Increased expression of IFN-γ in preeclampsia impairs human trophoblast invasion via a SOCS1/JAK/STAT1 feedback loop

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Abstract. The weakening of extravillous trophoblast (EVT) invasion results in shallow placenta implantation. In HTR8/SVneo cells, IFN-γ can activate STAT1 and reduce cell invasion, and suppressor of cytokine signaling (SOCS) is an important negative regulatory protein in the Janus kinase (JAK)/STAT activator pathway and has a negative feedback function on JAK/STAT1. The aim of the present study was to elucidate how SOCS1 feedback regulates JAK/STAT1 and affects EVT cell invasion, which in turn affects the development of preeclampsia (PE). MTT and Annexin V/phosphatidylserine (PS) assays were performed to evaluate the viability and apoptosis of HTR8/SVneo cells treated with IFN-γ, respectively. Wound healing and invasion assays were also conducted to measure the migratory and invasive abilities of IFN-γ-treated HTR8/SVneo cells. The mRNA and protein expression levels of genes were detected using reverse transcription-quantitative PCR and western blot analysis. Small interfering RNA knockdown of SOCS1 was used to verify the role of feedback regulation in the IFN-γ-activated JAK/STAT1 signaling pathway. IFN-γ can inhibit HTR8/SVneo migration and invasion, and promote apoptosis by increasing the expression of phosphorylated (p)-JAK, p-STAT1 and caspase3, and reducing the expression of platelet-derived growth factor receptor A and Ezrin. Furthermore, SOCS1 may negatively regulate JAK/STAT1 and affect HTR-8/SVneo invasiveness. Evaluation of clinical samples demonstrated that the expression levels of SOCS1 and IFN-γ were higher in patients with PE compared with the healthy group. Collectively, the present results indicated that IFN-γ reduced the invasion of HTR-8/SVneo cells by activating JAK/STAT1, concurrently leading to an increase in SOCS1, which negatively regulates JAK/STAT1 and eliminates the pro-inflammatory effects of IFN-γ, thus forming a feedback loop.

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Introduction

Preeclampsia (PE) is medical condition that begins after week 20 of pregnancy, with hypertension and proteinuria as the main clinical manifestations (1). In the United States, the prevalence of preeclampsia has increased from 2.4% in 1980 to 3.8% in 2010 in all pregnancies over the past three decades (2,3). Several studies have reported that PE pathogenesis is associated with insufficient recasting of the uterine spiral artery, inflammatory immune overactivation, vascular endothelial cell damage, genetic factors and nutritional deficiencies (4). Other theories include the weakening of extravillous trophoblast (EVT) invasion, leading to shallow placental implantation (5).

IFN-γ is produced by decidual natural killer (NK) cells and can induce apoptosis of primary human trophoblasts (6). Previous studies have revealed that IFN-γ increases EVT apoptosis, reduces active protease and insulin growth factor receptor-2 levels and inhibits EVT invasion (7). Additionally, IFN-γ can reduce abnormal expression of MMP1, MMP3 and MMP9 in decidual cells, and reverses the effects of TNF-α on EVT during decidual invasion (8). Therefore, IFN-γ can avoid excessive EVT invasion and reduce PE.

IFN-γ regulates the expression of different genes by activating the JAK/STAT pathway (9). Previous studies on STAT1-deficient fibrosarcoma bone cancer cells observed that STAT1 phosphorylation at tyrosine (Tyr)701 and serine (Ser)727 residues are essential for achieving transcriptional regulation (10). In HTR8/SVneo cells, IFN-γ can activate STAT1 at Ser1727 and Tyr701 residues and reduce basic leucine zipper transcriptional factor ATF-like 2 (BATF2) expression to reduce cell invasion, thus inhibiting JUN. By regulating the expression of JUN, cell invasion is diminished. Moreover, STAT1 and BATF2 function dependently or independently of each other (11).

Suppressor of cytokine signaling (SOCS) is an important negative regulatory protein in Janus kinase (JAK)/STAT activator pathways, and also inhibits the signaling pathways of various cytokines (12). Previous studies have discovered

seven SOCS molecules (SOCS1-7) and cytokine-inducible src homology 2 (SH-2) proteins, and cytokines and growth factors can produce most of these molecules (13). By regulating the Tyr phosphorylation levels of STAT protein, SOCS may negatively regulate the signaling pathways (14). Furthermore, the interaction of SOCS molecules and other signaling pathways may be key to maintain cytokine balance and fetal immune tolerance (15). Numerous cytokines, including the T-helper (Th) 1 cytokines IFN-γ and IL-2, and the Th2 cytokine TNF-α, could rapidly induce SOCS (16,17), and in turn, inhibit cytokine signal transduction.

The present study aimed to investigate IFN-γ-mediated activation of JAK/STAT1 in HTR-8/SVneo cells and its effects on cell viability, migration, invasion and apoptosis. Additionally, the present study examined how SOCS1 feedback regulated JAK/STAT1 and affected EVT invasion. Immortalized human chorionic trophoblast cell HTR-8/SVneo cells were used instead of EVTs.

Materials and methods

Tissue collection. Between January 2018 and June 2018, 30 patients with PE (age range, 21.0-42.0 years; mean age, 31.80±5.91 years) were selected for obstetric examinations and deliveries in The Second Hospital of Shanxi Medical University (Taiyuan, China). The Ethics Committee of The Second Hospital of Shanxi Medical University approved the research protocol, and written informed consent was obtained from each patient. The criteria to diagnose PE was a systolic blood pressure of ≥140 mmHg, a diastolic blood pressure of ≥90 mmHg and 0.3 g protein in urine samples from females that were ≥20 weeks pregnant and had previously healthy blood pressure and 24-h urine samples. In total, 10 healthy pregnant women (age range, 17.0-36.0 years; mean age, 28.90±6.12 years) who gave birth in the Second Hospital of Shanxi Medical University at the same time were selected as a control group. The gestational weeks of pregnant women were 34-39 weeks. Both groups of pregnant women (PE and control) underwent single primary labor, and none of these women had a history of repeated abortions or *in vitro* fertilization. The exclusion criteria were as follows: Patients that presented complications such as pregnancy with hypertension, gestational diabetes, pregnancy with diabetes and pregnancy with heart disease. A total of 3 min after delivery of the placenta, ~1x1x1 cm of placental tissue was collected, which was frozen and stored at -80˚C for further use.

Cell culture. HTR-8/SVneo cells (American Type Culture Collection) were cultured in RPMI-1640 medium (Wuhan Boster Biological Technology, Ltd.) supplemented with 10% FBS (Biological Industries), 100 mg/ml streptomycin and 100 U/ml penicillin. Cells were cultured in a humid atmosphere of 5% CO_2 and 37˚C. Cells were extracted after reaching 80% confluence. Drug intervention cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA) supplemented with 100 ng/ml IFN-γ, while control cells were cultured in conventional RPMI-1640 in a humid atmosphere of 5% CO_2 and 37˚C for 48 h. For inhibition experiments, cells were subjected to fluorescein amidites-labeled small interfering RNA (siRNA) transfection and cultured in RPMI-1640 medium for 48 h. Cells were collected for analysis 24 h after transfection.

MTT assay. HTR-8/SVneo cells in the logarithmic growth phase were plated in 96-well plates with 100 µl cell suspension in each well, and a blank group, with 100 µl sterile PBS added to the wells surrounding the cells was also established. Cells were cultured at 37˚C overnight. Cells were treated with 0, 0.1, 1, 10, 100 and 1,000 ng/ml IFN-γ and were cultured at 37˚C and 5% CO_2 saturated humidity for 48 h. A total 10 µl MTT solution was then added to each well and incubated at 37˚C for 4 h. The media was aspirated, followed by addition of 150 µl DMSO and agitation for 10 min at 37˚C. The absorbance at 568 nm in each well was determined using a microplate reader. All experiments were repeated three times.

