0.001 *vs.* Nor groups.

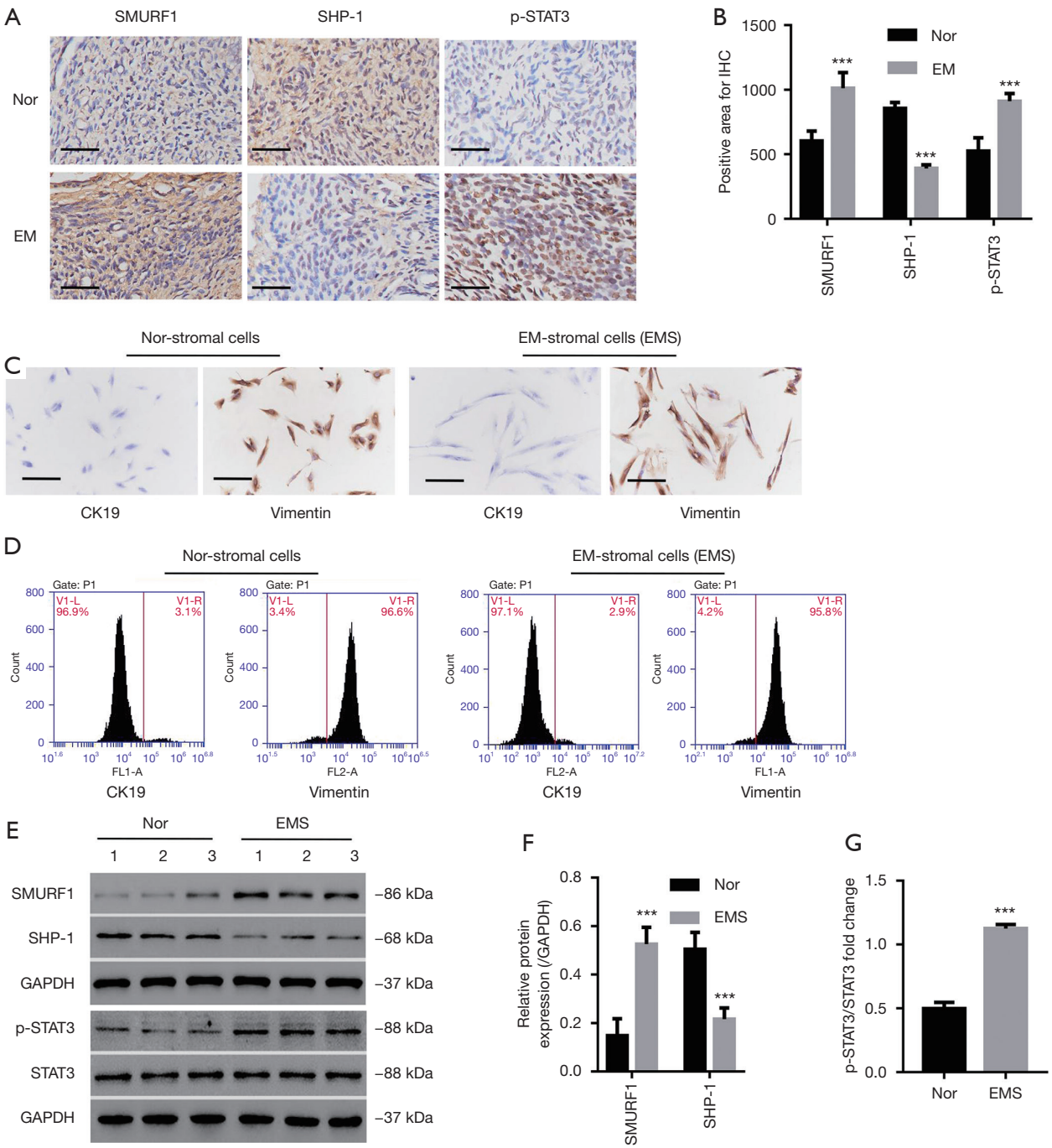
