Effect of SOCS3 on apoptosis of human trophoblasts via adjustment of the JAK2/STAT3 signaling pathway in preterm birth

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Background: The expression of suppressor of cytokine signaling 3 (SOCS3) was induced by interleukin-6 (IL-6) in preterm placental tissues. However, its role in IL-6 induced apoptosis of trophoblast cells derived from preterm placental tissues remains to be elucidated.

Methods: Primary cytotrophoblasts from human preterm placental tissues were used to stably knock down and overexpress the level of SOCS3 by corresponding lentiviral vectors and the expression of SOCS3 was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot. The effect of SOCS3 overexpression or knockdown on the proliferation and apoptosis of IL-6 treated human cytotrophoblasts were determined by Cell Counting Kit-8 (CCK8) assay and Annexin-V/Propidium Iodide (PI) double-staining assay, respectively. Based on it, we detected the proteins associated with the Janus Tyrosine Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway and apoptosis, such as JAK2, p-JAK2, STAT3, p-STAT3, B-cell lymphoma-2 (Bcl-2) and BCL2-associated X (Bax) by Western blot.

Results: IL-6-treatment resulted in significant apoptosis of human cytotrophoblasts. Overexpressing SOCS3 in the cytotrophoblasts reduced cell apoptosis, while the knockdown of SCOS3 had the opposite effects. Further analyses showed that SOCS3 overexpression inhibited JAK2 and STAT3 phosphorylation, which was induced by IL-6 stimulation.

Conclusions: SOCS3 plays a protective role in human preterm placental tissue-derived cytotrophoblasts from IL-6 induced apoptosis by feedback inhibition of JAK2/STAT3 signaling.

Keywords: Preterm birth; suppressor of cytokine signaling 3 (SOCS3); interleukin-6 (IL-6); apoptosis; JAK2/STAT3 signaling

Introduction

Preterm birth, which refers to an actual gestation of between 20 and 37 weeks, is a major obstetric and global health problem and is the leading cause of child mortality in those under 5 years of age worldwide (1). Globally, the morbidity rate of preterm birth is 5–15%, with approximately 15 million babies being born prematurely and more than 1 million deaths due to related complications every year (2). Also, preterm birth leads to adverse health outcomes for the mother and the baby and significant healthcare costs (3). According to the March of Dimes, a premature baby spends an average of 25.4 days in a specialty care nursery. The costs associated with premature birth increase the United States

healthcare expenses by 26.2 billion dollars annually (4). However, preterm birth pathogenesis is poorly understood, and accumulating evidence suggests that it is a complex process with multiple attributable causes (5-7).

Preterm birth is classified into two groups: spontaneous and iatrogenic premature births, with ~75% occurring spontaneously. Iatrogenic preterm birth is caused by human intervention, while a solid body of evidence indicates that systemic inflammation or infection are causally linked to early spontaneous preterm birth (8-10). It is generally believed that infection induces uterine contractions, premature rupture of membranes, or cervical softening by promoting changes in prostaglandins, matrix metalloproteinases, or oxytocin receptors through cytokines secretion. Additionally, an important mechanism involved in the pathogenesis of premature birth is cytokine-mediated trophoblast cell apoptosis (11-13). Abrahams *et al.* have demonstrated that ligation of toll-like receptor 4 (TLR4) induces cytokine production, while TLR2 activation induces trophoblast cell death during certain intrauterine infections. They also showed that the apoptosis was dependent upon the inhibition of the X-linked apoptosis protein (XIAP) and the activation of Caspase-8, -9, and -3 (14). More recently, numerous studies have also indicated that cytokines, such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-1beta (IL-1β) will cause physical and biochemical changes in trophoblast cells by inducing apoptosis and inflammatory reactions (15). Therefore, it is believed that excessive apoptosis of trophoblast cells plays a pivotal role in preterm birth pathogenesis; however, the detailed molecular mechanisms of apoptosis require further elucidation.