Wound healing assay. HTR-8/SVneo cells were seeded in 6-well plates with 2 ml cell suspension/well ($2x10^5$ cells/ml) and cultured overnight at 37˚C. The cells were then treated with 0 and 100 ng/ml IFN-γ (Sigma-Aldrich; Merck KGaA) at 37˚C for 48 h. Once cells reached 90% confluence, a scratch was made using a pipette tip to create a 1-mm-wide scratch, at the same time, cell culture medium was replaced with FBS-free medium. After 0 and 24 h, following three washes with PBS, images were captured using an Olympus IX51 Inverted fluorescence microscope (Olympus Corporation) at x100 magnification. The control and experimental group scratch widths were measured using ImageJ 1.42 (National Institutes of Health) and normalized to their values at 0 h. The migration distance is defined as follows: Migration distance (%) = (At 0 h-At 24 h)/At 0 h x100%. All experiments were repeated three times.

Cell apoptosis assay. Cell apoptosis analysis was performed to detect the percentage of early and late apoptotic cells using the Annexin V-APC apoptosis analysis kit (cat. no. AO2001-11A-G; Tianjin Sungene Biotech Co., Ltd.) were used to measure the apoptotic-inducing ability of IFN-γ. In total, ~$4x10^5$ cells were seeded on a 6-well plate, cultured at 37˚C overnight, treated with complete medium containing 0 and 100 ng/ml IFN-γ at 37˚C for 48 h and digested with 0.25% trypsin without EDTA to terminate digestion. Cells were subsequently collected, washed twice with PBS and centrifuged at 300 x g for 3 min at 37˚C. A total of 500 µl binding buffer was added and cells were resuspended. Following the addition of 5 µl APC, 5 µl 7-AAD was added to the mix and left to react at room temperature for 5 min in the dark. A CytoFLEX flow cytometer and CytExpert software v2.3 (Beckman Coulter, Inc.) were used for apoptosis analysis. All experiments were repeated three times.

Invasion assay. Cells were treated with 0 and 100 ng/ml IFN-γ complete medium at 37˚C for 48 h, and cells ($2x10^5$/ml) were then seeded into 24-well plates (Corning, Inc.). Transwell chambers (5 µm pore size, Corning, Inc.) with Matrigel (cat. no. 356234; Corning, Inc.) were used in the cell invasion assay. Matrigel was melted at 4˚C 1 day in advance, which was used to precoat the Transwell chambers at 37˚C for 30 min. The upper inserts (containing 200 µl cell suspension) were filled with serum-free medium at a concentration of $2x10^5$ cells/ml,

while the medium (800 μl) in the lower chamber contained 10% FBS and RPMI-1640 complete medium supplemented with 1% penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h incubation, the chambers were carefully washed with PBS. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.5% crystal violet at room temperature for 20 min. The non-migratory cells were then removed, the membrane was imaged with an Olympus IX51 inverted light microscope (Olympus Corporation) at x200 magnification and the cell numbers was measured randomly from five visual fields per well. All experiments were repeated three times.

Western blotting. RIPA lysis buffer (Beyotime Institute of Biotechnology) and loading buffer (1 μg/μl, 50 μg) were used to extract total protein from the placenta. Protein concentration was determined using a bicinchoninic acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Total proteins (20 μg) were loaded per lane and separated using 12% SDS-PAGE, and transferred to a PVDF membrane (EMD Millipore) overnight at 4˚C. The membrane was blocked with 10% skim milk in TBS-0.1% Tween (TBS-T) for 2 h at room temperature, followed by three TBS-T washes. Membranes were incubated overnight at 4˚C with the following primary antibodies: Murine anti-STAT1 (dilution 1:2,000; cat. no. 66545-1-Ig; Proteintech Group, Inc.), rabbit anti-phosphorylated (p)-STAT1 (dilution 1:1,000; cat. no. 9177T; Cell Signaling Technology, Inc.) and murine anti-JAK1 (dilution 1:2,000; cat. no. 66466-1-Ig; Proteintech Group, Inc.) in 10% milk; rabbit anti-p-JAK1 (dilution 1:1,000; cat. no. 74129S; Cell Signaling Technology, Inc.), mouse anti-PDGFRA (dilution 1:500; cat. no. sc-398206; Santa Cruz Biotechnology, Inc.), rabbit anti-cleaved caspase3 (dilution 1:1,000; cat. no. 19677-1-AP; Proteintech Group, Inc.), rabbit anti-caspase3 (dilution 1:1,000; cat. no. 19677-1-AP; Proteintech Group, Inc.), rabbit anti-Ezrin (EZR; dilution 1:2,000; cat. no. ab75840; Abcam), rabbit anti-GAPDH (dilution 1:1,000; cat. no. AB-P-R 001; Hangzhou Xianzhi Biotechnology Co., Ltd.) and goat anti-SOCS1 (dilution 1:250; cat. no. ab9870; Abcam). Membranes were then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:50,000; cat. nos. BA1051 and BA1054; Wuhan Boster Biological Technology, Ltd.) for 2 h at room temperature. An enhanced chemiluminescence detection kit (Applygen Technologies, Inc.) was used to detect protein signals. An X-ray film was pressed into the developing solution, a fixing solution was used at room temperature for 5 min and the film was washed. Glyko BandScan 5.0 software (ProZyme, Inc.; Agilent Biotechnologies, Inc.) was used to analyze film gray values. All experiments were repeated three times.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted using TRIzol® reagent (cat. no. 15596-026; Thermo Fisher Scientific, Inc.), and the RNA pellet was resuspended in Millipore water and temporarily stored at -80˚C. NanoDrop was used to assess RNA quantity and purity. RNA (1 μg) was reverse transcribed into cDNA using a Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The temperature protocol for reverse transcription was as follows: 25˚C for 5 min, 50˚C for 15 min, 85˚C for 5 min and 4˚C 10 min. cDNA (20 ng) was used to amplify the reference gene (GAPDH) or target genes (SOCS1, STAT1 and JAK1). The efficiency of primer-amplified amplicons was ~100%. The following primer pairs were used for the qPCR: GAPDH forward, 5'-GGATTTGGTCGTATTGGGCG-3' and reverse, 5'-GGATTTGGTCGTATTGGGCG-3'; SOCS1 forward, 5'-CGACACGCACTTCCGCACATT-3' and reverse, 5'-TGGGTCCCGAGGCCATCTTCAC-3'; STAT1 forward, 5'-TGAACTTACCCAGAATGCC-3' and reverse, 5'-TCTTTCCACCACAAACGAG-3'; and JAK1 forward, 5'-CCTGCCGTGCCCACCTAACT-3' and reverse, 5'-GCTTGTCCGATTGGATGGTT-3'. The reaction mixture consisted of 4 μl cDNA, 0.4 μl (10 μM) forward and reverse primers, 4.8 μl RNase/DNase-free water, 10 μl SYBR-Green buffer (Vazyme Biotech Co., Ltd.) and 0.4 μl 50X ROX (Vazyme Biotech Co., Ltd). The final total reaction volume was 20 μl. ABI QuantStudio 6 Pro systems V2.4.3 (Thermo Fisher Scientific, Inc.) was used for quantitative analysis of mRNA levels. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of 95˚C for 30 sec and annealing/extension at 60˚C for 60 sec. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18), and all experiments were repeated three times.