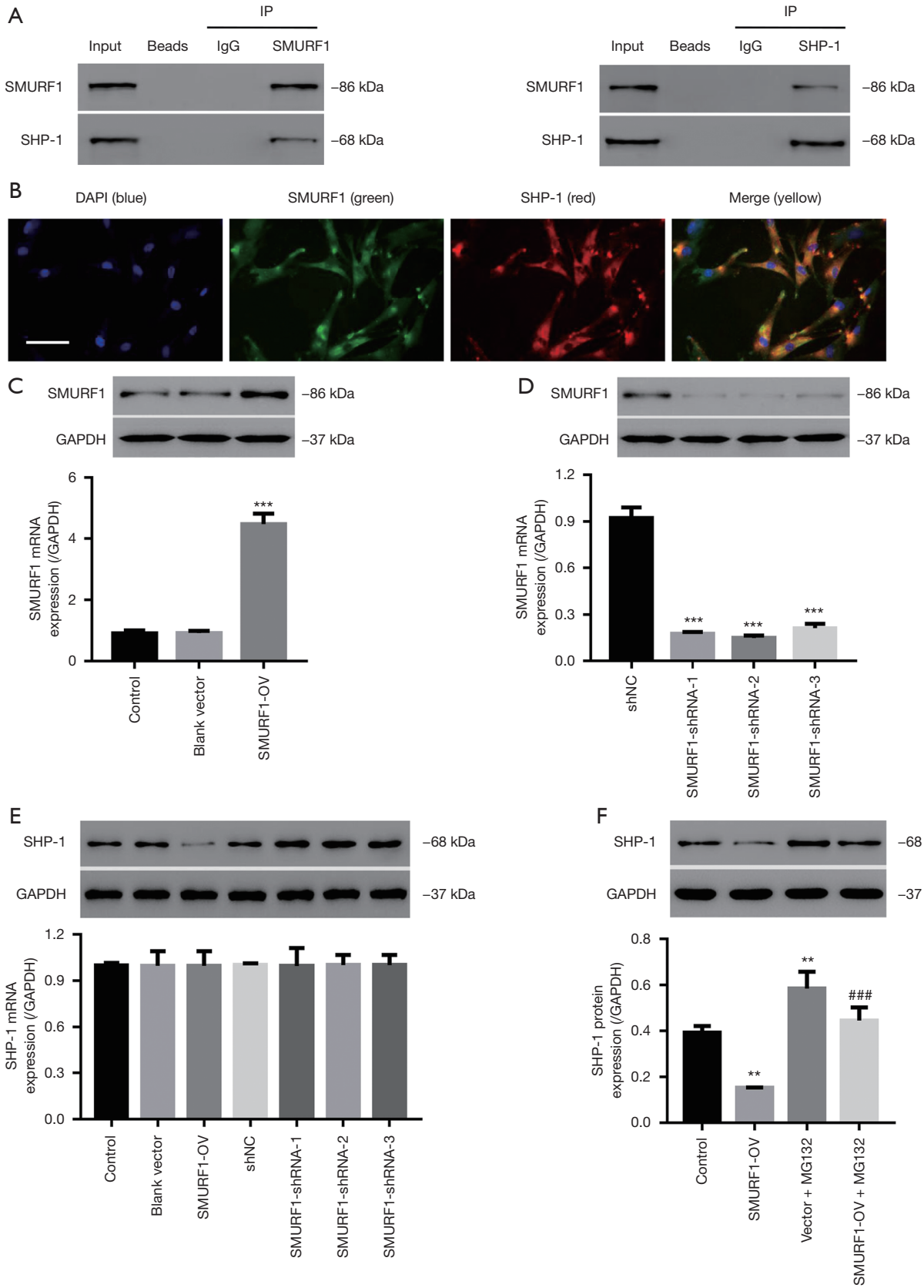