SOCS3 is a member of the SOCS family, including SOCS1–7 and cytokine-inducible SH2 containing protein (CISH), and negatively regulates cytokine signaling. The structural features of SOCS proteins include a conserved motif (termed the SOCS box of C terminal), a central Src homology-2 (SH2) area, and an N-terminal variable area (16-18). Roberts *et al.* reported that SOCS3 deficiency leads to midgestational fetal demise due to placental insufficiency (19). It is well accepted that SOCS3 is primarily induced by IL-6 family cytokines, such as IL-6, IL-11, leptin, and leukemia inhibitory factor (LIF), via the glycoprotein 130 (gp130)/JAK/STAT3 signaling pathway and inhibits the phosphorylation of JAK and STAT3 (20-22). The activation of JAK/STAT3 signaling is related to multiple biological functions, including immune regulation, cell proliferation, differentiation,

hematopoiesis, and apoptosis (including SOCS3 gene transcription) (23). SOCS3 regulates the JAK/STAT3 signaling pathway by inhibiting inflammation and is also related to apoptosis. Some studies have shown that the knockdown of SOCS3 persistently activates STAT3 by up-regulating the expression of growth regulatory proteins (Cyclin D1, c-Myc, c-Fos) or anti-apoptotic proteins [Bcl-2, myeloid cell leukemia 1 (Mcl-1), B-cell lymphoma extra-large (Bcl-XL)], thereby inducing hyperproliferation and preventing apoptosis (24,25). Recent studies have demonstrated that hypermethylation of the CpG islands of the SOCS3 gene leads to SOCS3 silencing and promotes the growth and migration of human hepatocellular carcinoma, head and neck squamous cell carcinoma, and lung cancer (26,27). As a negative cytokine signaling feedback inhibitor, SOCS3 is highly expressed in preterm placental trophoblast cells (20,28). However, there are few studies on the mechanism underlying the apoptosis of SOCS3 in trophoblast cells.

Therefore, this study aimed to explore the role of SOCS3 in the apoptosis of placental trophoblast cells and its effects on the JAK2/STAT3 signaling pathway. Collectively, our data confirmed that IL-6 treatment of trophoblasts significantly promoted the expression of SOCS3 through the JAK2/STAT3 pathway. The expression of SOCS3 was required to inhibit IL-6-induced inflammation and trophoblast apoptosis. Our findings could strengthen the understanding of preterm birth pathogenesis mechanisms and provide theoretical guidance for subsequent studies.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tp-21-39).

Methods

Placenta collection

Placentas were collected from cesarean sections of non-smoking, healthy women with a pregnancy less than 37 weeks' gestation (hereafter called "preterm labor"). The placentas were obtained from the Jiangsu Women and Children Health Hospital. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (No. 2020-SR-434) and informed consent was taken from all the patients.

Cytotrophoblast purification and culture

After placenta collection, the tissues were washed in phosphate buffered saline (PBS). Next, chorionic villi were gently scraped free from vessels and connective tissues, and were subsequently dissected into small fragments. The time for placental dissection was kept under 30 minutes to prevent tissue degradation. After dissection, the chorionic villi were washed twice with PBS and then digested by medium containing 0.1% Trypsin, 0.1 M magnesium sulfate ($MgSO_4$), 0.1 M calcium chloride ($CaCl_2$), 4% bovine serum albumin (BSA), and 50 Kunitz/mL Deoxyribonuclease I (DNase I) for 30 minutes at 37 ℃ without agitation. The supernatant-containing mononucleated villous cytotrophoblasts (VCTs) were collected after tissue sedimentation, filtered with a 40-μm cell strainer, and incubated with fetal bovine serum (FBS) to stop trypsin activity. After purification by Percoll gradient centrifugation, the VCTs were resuspended and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After around 16 hours of culturing (overnight), the VCTs were carefully washed to eliminate non-adherent cells. The VCTs were characterized for each culture to ensure the purifications by microscopic visualization or collected and kept at –80 ℃ for total ribonucleic acid (RNA) or protein extraction and fixed in cold methanol for further immunolabeling.