SOCS1 siRNA transient transfection. siRNA against SOCS1 and NC siRNA were all produced by Shanghai GenePharma Co., Ltd. Cells were extracted at the logarithmic growth phase, and the cell density was adjusted to $2x10^5$ cells/ml via the addition of RPMI-1640 complete medium. Cells were seeded in 6-well plates with 2 ml cell suspension/well and cultured overnight at 37˚C. The following sequences were used: SOCS1 siRNA, 5'-UCGCCCUUAGCGUGAAGAUTT-3' and NC siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'. Cells were transfected with fluorescein amidites-labeled siRNA and cultured in a 37˚C incubator for 24 h and then subjected to the following analysis. A total of 10 μl siRNA was diluted in 100 μl serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.). The mixture was gently mixed with a pipette tip and left to stand for 5 min at room temperature. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was gently mixed before use. A total of 5 μl Lipofectamine 2000 was diluted in 100 μl Opti-MEM and left to stand for 5 min at room temperature. Lipofectamine and siRNA dilutions were mixed gently and left to stand for a further 20 min at room temperature. A total of 200 μl Lipofectamine and siRNA solution was added to each culture well plate and the plate was gently agitated in order for the solution to mix. The cells were cultured in a 37˚C CO_2 incubator. After 6 h, the mixture was aspirated and replaced with standard medium. Cells were cultivated in a 5% CO_2 incubator at 37˚C for 24 h, and photomicrographs were captured using the Olympus IX51 inverted fluorescence microscope (Olympus Corporation) at x100 magnification.

Hematoxylin & eosin (H&E) staining. The placenta tissue was fixed in 4% paraformaldehyde for 24 h at room temperature and the sections (thickness, 5 μm) were stained with hematoxylin (37˚C for 5 min) and eosin (37˚C for 1 min) using a HE Staining kit (Beijing Solarbio Science & Technology Co., Ltd.). For each placental sample, two randomly selected fields of view were captured at x100 or 400 magnification

using the Olympus IX51 inverted light microscope (Olympus Corporation).

ELISA. Placental tissue was washed with cold PBS (0.01 M; pH=7.2-7.4) to remove blood. The clean tissue was cut into small pieces and homogenized in PBS on ice. The placenta homogenate was collected and centrifuged at 5,000 x g for 5 min at 4˚C. Supernatants were tested using ELISA kits for IL-10 (cat. no. E-EL-H0103c; Elabscience, Inc.) and IFN-γ (cat. no. E-EL-H0108c; Elabscience, Inc.) according to the manufacturer's protocol.

Immunohistochemistry. Placental tissue was fixed in 4% formalin overnight and embedded in paraffin at room temperature. Placental sections (thickness, 5 μm) placental sections were cut and SOCS1 was applied using the 3,3'-diaminobenzidine staining method (cat. no. PV-6000; OriGene Technologies, Inc.). Following deparaffinization using xylene followed by a descending ethanol gradient, the antigen retrieval was performed using EDTA buffer (1 mM EDTA; pH 8.0) in a high-pressure steamer at 37˚C for 15 min. Samples were incubated with 3% peroxidase and protein blocking solution at 37˚C for 10 min and then incubated with an anti-SOCS1 antibody (dilution 1:500; cat. no. ab9870; Abcam) overnight at 4˚C. The following day, samples were incubated with secondary antibody using the Universal SP kit (mouse/rabbit streptavidin-biotin method detection system, ready for use; cat. no. SP-9000; ZSGB-BIO; OriGene Technologies, Inc.) at 37˚C for 30 min. The sections were then washed and incubated with chromogen (liquid diaminobenzidine and peroxide buffer; dilution 1:200) at 37˚C for 5 min until a reaction was observed. The slides were counterstained with hematoxylin at 37˚C for 5 min to provide nuclear and morphological details and fixation. For each placental sample, two randomly selected fields of view were captured at x100 or 400 magnification using the Olympus IX51 inverted light microscope (Olympus Corporation).

Statistical analysis. Each experiment was repeated three times. Data are presented as the mean ± SD. GraphPad Prism 5 (GraphPad Software, Inc.) was used for statistical analysis. An unpaired t-test was used for comparison between two groups. A one-way ANOVA was performed followed by Dunnett's test to compare differences between >3 groups. A Spearman's rank correlation coefficient was used for correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Placental tissue pathology. In the control group, the placental villi were rich in blood vessels, with a clear structure and fewer cytotrophoblast cells compared with the PE group (Fig. 1A). By contrast, in the PE group, the number of placental villi was reduced, the structure was irregular and atrophic, with parts of the villus cellulose necrotic. The placental villi trophoblast nodules were increased, and most of the villi were immature (Fig. 1A).

Expression levels of SOCS1 and IFN-γ in villi and outer villous trophoblasts. SOCS1 in patients with PE demonstrated weak syncytial staining with some nuclei and cytotrophoblasts strongly stained. Immunostaining results are presented in Fig. 1A. SOCS1 protein expression in PE placental tissue was measured using western blot analysis (Fig. 1B), which indicated that SOCS1 protein expression was increased in the PE group (Fig. 1B; 0.50±0.28). Compared with the control group, tissue expression levels of IFN-γ were increased in the PE group (Fig. 1C). The correlation between SOCS1 expression and IFN-γ was evaluated, and it was found that SOCS1 expression was strongly, positively correlated with IFN-γ in healthy placental and PE groups (Fig. 1D).

IFN-γ activates JAK/STAT, inhibits HTR8/SVneo cell viability, migration and invasion and promotes apoptosis. MTT experiments were performed to detect the cell viability of HTR-8/SVneo cells. The results showed a dose-dependent inhibitory effect of IFN-γ on the cell viability of HTR-8/SVneo cells (Fig. 2A). The data demonstrated a significant reduction in the number of live cells treated with IFN-γ (100 ng/ml) compared with the untreated control group (Fig. 2A), therefore 100 ng/ml was selected as the concentration for the subsequent analysis. Furthermore, the migratory and invasive abilities were significantly reduced (Fig. 2B-E), while apoptosis (Fig. 2F and G) was significantly increased in HTR-8/SVneo cells treated with 100 ng/ml IFN-γ compared with untreated cells.

SOCS1 may be involved in IFN-γ mediated reduction of HTR8/SVneo cell invasion. RT-qPCR results demonstrated that compared with untreated controls, HTR-8/SVneo cells treated with IFN-γ (100 ng/ml) for 48 h had significantly increased mRNA expression levels of SOCS1 (Fig. 3A). Additionally, a significant increase in SOCS1 protein expression was observed (Fig. 3D and E). RT-qPCR results also indicated that, with GAPDH was used as a reference, JAK and STAT1 mRNA expression levels were significantly increased compared with the untreated control group (Fig. 3B and C). Western blot analysis found that p-JAK and p-STAT1 expression levels were significantly increased compared with the control group, while JAK and STAT1 expression levels were not significantly altered (Fig. 3H and I). In the IFN-γ treatment group, cell migration and invasion-related markers PDGFRA and EZR were significantly reduced compared with the control group, and the apoptosis-related gene cleaved caspase3 expression was significantly increased (Fig. 3F and G).

SOCS1 silencing further inhibits IFN-γ-mediated HTR8/SVneo cell invasion. SOCS1 siRNA was transfected into HTR8/SVneo cells to investigate the role of SOCS1 in the reduction of IFN-γ-mediated invasion. RT-qPCR and fluorescence microscopy were used to assess SOCS1 silencing at the transcript level. Compared with cells transfected with siRNA negative control (siNC), the expression of SOCS1 in SOCS1 siRNA-transfected cells was significantly reduced (Fig. 4A and B).