Figure 3 SMURF1, SHP-1, and STAT3 expression and p-STAT3 levels in ectopic endometrial stromal cells from patients with endometriosis. (A,B) SMURF1, SHP-1, and p-STAT3 levels in ectopic endometrium from women with endometriosis (EM) or in normal endometrium from women without endometriosis (Nor) were measured by IHC. Scale bar: 25 μm. (C) Intracellular expression of cytoskeleton proteins CK19 and vimentin was measured with immunocytochemistry. Scale bar: 10 μm. (D) Stromal cells expressed vimentin and CK19 were measured by flow cytometric analysis. (E,F,G) Representative SMURF1, SHP-1, and STAT3 expression as well as p-STAT3 levels in endometrial stromal cells from ectopic endometrium of women with endometriosis (EMS1–3) or that from normal endometrium of women without endometriosis (Nor1–3) was measured by Western blotting. Results are presented as mean ± SD of three samples in triplicate and means comparisons were performed using two-way ANOVA followed by Tukey's test (B,F) or unpaired *t*-test (G). ***, P<0.001 *vs.* Nor groups. IHC, immunohistochemistry; ANOVA, analysis of variance.



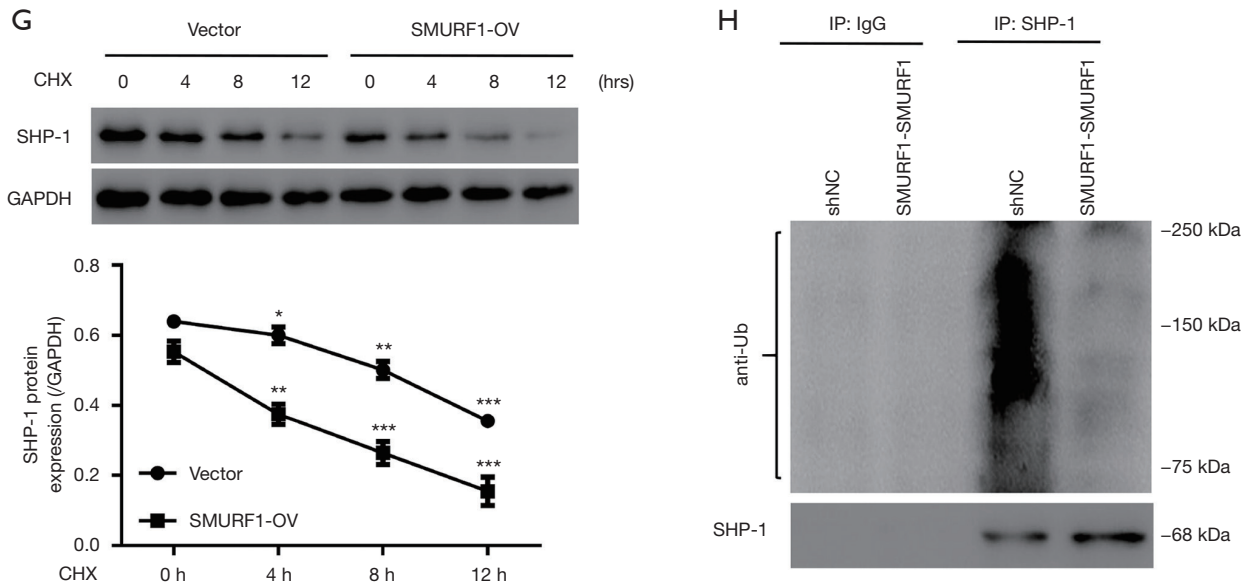


Figure 4 SMURF1 binds to and promotes ubiquitylation of SHP-1. (A) Co-immunoprecipitation showed that SMURF1 interacted with SHP-1. (B) The subcellular localizations of SMURF1 (green) and SHP-1 (red) were measured by immunofluorescence. DAPI (blue) was used for nuclear counter stain. Scale bar: 10 μm. (C-F) Ectopic endometrial stromal cells were infected with pLVX-Puro-SMURF1 or pLKO.1-SMURF1-shRNA in the absence or presence of 10 μM MG132, and SMURF1 and SHP-1 expression was measured by qPCR and Western blotting. (G) Ectopic endometrial stromal cells infected with pLVX-Puro-SMURF1or blank vector were treated with CHX (0.1 mg/mL) and harvested at the indicated times. Cells were lysed and the lysates were then blotted with the indicated antibodies. (H) Ectopic endometrial stromal cells were infected with pLKO.1-SMURF1-shRNA, and SHP-1 was immunoprecipitated and immunoblotted using the indicated antibodies. Results are presented as mean ± SD of three samples in triplicate and means comparisons were performed using one-way (C-F) or two-way (G) ANOVA followed by Tukey's test. *, P<0.05, **, P<0.01, ***, P<0.001 *vs.* vector, shNC or 0 h groups; ###, P<0.001 *vs.* SMURF1-OV (SMURF1 overexpression) groups. qPCR, quantitative polymerase chain reaction; CHX, cycloheximide; ANOVA, analysis of variance.

mediated decrease in MMP2 and MMP9 expression as well as p-STAT3 levels. However, STAT3 expression showed no changes among the four groups.

Overexpression of SMURF1 and/or SHP-1 regulates normal endometrial stromal cell proliferation and invasion

Our findings prompted us to examine whether SMURF1 and SHP-1 also regulate the proliferation and invasion of normal endometrial stromal cells from women without endometriosis. Endometrial stromal cells were infected with pLVX-Puro-SMURF1 and/or pLVX-Puro-SHP-1 and cell proliferation and invasion were measured. pLVX-Puro-SHP-1 infection significantly increased the expression of SHP-1 compared with that seen with the blank vector (*Figure 6A,B*). Furthermore, pLVX-Puro-SMURF1 infection significantly increased cell proliferation and cell invasion compared with pLVX-Puro-SMURF1 at 48 h, whereas pLVX-Puro-SHP-1 infection demonstrated an inverse effect (*Figure 6C,D*). Interestingly, pLVX-Puro-SHP-1 infection also significantly inhibited pLVX-Puro-SMURF1-mediated increases of cell proliferation and invasion. The number of invading cells in the blank vector, pLVX-Puro-SMURF1, pLVX-Puro-SHP-1, and pLVX-Puro-SMURF1+pLVX-Puro-SHP-1 groups were 49.7±4.04, 99.3±4.51, 14±1.01, and 29.3±2.52, respectively. Furthermore, pLVX-Puro-SMURF1 infection significantly increased the expression of MMP2 and MMP9 as well as p-STAT3 levels compared with that seen with the blank vector, whereas pLVX-Puro-SHP-1 infection demonstrated an inverse effect (*Figure 6E,F,G*). Interestingly, pLVX-Puro-SHP-1 also significantly inhibited pLVX-Puro-SMURF1-