Construction of SOCS3-overexpression or SOCS3-knockdown vectors

Vector pCDH-MSCV-EF1-copGFP-T2A-Puro was used to construct the SOCS3 overexpressing plasmid (SOCS3-OE). Briefly, coding sequences of human SOCS3 were amplified from human complementary DNA (cDNA) by polymerase chain reaction (PCR) using oligonucleotide to create restriction sites for EcoR I and BamH I of the SOCS3 sequence. The upstream (5'-AGCGCTAGCACCA TGGTCACCCACAGCAAGTTTCC-3') and downstream (5'-GGTGGTACCCAAAGCGGGGCATCGTACTG-3') primers were designed according to the National Center for Biotechnology Information (NCBI) and were synthesized by Sangon Biotech (Shanghai, China). PCR was performed using Pfu DNA polymerase (GENEWIZ, China). The PCR product and the pCDH-MSCV-EF1-copGFP-T2A-Puro were digested with EcoR I and BamH I (NEB, Ipswich, MA). After ligation, competent DH5a *E. coli*

cells were transformed using the plasmid, and the selected positive clones were amplified and verified by RuiBiotech (Beijing, China).

Vector pLKO.1-EGFP-puro was used to construct the SOCS3 knockdown plasmid (SOCS3-sh). SOCS3 short hairpin RNA (shRNA) and non-targeting shRNA control (sh-scramble) oligonucleotides were designed according to the Sigma website (https://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html) and were synthesized by Sangon Biotech (Shanghai). Oligonucleotides were annealed, and the pLKO.1-EGFP-puro were digested with EcoR I and Age I (NEB, Ipswich, MA). After ligation, competent DH5a *E. coli* cells were transformed using the plasmid and selected positive clones were amplified and verified by RuiBiotech (Beijing, China).

Knockdown and overexpression of SOCS3 in human cytotrophoblasts

Lentivirus was produced by co-transfecting HEK293T cells with helper plasmids, including psPAX2 and pMD2G, and overexpressing (pCDH-MSCV-SOCS3-EF1-copGFP-T2A-Puro) or knockdown (pLKO.1-shSOCS3-EGFP-puro) plasmids using polyethylenimine (PEI) reagent. Culture supernatants were collected after 48 hours, filtered using a 0.45-μm filter, and the lentivirus collected from the supernatant was directly used to infect cells. To validate the efficiency of infection, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis were used to examine the expression level of SOCS3 in human cytotrophoblasts.

RNA extraction and qRT-PCR

Total RNA was extracted from human cytotrophoblasts using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was reversely transcribed into cDNA using a reverse transcription kit and then stored at –80 ℃ until use. Also, qRT-PCR was performed using SYBR Green Mix (Transgen Biotech, Beijing, China). The PCR products were subjected to melting curve analysis to confirm the amplification. Messenger RNA (mRNA) with an amount of 20 ng was normalized to β-actin and evaluated using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate. The primer sequences were used as follows:

SOCS3-Forward: 5'-CATCTCTGTCGGAAGACCGT

CA-3';

SOCS3-Reverse: 5'-GCATCGTACTGGTCCAGGAA CT-3';

β-actin-Forward: 5'-CACCATTGGCAATGAGCGGT TC-3';

β-actin-Reverse: 5'-AGGTCTTTGCGGATGTCCAC GT-3'.