The results demonstrated that IFN-γ-treated SOCS1 siRNA-transfected cells had a significantly lower migratory capacity (Fig. 5A and B). The data showed that the invasion of siSOCS1-transfected cells was significantly reduced in comparison with the siNC group (Fig. 5C). Moreover, the invasion of cells transfected with SOCS1 siRNA and treated with IFN-γ was further reduced compared with cells transfected

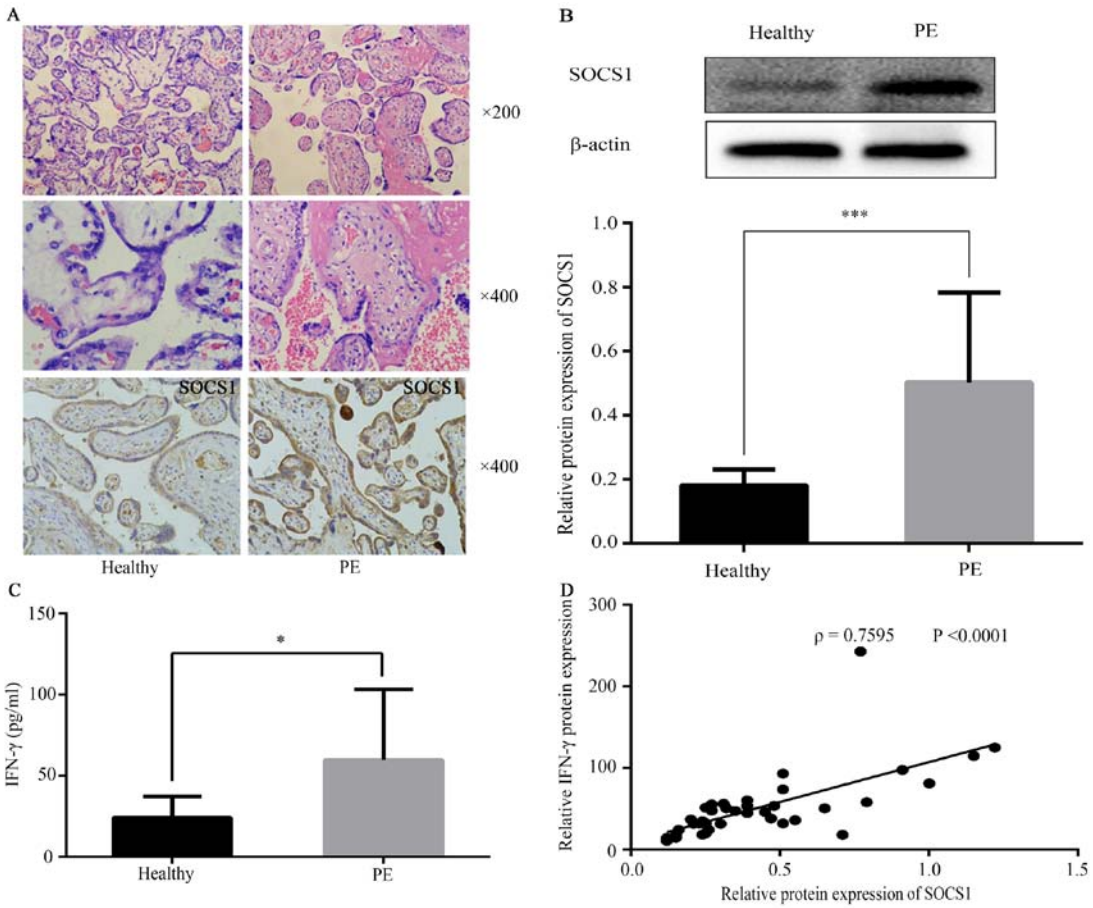

Figure 1. SOCS1 and IFN-γ are highly expressed in the placenta of patients with PE. (A) Compared with healthy maternal patients, the placenta of patients with PE has more synaptic cell nodules, capillary congestion, fibrous necrotic villi and reduced vascular syncytial membrane villi. SOCS1 is mainly located in the trophoblast of the placenta. Compared with the healthy control, the immunostaining of SOCS1 in the trophoblast of the PE group placenta was increased. (B) SOCS1 protein expression in the placenta. The expression of SOCS1 in PE was higher compared with healthy control. ***P<0.001. (C) Level of IFN-γ protein in placental tissue. IFN-γ levels were detected using ELISA in patients with PE. *P<0.05. (D) A positive correlation was found between SOCS1 and IFN-γ in the placenta. ρ=0.7595. PE, PE; SOCS1, suppressor of cytokine signaling 1; IFN-γ, interferon-γ.

with siNC and treated with IFN-γ (Fig. 5C and D). In addition, IFN-γ-treated SOCS1 siRNA-transfected cells significantly increased apoptosis compared with that in the IFN-γ-treated siNC-transfected group (Fig. 5E and F). Concurrently, RT-qPCR (Fig. 6A) and western blotting (Fig. 6B-E) results demonstrated that knockdown of SOCS1 reduced SOCS1 expression in HTR8+ siSOCS1 group compared with that in the HTR + NC group, but IFN-γ intervention increased SOCS1 expression in HTR8 + IFN-γ + siSOCS1 group compared with that in the HTR + siSOCS1 group. Compared with the siNC group, western blot analysis indicated that EZR and PDGFRA expressions, which is related to cell invasion and migration respectively (19,20), were reduced in the siSOCS1 group; whilst the expression of cell apoptosis marker cleaved caspase3 was increased in the siSOCS1 group (Fig. 6B and C). Furthermore, western blot results showed that EZR and PDGFRA expressions were reduced in the IFN-γ + siSOCS1 group compared with the IFN-γ + siNC group, whilst the expression of cell apoptosis marker cleaved caspase3 was increased in the IFN-γ and siSOCS1 group compared with that in the IFN-γ and siNC group (Fig. 6D and E). These results indicated that IFN-γ-treated SOCS1 siRNA-transfected cells had a significantly lower migratory capacity and increased apoptosis.

Discussion

Placental trophoblast cell differentiation is a complex process (21). Some of the trophoblast cells differentiate into trophoblastic cells, which have a high degree of infiltration ability and assists in implantation (22). Following implantation, trophoblast cells are further separated into villous trophoblastic and EVTs. During placental implantation, EVTs migrate into the uterus and change the blood vessels. EVT enters the lumen of the uterine spiral arterioles and gradually replaces the smooth muscle cells and endothelial cells of the vascular wall (23,24). This transforms the artery from a high-resistance low-volume blood vessel to a low-resistance high-volume blood vessel to increase the blood flow of the placenta and ensure the normal exchange of materials between mother and fetus (23,24). The present study investigated the relationship between IFN-γ and trophoblast invasion using HTR-8/SVneo cells as the *in vitro* model due to its highly similar physiological phenotype with EVTs in early pregnancy (25,26).