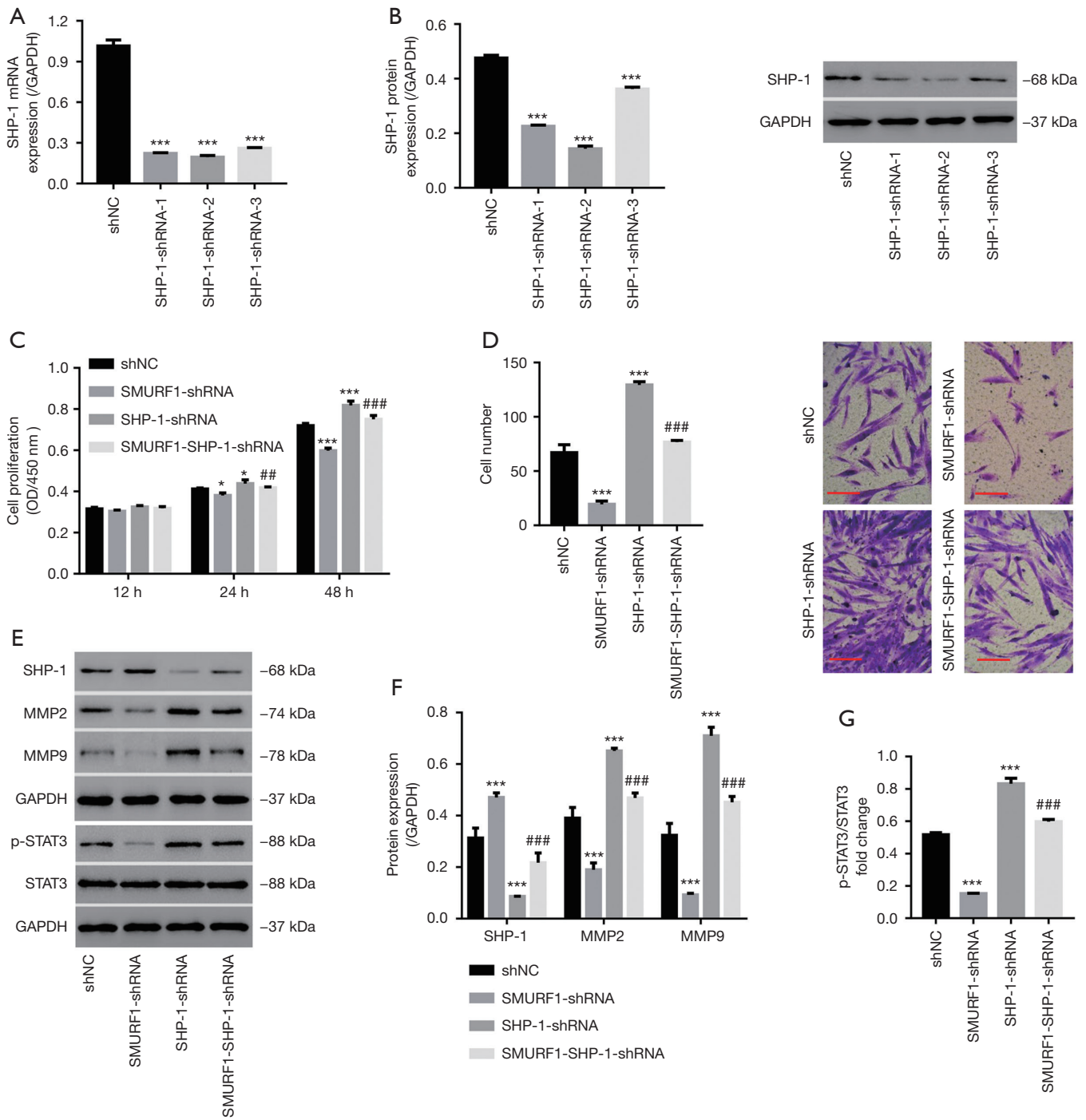


Figure 5 Silencing of SMURF1 and/or SHP-1 regulates the proliferation and invasion of ectopic endometrial stromal cells. Ectopic endometrial stromal cells were infected with pLKO.1-SMURF1-shRNA, pLKO.1-SHP-1-shRNA, or pLKO.1-scramble shRNA as a negative control, and the expression of SHP-1, MMP2, MMP9, and STAT3 as well as p-STAT3 levels were determined by qPCR (A) or Western blot analysis (B,E-G), and cell proliferation (C) and invasion (D) were measured by CCK-8 and Transwell analysis, respectively. Scale bar: 10 μm. Results are presented as mean ± SD of three samples in triplicate and means comparisons were performed using one-way (A,B,D,G) or two-way (C,F) ANOVA followed by Tukey's test. *, P<0.05, ***, P<0.001 *vs.* shNC; ##, P<0.01, and ###, P<0.001 *vs.* SMURF1-shRNA groups. qPCR, quantitative polymerase chain reaction; MMP, matrix metalloproteinase; CCK-8, Cell Counting Kit-8; ANOVA, analysis of variance.