Western blot analysis

Whole cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor and phosphatase inhibitor for 30 minutes on ice, centrifuged at 12,000 rpm, and harvested proteins. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Burlington, MA, USA). The membranes were then blocked in 5% non-fat dry milk for 1 hour at room temperature in Tris-Buffered Saline and Tween 20 (TBST, 0.1% Tween 20). The membranes were subsequently incubated with primary antibodies against SOCS3 (Cell Signaling Technology, Cat No. 52113S, 1:1,000), Bcl-2 (Proteintech, Cat No. 12789-1-AP, 1:1,000), Bax (Proteintech, Cat No. 50599-2-Ig, 1:1,000), p-JAK2 (Tyr1007/1008) (Cell Signaling Technology, Cat No. 3776S, 1:500), JAK2 (Proteintech, Cat No. 17670-1-AP, 1:1,000), p-STAT3 (Ser727) (Cell Signaling Technology, Cat No. 9136S, 1:1,000), STAT3 (Cell Signaling Technology, Cat No. 9139S, 1:1,000), and β-Actin (Proteintech, Cat No. 66009-1-Ig, 1:2,000) overnight at 4 ℃. The membranes were then washed with TBST three times and incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-Rabbit immunoglobulin G (IgG) (H + L) (Cell Signaling Technology, Cat No. 7074S, 1:2,000) or secondary HRP-conjugated horse anti-Mouse IgG (H + L) (Cell Signaling Technology, Cat No. 7076S, 1:2,000) at room temperature for 1 hour. All membranes were detected using an enhanced chemiluminescence kit (Millipore, Burlington, MA, USA), and the images were obtained using Image Lab software (Bio-Rad, USA). Blots were semi-quantified using ImageJ software (National Institutes of Health).

Flow cytometry

The rate of cell apoptosis was detected by flow cytometry. After 24 h treatment with IL-6, lentivirus infected human cytotrophoblasts were washed with PBS solution and then dissociated with trypsin without edetate disodium (EDTA), and FBS was used to stop trypsin activity. After centrifugation at 2,000 rpm for 5 minutes, the supernatant was removed, and the cells were resuspended with 1× binding buffer. After the addition of 5 μL Annexin V-APC and 5 μL PI, the cells were incubated at 4 ℃ in the dark for 20 minutes, followed by flow cytometry. A total of 10,000 cells were counted, and the rate of cell apoptosis was calculated accordingly. Simultaneously, cells that were not incubated with Annexin V-APC and PI served as the negative control. The data was analyzed using FlowJo software (version 7.6, FlowJo LLC, Ashland, OR).

Statistical analysis

Prism software (version 8.0, GraphPad Software, San Diego, CA) was used for statistical analysis. One- or two-way ANOVAs were performed to determine significance. Statistical differences were indicated as $*P<0.05$, $**P<0.01$, $***P<0.001$, and $***P<0.0001$. All quantitative data were presented as the mean ± the standard error of the mean (SEM) from three independent experiments.