In the present study, treatment of HTR-8/SVneo cells with IFN-γ reduced the invasion of HTR-8/SVneo cells, while cell viability was not affected. Laganà *et al* (27) reported that endothelial progenitor cells were significantly lower, whereas

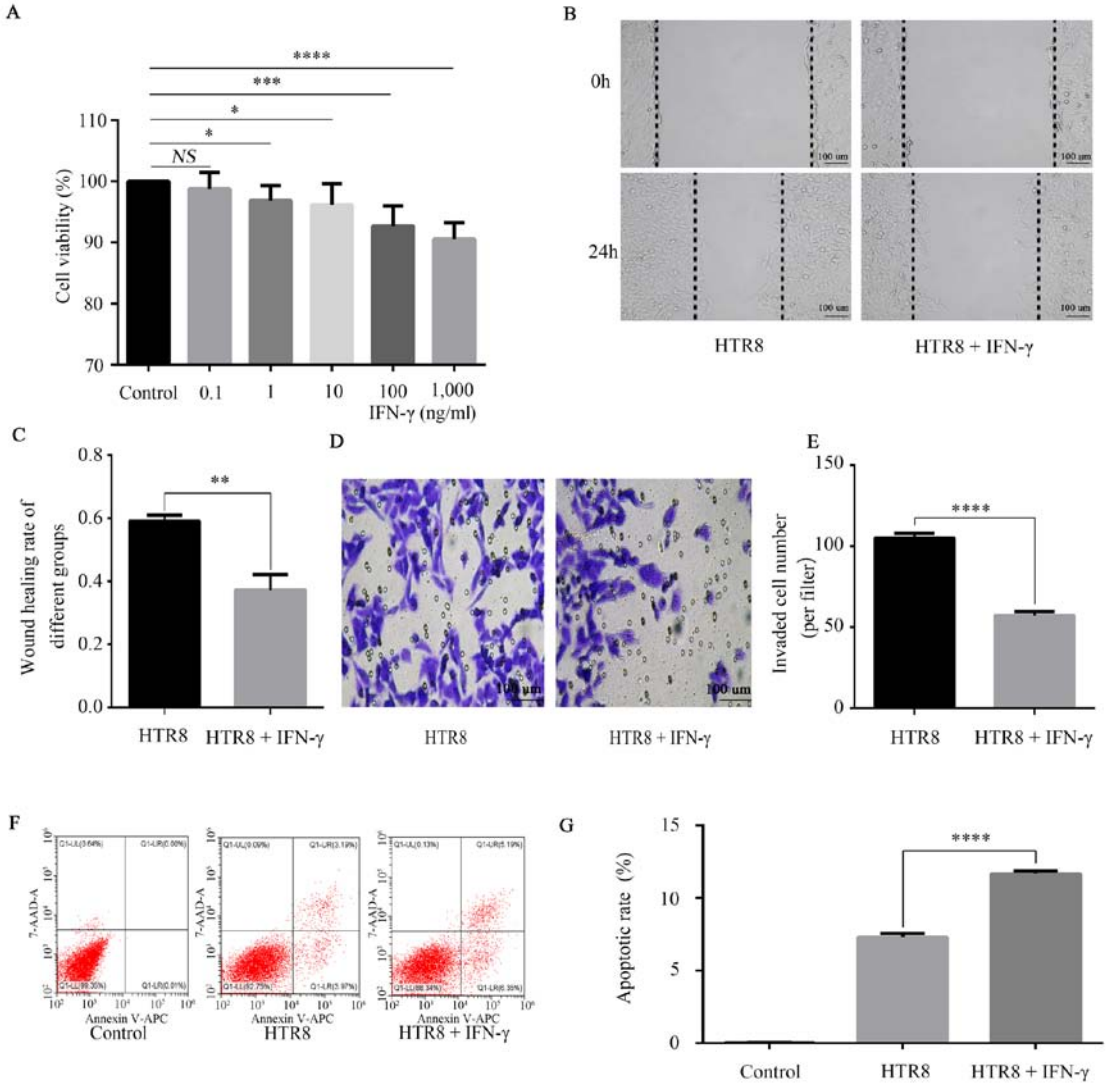

Figure 2. Effects of IFN-γ on cell viability, migration, migration and apoptosis of HTR8/SVneo cells. (A) HTR8/SVneo cells were treated with 0, 0.1, 1, 10, 100 and 1,000 ng/ml IFN-γ. MTT assay was conducted to detect cell viability, with the absorbance measured at a wavelength of 568 nm. HTR8/SVneo cells were treated with 0 and 100 ng/ml IFN-γ for 48 h. NS, not significant; *P<0.05; ***P<0.001; ****P<0.0001. (B) Wound healing results indicated that (C) IFN-γ decreased HTR8/SVneo migration. **P<0.01. (D) Transwell assay results demonstrated that (E) IFN-γ decreased HTR8/SVneo invasion. ****P<0.0001. (F) Flow cytometry findings suggested that (G) IFN-γ increased the apoptotic rate. ****P<0.0001. Data are presented as the mean ± SD of three independent experiments. IFN-γ, interferon-γ.

NK cells were significantly higher in a PE group compared with uncomplicated pregnancies during the first trimester. NK cells in the maternal endometrium produce high amounts of IFN-γ (28). Previous studies (29-32) have aimed to identify precise and unique biochemical markers in the serum to predict PE; however, there has been limited success (33). In early pregnancy, high levels of IFN-γ are beneficial for pregnancy (34), but excessive IFN-γ can inhibit cell reproduction and metabolic activities, as well as induce apoptosis. Simultaneously, during the second to third trimester of pregnancy, IFN-γ secretion decreases, regulating the number and invasiveness of trophoblast cells (35). There have been reports of persistently high levels of IFN-γ secretion in the plasma of patients with PE (36,37), and the present clinical data also demonstrated that patients with PE secrete higher levels of IFN-γ in the placenta. Pinheiro *et al* (38) reported that elevated levels of pro-inflammatory cytokines in the maternal circulation with a deviation in the IL-8/IL-6 axis towards IFN-γ may drive the cytokine network in women with PE towards an excessive systemic inflammatory state. Collectively, these studies suggest that IFN-γ is required during implantation, but levels above or below the threshold may be harmful to pregnant women.

Previous studies have reported that IFN-γ can activate JAK/STAT1 and innate and adaptive immune responses, as well as promote apoptosis. For example, it was revealed that IFN-γ binds to its receptor and then activates JAK1/2 (39). Subsequently, cytoplasmic STAT1 is recruited to the receptor complex and phosphorylated at Tyr701 and Ser727 (40). Following phosphorylation, STAT1 forms a homodimer and translocates to the nucleus where it binds to the conserved DNA sequence in the promoter region of the downstream target gene and activates its transcription (9). Previous studies have shown that IFN-γ could activate the JAK/STAT pathway in HTR-8/SVneo cells (41,42). STAT1 is phosphorylated at Ser727 and Tyr701 residues (43). In the present