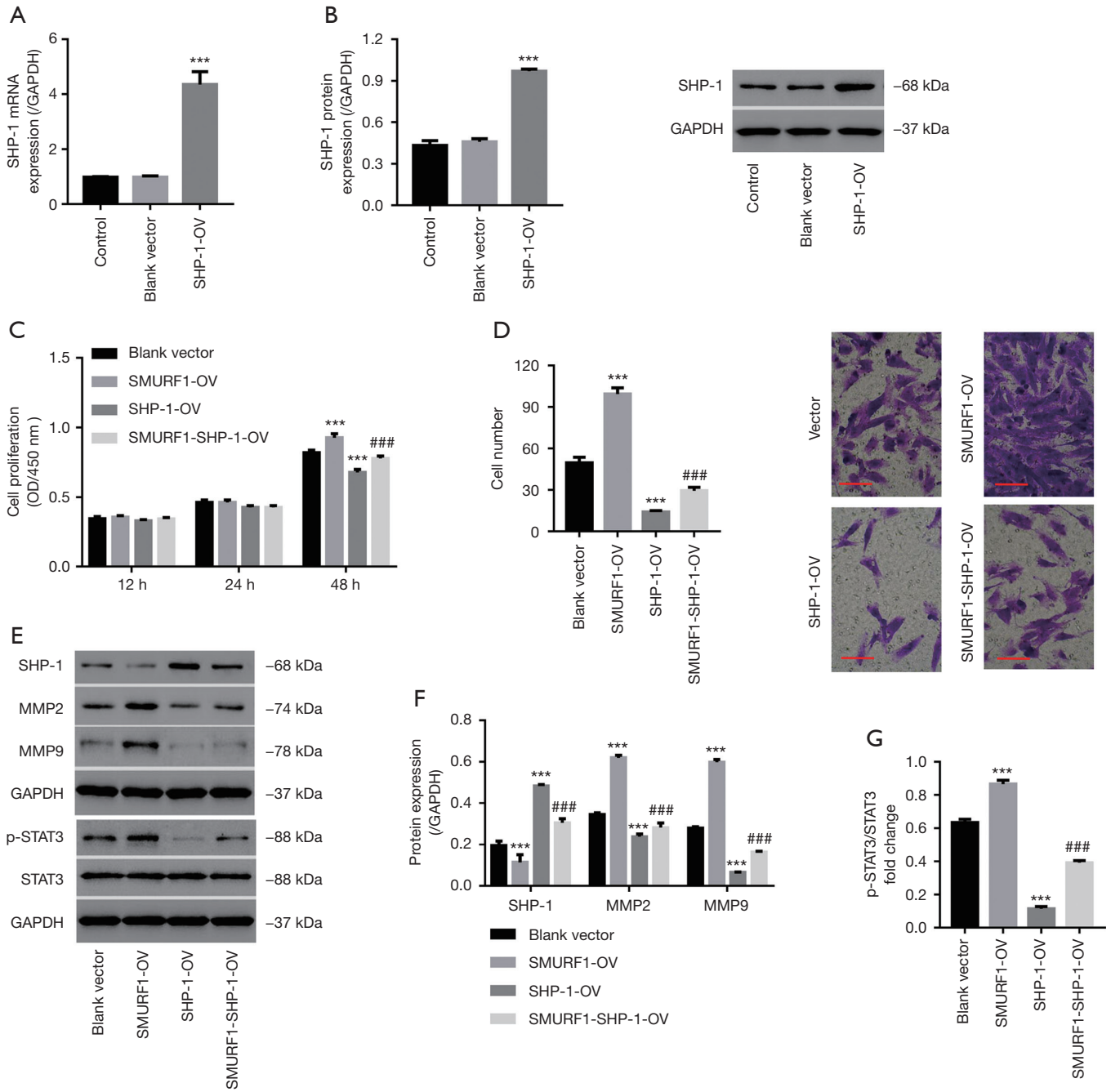


Figure 6 Overexpression of SMURF1 and/or SHP-1 regulates the proliferation and invasion of normal endometrial stromal cells. Normal endometrial stromal cells were infected with pLVX-Puro-SMURF1, pLVX-Puro-SHP-1, or blank pLVX-Puro as a negative control, and the expression of SHP-1, MMP2, MMP9, and STAT3 as well as p-STAT3 levels were determined by qPCR (A) or Western blot analysis (B,E-G), and cell proliferation (C) and invasion (D) were measured by CCK-8 and Transwell analysis, respectively. Scale bar: 10 μm. Results are presented as mean ± SD of three samples in triplicate and means comparisons were performed using one-way (A,B,D,G) or two-way (C,F) ANOVA followed by Tukey's test. ***, P<0.001 *vs.* blank vector; ###, P<0.001 *vs.* SMURF1-OV (SMURF1 overexpression) groups. qPCR, quantitative polymerase chain reaction; MMP, matrix metalloproteinase; CCK-8, Cell Counting Kit-8; ANOVA, analysis of variance.

mediated increase in MMP2 and MMP9 expressions as well as p-STAT3 levels, whereas STAT3 expression showed no changes among the four groups.

Discussion

While endometriosis is not a cancer, it displays features of tumor cells such as angiogenesis, adhesion, growth, invasion, and migration (36), which contribute to its development and maintenance. Endometrial stromal cells are increasingly recognized as an essential component in the development of endometriosis show increased proliferation, invasion and migration, and decreased apoptosis in patients with endometriosis (37). STAT3 activity is negatively regulated by SHP-1 and is important for normal uterine function and associated with the pathogenesis of endometriosis (18,19). However, SHP-1 expression in endometriosis and the cellular and molecular mechanisms underlying the proliferation and invasion of endometrial stromal cells induced by the SHP-1-STAT3 signaling axis remain unclear.