Results

Overexpression or knockdown of SOCS3 in human cytotrophoblasts

To investigate the role of SOCS3 in human placental cells, we first performed lentivirus-mediated gene delivery in primary human cytotrophoblasts to overexpress or knockdown the *SOCS3* gene with pCDH-MSCV-SOCS3-EF1-copGFP-T2A-Puro or pLKO.1-shSOCS3-EGFP-puro, respectively. After 24 hours of infection, enhanced green fluorescent protein (EGFP) expression was detected by fluorescent microscopy, and the transfection efficiency was approximately 90% (*Figure 1A*). At 48 hours after transfection, both qRT-PCR and Western blot assay showed that, compared with the EGFP control group, SOCS3 expression was significantly up-regulated or down-regulated in the pCDH-MSCV-SOCS3-EF1-copGFP-T2A-Puro
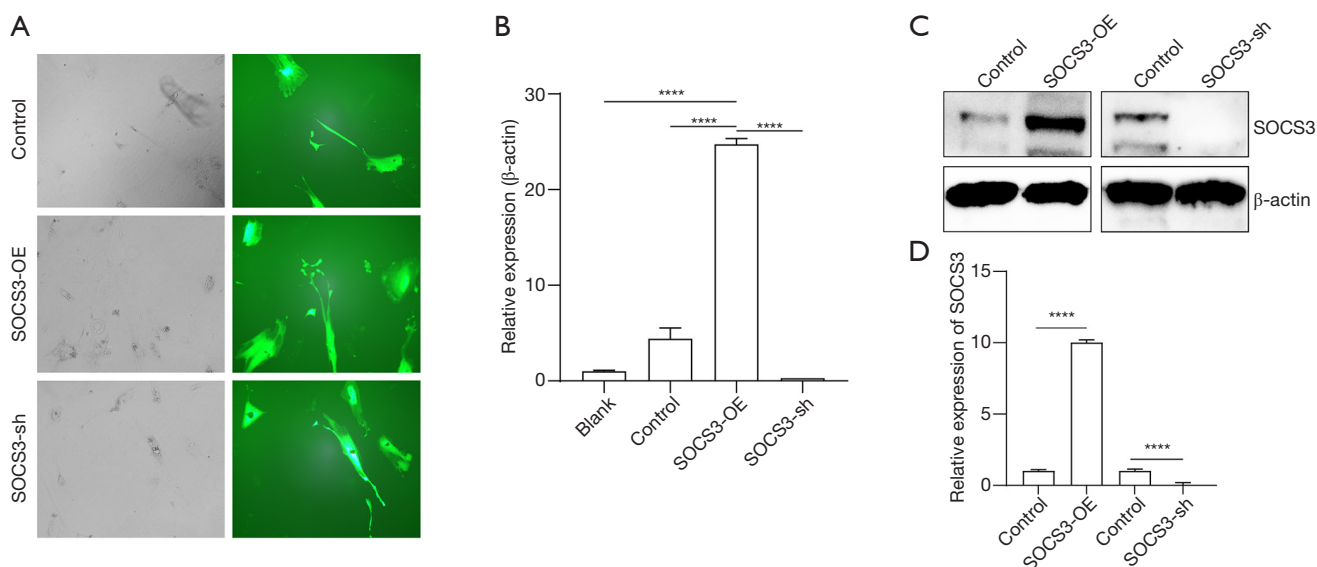
Figure 1 Successful overexpression or knockdown of SOCS3 in human cytotrophoblasts. (A) Representative photomicrographs demonstrating successful infection of human cytotrophoblasts with an empty vector (control), the PCDH-SCOS3 expression vector (SOCS3-OE), and PLKO-shSCOS3 knockdown vector (SOCS3-sh). Magnification, ×200. (B) The SOCS3 mRNA level in the blank (non-lentivirus infection), control, SOCS3-OE, and SOCS3-sh was measured by qRT-PCR, and actin was used as control (n=3 per group). One-way ANOVA, mean ± SEM, ****$P<0.0001$. (C) Representative blot demonstrating SOCS3 expression in human cytotrophoblasts following infection. β-actin was used as the loading control. (D) Blots were semi-quantified using ImageJ software. Data are expressed as the mean ± SEM of the mean of three independent experiments. ****$P<0.0001$.

(SOCS3-OE) or pLKO.1-shSOCS3-EGFP-puro (SOCS3-sh) groups, respectively (*Figure 1B,C,D*).

The role of SOCS3 in the survival of human cytotrophoblasts upon IL-6 exposure

To determine the role of SOCS3 in the proliferation of human cytotrophoblasts upon IL-6 exposure, we treated normal SOCS3-OE and SOCS3-sh cells with 100 ng/mL IL-6 for 24 hours. IL-6 exposure significantly inhibited normal human cytotrophoblasts' survival, and the knockdown of SOCS3 genes (SOCS3-sh) showed additional inhibitory effects (*Figure 2A*). However, cells in the SOCS3-OE group exhibited markedly superior survival (*Figure 2A*), which suggested the expression of SOCS3 might play a protective role in human cytotrophoblasts upon IL-6 exposure. Furthermore, we performed a cell counting kit-8 (CCK-8) assay to evaluate human cytotrophoblasts' viability from the various treatment groups *in vitro*. As shown in *Figure 2B*, the viability of cells exposed to IL-6 was significantly decreased compared with the normal group ($P<0.05$), and the deletion of SOCS3 (SOCS3-sh) could promote this consequence.