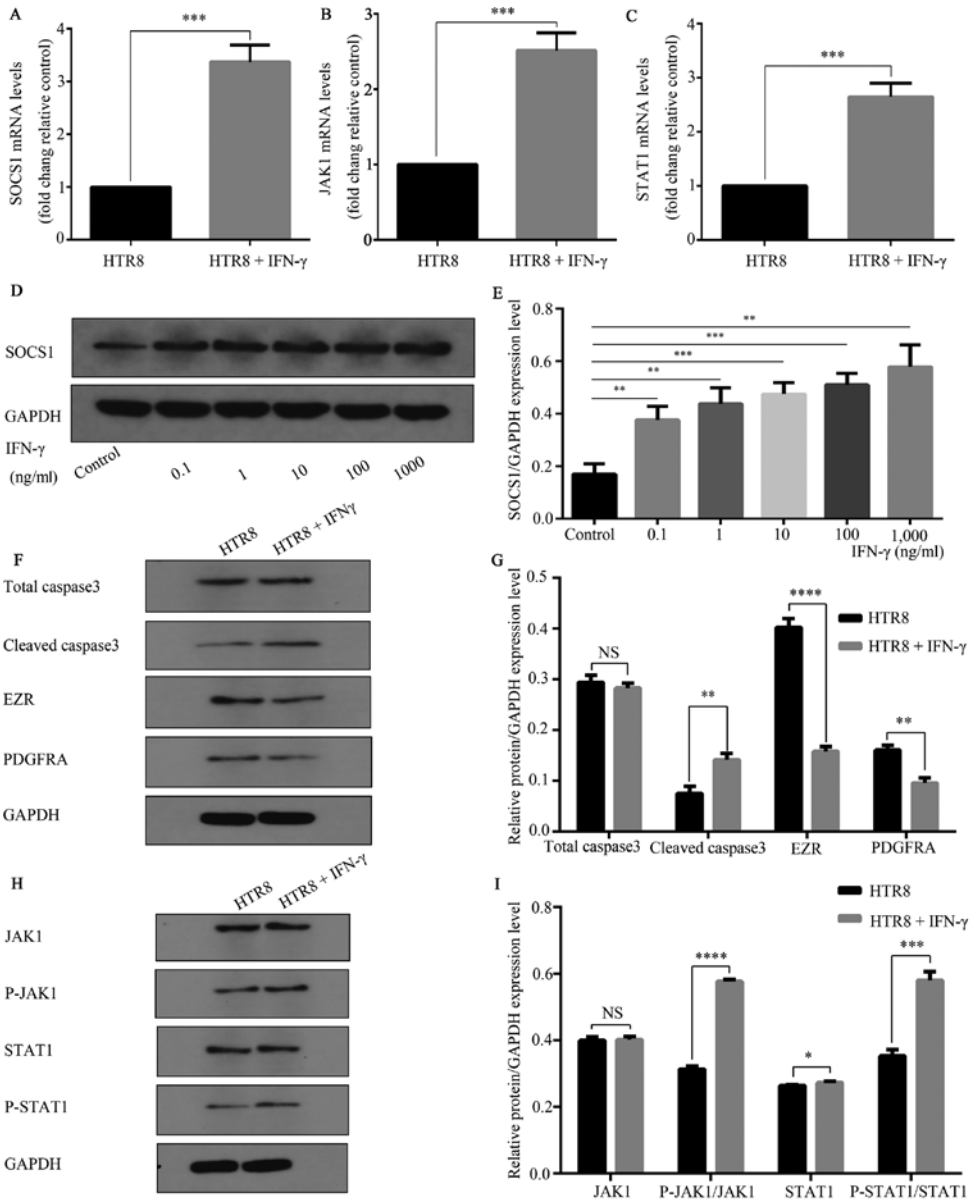

Figure 3. IFN-γ activates JAK1/STAT1 and increases SOCS1 expression. HTR8/SVneo cells were treated in 0 and 100 ng/ml complete medium for 48 h. Total cellular RNA was extracted. IFN-γ increased the mRNA expression levels of (A) SOCS1, ***P=0.0002; (B) JAK1, ***P=0.0004; and (C) STAT1, ***P=0.0003 mRNA. (D) HTR8/SVNeo cells were treated with 0, 0.1, 1, 10, 100 and 1,000 ng/ml and analyzed using western blotting. (E) Different concentrations of IFN-γ increased the expression of SOCS1 protein. **P<0.01; ***P<0.001. (F) Caspase3, EZR and PDGFRA are markers of apoptosis, migration and invasion, respectively. (G) IFN-γ (100 ng/ml) reduced the expression levels of total caspase3 (NS, not significant), cleaved caspase3 **P=0.01; EZR, ****P<0.0001; and PDGFRA, **P=0.01. (H) Western blotting results suggested that 100 ng/ml activated JAK/STAT1. (I) p-JAK1, ****P<0.0001; and p-STAT, ***P=0.0003, expression levels were increased, while JAK1 (NS, not significant) and STAT1 (*P=0.0434) expression levels did not significantly change. Data are presented as the mean ± SD of three independent experiments. SOCS1, suppressor of cytokine signaling 1; PDGFRA, platelet-derived growth factor receptor A; EZR, Ezrin; p-, phosphorylated; JAK, Janus kinase.

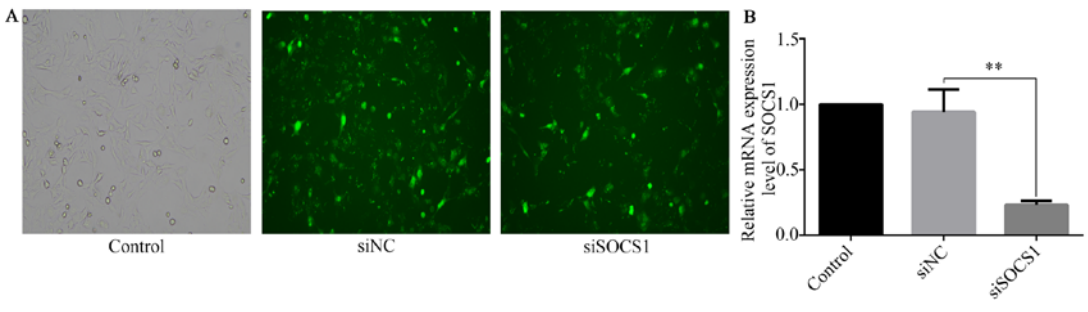

Figure 4. SiRNA transfection into HTR8/SVneo cells. (A) Fluorescein amidites-labeled siRNA was transfected in HTR8/SVneo cells for 24 h. Magnification, x100. (B) After 24 h, reverse transcription-quantitative PCR results identified lower expression of SOCS1 mRNA in the siSOCS1 group. **P=0.0021. Data are presented as the mean ± SD of three independent experiments. siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; siSOCS1, siRNA targeting SOCS1; NC, negative control.