Aberrant activation of STAT3 has been identified as both abnormal and oncogenic by stimulating cell proliferation, promoting angiogenesis, migration and invasion, and conferring resistance to apoptosis (38). Previous findings showed that levels of p-STAT3 in the endometrium were markedly increased in women with endometriosis compared with those without endometriosis (19), and several PTPs such as SHP-1 have been implicated in the negative regulation of the JAK/STAT3 signaling pathway (21), Therefore, we hypothesized that SHP-1 expression may be deregulated in endometriosis, and aberrant deletion of SHP-1 within the ectopic endometrium is relevant to endometriosis due to dysregulation of cell proliferation and invasion via STAT3 signaling. Despite an increase in SHP-1 protein levels in endometriosis, *SHP-1* mRNA levels did not change in the peritoneal fluid cells between endometriosis patients and controls (39). These results suggest that post-transcriptional regulation of SHP-1 may play an important role in endometriosis.

RING-finger protein 6 (RNF6) can activate the JAK/STAT3 pathway by modifying SHP-1 ubiquitylation (18). In the present study, SHP-1 was bioinformatically predicted to be a substrate that could interact with SMURF1, CBL, BMI1, MDM2, and SYVN1, with increased expression in ectopic endometrium. However, RNF6 expression did not differ between patients with or without endometriosis (data not shown) and only SMURF1 was negatively and positively correlated with the SHP-1 and p-STAT3 expression, respectively, in endometriosis, suggesting the involvement of SMURF1 in the regulation of SHP-1–STAT3 signaling in endometriosis. The role of these predicted E3 ubiquitin ligases in the pathogenesis of endometriosis has not been previously reported, except BMI1, which did not show differential expression between patients with or without endometriosis (35). Although few studies have investigated the role of SMURF1 in endometriosis, RhoA, a substrate that interacts with and is ubiquitylated by SMURF1, is mainly distributed in the cytoplasm of endometrial stromal cells and shows lower expression levels in endometriosis compared with normal endometrium (40,41), These findings are similar to our observation that SMURF1 negatively regulated SHP-1 protein levels in endometriosis via ubiquitylation of SHP-1. However, Smad7 ubiquitylation and degradation by SMURF1 was increased in the peritoneal fluid from women with endometriosis compared with those without endometriosis (42,43). These conflicting results suggest that substrate post-translational modifications by SMURF1 may be responsible for its different functions in endometriosis. Moreover, based on bioinformatics prediction by using UbiBrowser, K19, K277, and K391 of SHP-1 are predicted to be the ubiquitination sites mediated by SMURF1; hence, future studies are required to elucidate their roles in SMURF1-mediated SHP-1 polyubiquitination.

The initial phase of endometriosis is a motility invasion event that requires STAT3 activation and is associated with several mechanisms of cell invasion and migration, including the transcriptional regulation of the MMPs (33). TECK derived from endometriotic-associated cells promotes endometrial stromal cell invasion by inducing the expression of MMP2 and MMP9 (44). The significant reduction of MMP2 expression in ectopic endometrial stromal cells led to a significant reduction of ectopic lesions in mice (45). MMP9 expression was elevated in ectopic peritoneal fluid, plasma, and tissues in patients with endometriosis and induced the invasion and migration of endometrial epithelial and stromal cells (46). Interestingly, MMP2 and MMP9 expression in different cell types is critically mediated by the STAT3 pathway via binding to their promoters (47-49). Additionally, considerable evidence indicates that the SHP-1-STAT3-MMP2 and MMP9 signaling pathways are involved in regulating cell migration and invasion (50,51). Silencing SMURF1 repressed cell proliferation and invasion as well as MMP2 and MMP9 expression in ovarian cancer (52). Therefore,

it is reasonable to speculate that SMURF1 and the SHP-1-mediated STAT3 signaling may be involved in the proliferation and invasion of endometrial stromal cells via regulation of MMP2 and MMP9 expression. However, it was also reported that SMURF1 could regulate STAT3 activity by facilitating protein inhibitors of activated STAT3 (PIAS3) ubiquitination and degradation (27). Therefore, further studies are required to investigate the molecular mechanism by which SMURF1 regulates STAT3 signaling in endometriosis.

Conclusions

We report for the first time that SMURF1 regulates cell proliferation and invasion as well as STAT3 activation in endometrial stromal cells via ubiquitylation of SHP-1. Although further studies are required to elucidate the effects of SMURF1 and SHP-1 on the progression of endometriosis, downregulation of SMURF1 or upregulation of SHP-1 could represent a new strategy to improve physiological endometriosis by preventing the proliferation and invasion of endometriotic cells.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital, Tongji University (No. 2019tjdx351) and informed consent was taken from all the patients.

References

1. Khan KN, Kitajima M, Fujishita A, et al. Pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions. Hum Reprod 2013;28:109-18.
2. May KE, Villar J, Kirtley S, et al. Endometrial alterations in endometriosis: a systematic review of putative biomarkers. Hum Reprod Update 2011;17:637-53.
3. Vercellini P, Vigano P, Somigliana E, et al. Endometriosis: pathogenesis and treatment. Nat Rev Endocrinol 2014;10:261-75.
4. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. Fertil Steril 2017;108:19-27.
5. Fu X, Yao M, Ye C, et al. Osteopontin regulates endometrial stromal cell migration in endometriosis through the PI3K pathway: osteopontin regulates endometrial cell migration in endometriosis. Reprod Sci 2021;28:435-46.
6. Culley L, Law C, Hudson N, et al. The social and psychological impact of endometriosis on women's lives: a critical narrative review. Hum Reprod Update 2013;19:625-39.
7. Fan YY, Chen HY, Chen W, et al. Expression of inflammatory cytokines in serum and peritoneal fluid from patients with different stages of endometriosis. Gynecol Endocrinol 2018;34:507-12.
8. Sirohi D, Al Ramadhani R, Knibbs LD. Environmental exposures to endocrine disrupting chemicals (EDCs) and their role in endometriosis: a systematic literature review. Rev Environ Health 2020;36:101-15.
9. Metzemaekers J, Slotboom S, Sampat J, et al. Crossroad decisions in deep endometriosis treatment options: a

qualitative study among patients. Fertil Steril 2020. [Epub ahead of print].
10. Dunselman GA, Vermeulen N, Becker C, et al. ESHRE guideline: management of women with endometriosis. Hum Reprod 2014;29:400-12.
11. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. Fertil Steril 2012;98:511-9.
12. McKinnon B, Mueller M, Montgomery G. Progesterone resistance in endometriosis: an acquired property? Trends Endocrinol Metab 2018;29:535-48.
13. Weimar CH, Macklon NS, Post Uiterweer ED, et al. The motile and invasive capacity of human endometrial stromal cells: implications for normal and impaired reproductive function. Hum Reprod Update 2013;19:542-57.
14. Sundqvist J, Andersson KL, Scarselli G, et al. Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis. Hum Reprod 2012;27:2737-46.
15. Liu J, Zhang Z, Liu J, et al. LIM Kinase 1 Mediates estradiol effects on the phosphorylation of cofilin1 in eutopic endometrial stromal cells during the invasion and proliferation of endometriosis. Reprod Sci 2019;26:1499-505.
16. Chen Q, Hang Y, Zhang T, et al. USP10 promotes proliferation and migration and inhibits apoptosis of endometrial stromal cells in endometriosis through activating the Raf-1/MEK/ERK pathway. Am J Physiol Cell Physiol 2018;315:C863-72.
17. Liu H, Zhang W, Wang L, et al. GLI1 is increased in ovarian endometriosis and regulates migration, invasion and proliferation of human endometrial stromal cells in endometriosis. Ann Transl Med 2019;7:663.
18. Liang Q, Ma D, Zhu X, et al. RING-finger protein 6 amplification activates JAK/STAT3 pathway by modifying SHP-1 ubiquitylation and associates with poor outcome in colorectal cancer. Clin Cancer Res 2018;24:1473-85.
19. Kim BG, Yoo JY, Kim TH, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. Hum Reprod 2015;30:1069-78.
20. Liu Y, Qin X, Lu X. Crocin improves endometriosis by inhibiting cell proliferation and the release of inflammatory factors. Biomed Pharmacother 2018;106:1678-85.
21. Kim B, Lee KY, Park B. Crocin suppresses constitutively Active STAT3 through induction of protein tyrosine phosphatase SHP-1. J Cell Biochem 2017;118:3290-8.
22. Suresh HG, Pascoe N, Andrews B. The structure and function of deubiquitinases: lessons from budding yeast. Open Biol 2020;10:200279.
23. Li X, Dai X, Wan L, et al. Smurf1 regulation of DAB2IP controls cell proliferation and migration. Oncotarget 2016;7:26057-69.
24. Shimazu J, Wei J, Karsenty G. Smurf1 inhibits osteoblast differentiation, bone formation, and glucose homeostasis through serine 148. Cell Rep 2016;15:27-35.
25. Scott JL, Frick CT, Johnson KA, et al. Molecular analysis of membrane targeting by the C2 domain of the E3 ubiquitin ligase Smurf1. Biomolecules 2020;10:229.
26. Xia Q, Zhang H, Zhang P, et al. Oncogenic Smurf1 promotes PTEN wild-type glioblastoma growth by mediating PTEN ubiquitylation. Oncogene 2020;39:5902-15.
27. Jiao J, Zhang R, Li Z, et al. Nuclear Smad6 promotes gliomagenesis by negatively regulating PIAS3-mediated STAT3 inhibition. Nat Commun 2018;9:2504.
28. Zhang J, Wu H, Yi B, et al. RING finger protein 38 induces gastric cancer cell growth by decreasing the stability of the protein tyrosine phosphatase SHP-1. FEBS Lett 2018;592:3092-100.
29. Deng Z, Li W, Alahdal M, et al. Overexpression of ClC-3 chloride channel in chondrosarcoma: an in vivo immunohistochemical study with tissue microarray. Med Sci Monit 2019;25:5044-53.
30. Zheng S, Qiao G, Min D, et al. Heterogeneous expression and biological function of ubiquitin carboxy-terminal hydrolase-L1 in osteosarcoma. Cancer Lett 2015;359:36-46.
31. Mohammadi Z, Shariati L, Khanahmad H, et al. a lentiviral vector expressing desired gene only in transduced cells: an approach for suicide gene therapy. Mol Biotechnol 2015;57:793-800.
32. Wang KH, Kao AP, Chang CC, et al. Modulation of tumorigenesis and oestrogen receptor-alpha expression by cell culture conditions in a stem cell-derived breast epithelial cell line. Biol Cell 2010;102:159-72.
33. Ahn JH, Choi YS, Choi JH. Leptin promotes human endometriotic cell migration and invasion by up-regulating MMP-2 through the JAK2/STAT3 signaling pathway. Mol Hum Reprod 2015;21:792-802.
34. Yu H, Zhong Q, Xia Y, et al. MicroRNA-2861 targets STAT3 and MMP2 to regulate the proliferation and apoptosis of ectopic endometrial cells in endometriosis. Pharmazie 2019;74:243-9.
35. Forte A, Schettino MT, Finicelli M, et al. Expression pattern of stemness-related genes in human endometrial and endometriotic tissues. Mol Med 2009;15:392-401.