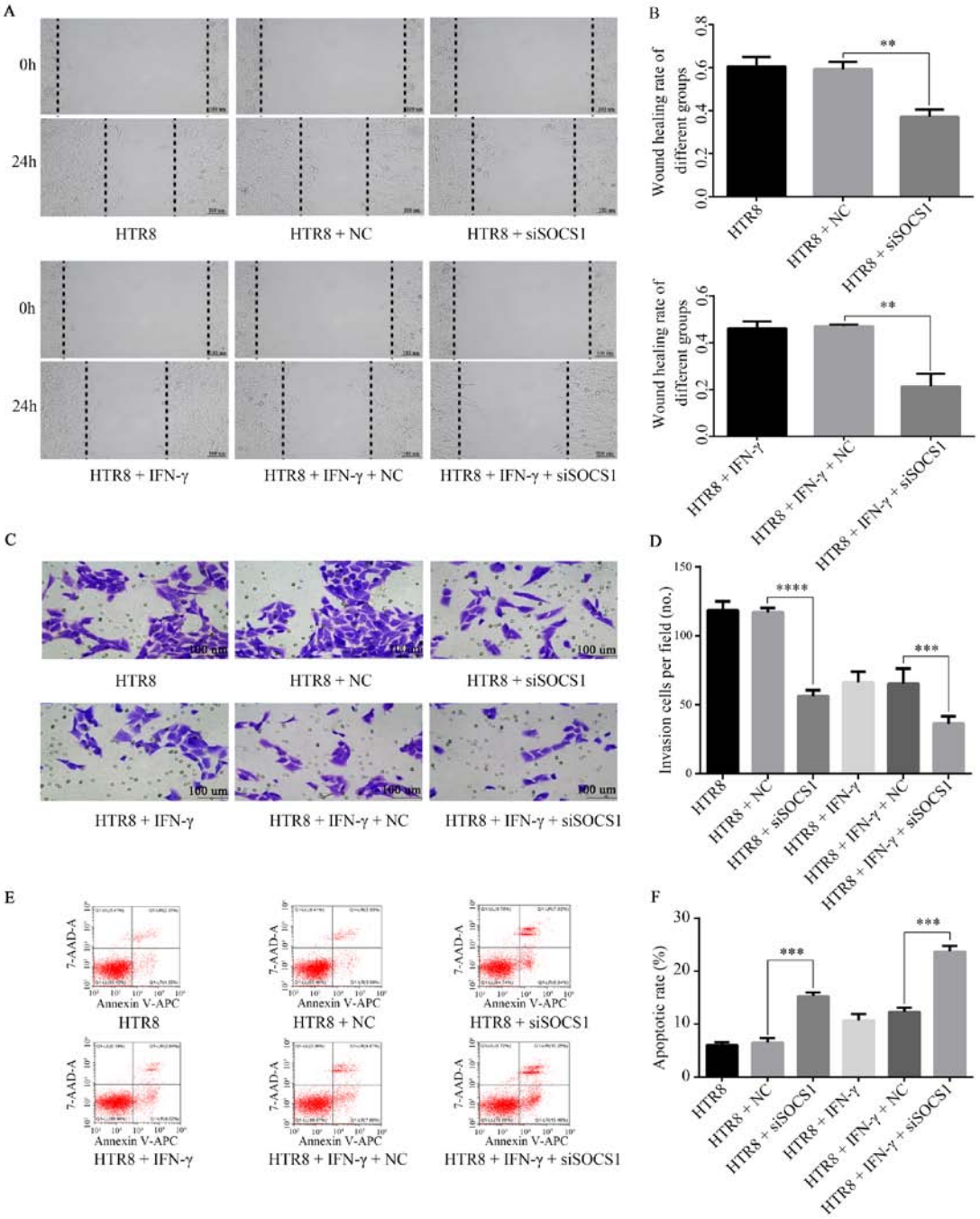

Figure 5. Role of SOCS1 in IFN-γ-mediated reduction of HTR8/SVneo cell invasion. (A) siSOCS1 was transfected into HTR8/SVneo cells, resulting in decreased cell migration. (B) Following co-treatment with 100 ng/ml IFN-γ and siSOCS1, cell migration was further reduced. **P=0.01. (C) siSOCS1 was transfected into HTR8/SVneo cells, resulting in decreased invasive abilities. (D) Following co-treatment with 100 ng/ml IFN-γ and siSOCS1, cell invasion were further reduced. ***P=0.0007, ****P<0.0001. (E) Flow cytometry results found that (F) in siSOCS1-transfected cells, the apoptotic rate increased. ***P=0.0001. After treatment with 100 ng/ml IFN-γ, the apoptotic rate was further increased. ***P=0.0001. SOCS1, suppressor of cytokine signaling 1; siSOCS1, siRNA targeting SOCS1; NC, negative control.

study, knockdown of STAT1 expression results in significant increase of HTR8/SVneo cell invasion. Moreover, clinical samples and cytological experiments indicated that IFN-γ could activate JAK/STAT, inhibit HTR8/SVneo cell migration and invasion, promote apoptosis, increase expression levels of p-JAK, p-STAT1 and caspase3 and decrease expression levels of PDGFRA and EZR.

SOCS is a newly-discovered cytokine-induced protein family composed of an N-terminal variable region, central SH-2 region and C-terminal SOCS box (44). SOCS can inhibit signal transduction by inhibiting JAK/STAT pathways and regulating the signaling of cytokine factors, such as INF-γ, prolactin and growth hormones (45,46). Among the family, SOCS1 potently suppresses cytokine actions by inhibiting JAK kinase activity (47). While SOCS expression levels are low under physiological conditions, these are upregulated in response to cytokine stimulation in a number of immune and inflammatory processes (48). Overexpression of SOCS1 in keratinocyte clones abrogates IFN-γ-induced expression of numerous pro-inflammatory genes and the release of related chemokines via blocking