36. Moggio A, Pittatore G, Cassoni P, et al. Sorafenib inhibits growth, migration, and angiogenic potential of ectopic endometrial mesenchymal stem cells derived from patients with endometriosis. Fertil Steril 2012;98:1521-30.e2.
37. Delbandi AA, Mahmoudi M, Shervin A, et al. Eutopic and ectopic stromal cells from patients with endometriosis exhibit differential invasive, adhesive, and proliferative behavior. Fertil Steril 2013;100:761-9.
38. Fagard R, Metelev V, Souissi I, et al. STAT3 inhibitors for cancer therapy: have all roads been explored? JAKSTAT 2013;2:e22882.
39. Kang YJ, Jeung IC, Park A, et al. An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression. Hum Reprod 2014;29:2176-89.
40. Zhang WB, Wu YI, Huang HD, et al. Preliminary research on the role of rho family RhoA, RhoB, RhoC mRNA expression in endometriosis. Chin J Exp Trad Med Formulae 2013;19:271-3.
41. Wang HR, Ogunjimi AA, Zhang Y, et al. Degradation of RhoA by Smurf1 ubiquitin ligase. Methods Enzymol 2006;406:437-47.
42. Kume S, Haneda M, Kanasaki K, et al. SIRT1 inhibits transforming growth factor beta-induced apoptosis in glomerular mesangial cells via Smad7 deacetylation. J Biol Chem 2007;282:151-8.
43. Hou Z, Sun L, Gao L, et al. Cytokine array analysis of peritoneal fluid between women with endometriosis of different stages and those without endometriosis. Biomarkers 2009;14:604-18.
44. Wang Y, Yu J, Luo X, et al. Abnormal regulation of chemokine TECK and its receptor CCR9 in the endometriotic milieu is involved in pathogenesis of endometriosis by way of enhancing invasiveness of endometrial stromal cells. Cell Mol Immunol 2010;7:51-60.
45. Li MQ, Wang Y, Chang KK, et al. CD4+Foxp3+ regulatory T cell differentiation mediated by endometrial stromal cell-derived TECK promotes the growth and invasion of endometriotic lesions. Cell Death Dis 2014;5:e1436.
46. Zhang C, Wu W, Zhu H, et al. Knockdown of long non-coding RNA CCDC144NL-AS1 attenuates migration and invasion phenotypes in endometrial stromal cells from endometriosis. Biol Reprod 2019;100:939-49.
47. Xie TX, Wei D, Liu M, et al. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 2004;23:3550-60.
48. Song Y, Qian L, Song S, et al. Fra-1 and Stat3 synergistically regulate activation of human MMP-9 gene. Mol Immunol 2008;45:137-43.
49. Kim JE, Kim HS, Shin YJ, et al. LYR71, a derivative of trimeric resveratrol, inhibits tumorigenesis by blocking STAT3-mediated matrix metalloproteinase 9 expression. Exp Mol Med 2008;40:514-22.
50. Wang J, Zhang L, Chen G, et al. Small molecule 1'-acetoxychavicol acetate suppresses breast tumor metastasis by regulating the SHP-1/STAT3/MMPs signaling pathway. Breast Cancer Res Treat 2014;148:279-89.
51. Jia WQ, Wang ZT, Zou MM, et al. Verbascoside inhibits glioblastoma cell proliferation, migration and invasion while promoting apoptosis through upregulation of protein tyrosine phosphatase SHP-1 and inhibition of STAT3 phosphorylation. Cell Physiol Biochem 2018;47:1871-82.
52. Fan X, Wang Y, Fan J, et al. Deletion of SMURF 1 represses ovarian cancer invasion and EMT by modulating the DAB2IP/AKT/Skp2 feedback loop. J Cell Biochem 2019;120:10643-51.

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