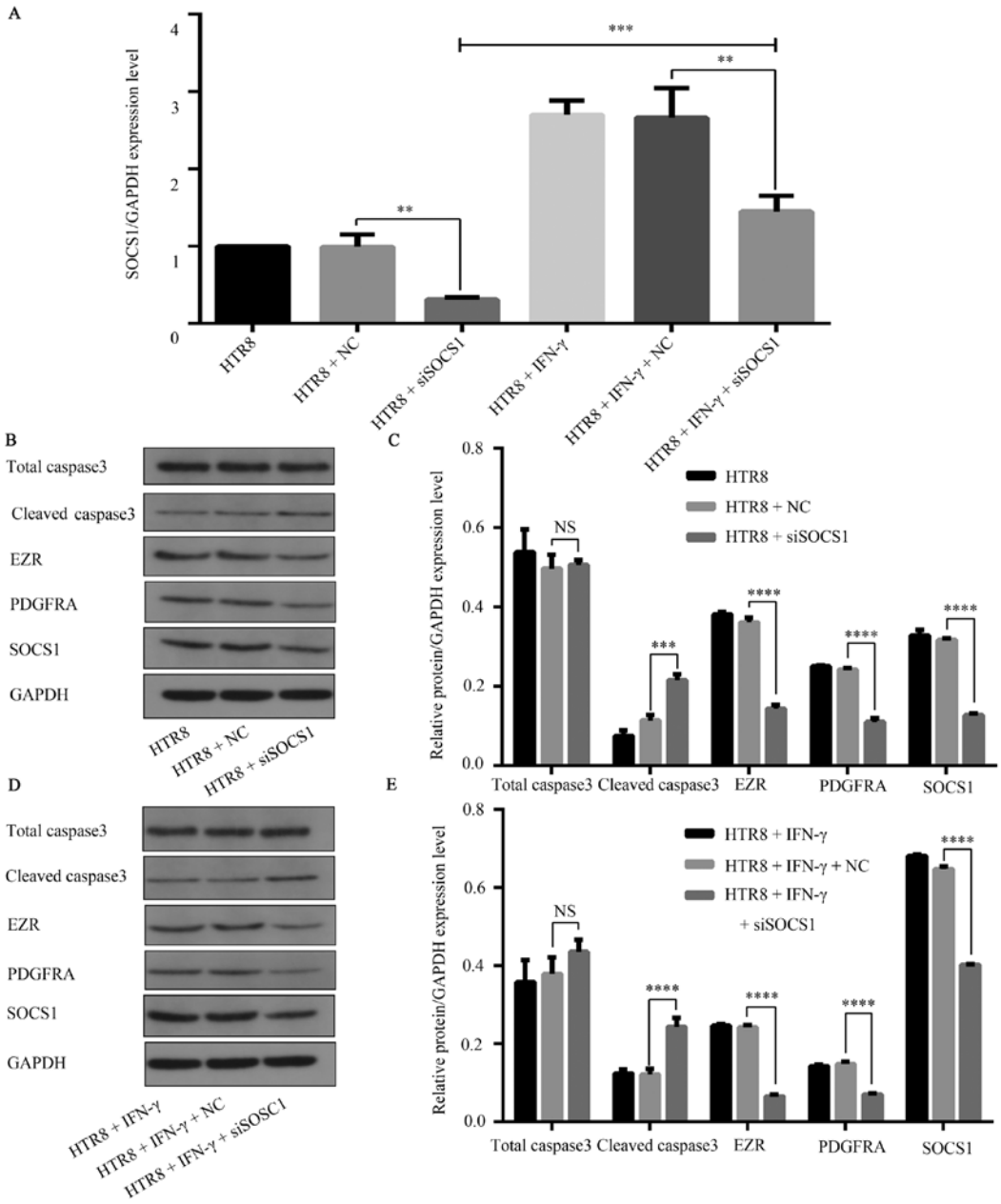

Figure 6. SOCS1 feedback regulation of the JAK1/STAT1 signaling pathway in IFN-γ-activated HTR8/SVneo cells. (A) siSOCS1 was transfected into HTR8/SVneo cells, resulting in decreased SOCS1 mRNA (**P=0.0018) levels. Upon co-treatment with 100 ng/ml IFN-γ, the mRNA levels of SOCS1 decreased compared with the NC group. **P=0.0083. The mRNA levels increased compared with the HTR8 + siSOCS1 group. ***P=0.0007. (B) Representative western blotting images. (C) In siSOCS1-transfected cells, the expression of the apoptosis marker cleaved caspase3 increased (***P=0.0002), while expression levels of the migration and invasion markers EZR (****P<0.0001) and PDGFRA, as well as (****P<0.0001) SOCS1 (****P<0.0001) were decreased. (D) Representative western blotting images. (E) In HTR8/SVneo cells transfected with siSOCS1 and 100 ng/ml IFN-γ, Caspase3 expression was increased (****P<0.0001), while EZR and PDGFRA expression levels were further decreased (****P<0.0001). SOCS1 expression was lower compared with the NC group in drug intervention (****P<0.0001), but increased compared with siSOCS1 without drug intervention. SOCS1, suppressor of cytokine signaling 1; siSOCS1, small interfering RNA targeting SOCS1; PDGFRA, platelet-derived growth factor receptor A; NC, negative control; EZR, Ezrin; JAK, Janus kinase.

the JAK/STAT pathway (49). In addition, SOCS1 inhibits JAK2 kinase activity by binding the catalytic site of JAK2, with its kinase-inhibitory region acting as a pseudo-substrate of the enzyme (49). Skjesol *et al* (50) were the first to demonstrate that SOCS1 is a potent inhibitor of IFN-γ-mediated JAK/STAT signaling in teleost fish. Previous studies (51,52) have also reported that SOCS1 is one of the most effective IFN-γ signaling inhibitors (53).

In the present study, SOCS1 was upregulated in patients with PE, and HTR-8/SVneo cells treated with IFN-γ also had increased SOCS1 expression. However, the role of SOCS1 in the regulation of trophoblast invasion remains to be elucidated. Therefore, the present study investigated how SOCS1 in IFN-γ led to reduced HTR-8/SVneo invasion. SOCS1 siRNA was transfected in HTR-8/SVneo cells and the effects on cell invasion with or without IFN-γ treatment were examined. Knockdown of SOCS1 further reduced the invasion and migration of HTR-8/SVneo cells, reduced the expression levels of PDGFRA and EZR, increased apoptosis and enhanced the expression of caspase3. Therefore, the current findings

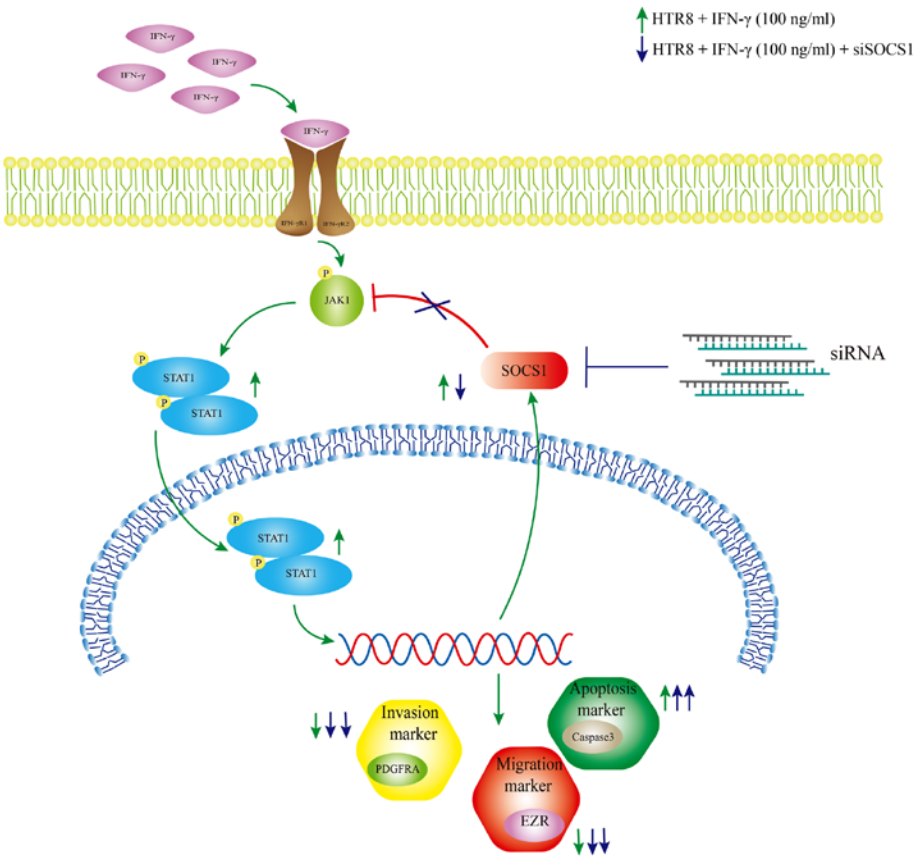

Figure 7. Schematic effect of SOCS1 feedback regulation of IFN-γ-activated JAK1/STAT1 in HTR8/SVneo cells, resulting in reduced cell invasion. IFN-γ increased the phosphorylation levels of JAK1 and STAT1 in HTR8/SVneo cells, leading to a decrease in cell invasion and migration, an increase in apoptosis rate, decrease in PDGFRA, EZR and caspase3 protein expression levels and an increase in SOCS1 protein expression. Following knockdown of SOCS1, the cell invasive and migratory abilities were further reduced, the apoptotic rate was increased, PDGFRA and EZR protein expression levels were reduced and cleaved caspase3 protein expression was increased. SOCS1, suppressor of cytokine signaling 1; PDGFRA, platelet-derived growth factor receptor A; EZR, Ezrin; p, phosphate; JAK, Janus kinase.

indicated that SOCS1 may negatively regulate JAK/STAT1 and affect HTR-8 SVneo invasiveness (Fig. 7).

There are limitations to the current study. For instance, the present report is only a preliminary investigation into the role of IFN-γ in PE, and additional studies are required to further illustrate the functions and underlying mechanism of IFN-γ in PE. For example, increasing evidence suggests that microRNAs (miRNAs), a type of non-coding small RNA that consists of 20-26 nucleotides and regulates gene expression by targeting the 3'-untranslated region, are involved in various pregnancy-related disorders, including PE and fetal growth restriction (53,54). Thus, would be worth investigating whether miRNAs are in involved in the regulation of the IFN-γ/SOCS1/JAK/STAT1 feedback loop. Moreover, additional siRNAs for SOCS1 could be used to exclude off-target effects in future studies. Finally, the present study only conducted experiments on HTR-8/SVneo cells and clinical tissue samples. Since HTR-8/SVneo is different from primary EVTs, the pathogenesis of IFN-γ and PE requires further research.

In conclusion, IFN-γ reduced the invasion of HTR-8/SVneo cells by activating JAK/STAT1, leading to an increase in SOCS1 expression, which negatively regulated JAK/STAT1 and eliminated the pro-inflammatory effects of IFN-γ, thus forming a feedback loop. These findings could explain the higher expression of IFN-γ and SOCS1 in the placental tissue of patients with PE compared with the healthy control group.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL and CL conceived and designed the present study. HL and WW performed the experiments and analyzed the data. HL

and CL interpreted the data and wrote the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University. Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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