Meanwhile, SOCS3 overexpression (SOCS3-OE) promoted the proliferation of human cytotrophoblasts, even in the presence of IL-6 (*Figure 2B*). These results suggested that enhanced cellular SOCS3 expression could reverse IL-6-induced growth inhibition in human cytotrophoblasts.
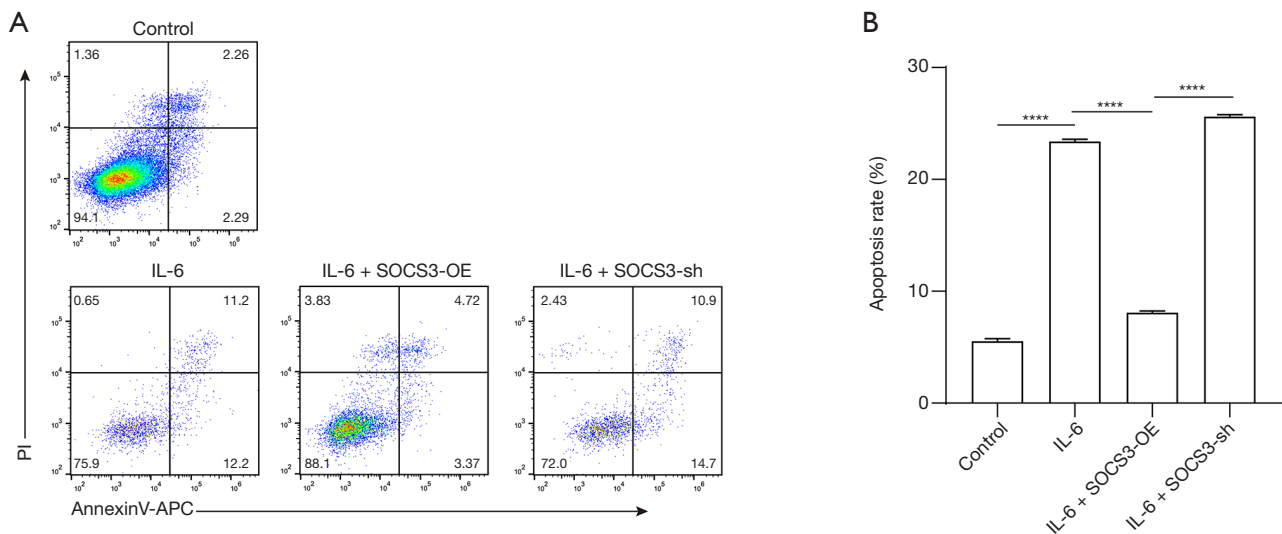
SOCS3 overexpression protected human cytotrophoblasts from IL-6-induced apoptosis

To determine the role of SOCS3 in IL-6-induced apoptosis of human cytotrophoblasts, we exposed both SOCS3-OE and SOCS3-sh cells to 100 ng/mL IL-6 for 24 hours. The fraction of apoptotic cells in the SOCS3-OE group was significantly reduced compared to the control group (*Figure 3*). Also, IL-6-induced Bax up-regulation and Bcl2 down-regulation were reversed in SOCS3-OE cells (*Figure 4*). These results indicated that overexpression of SOCS3 could reverse IL-6-induced apoptosis in human cytotrophoblasts.

On the other hand, compared with the control group, the proportion of apoptosis in the SOCS3-sh group increased slightly (*Figure 3*), and IL-6-induced Bax up-regulation and
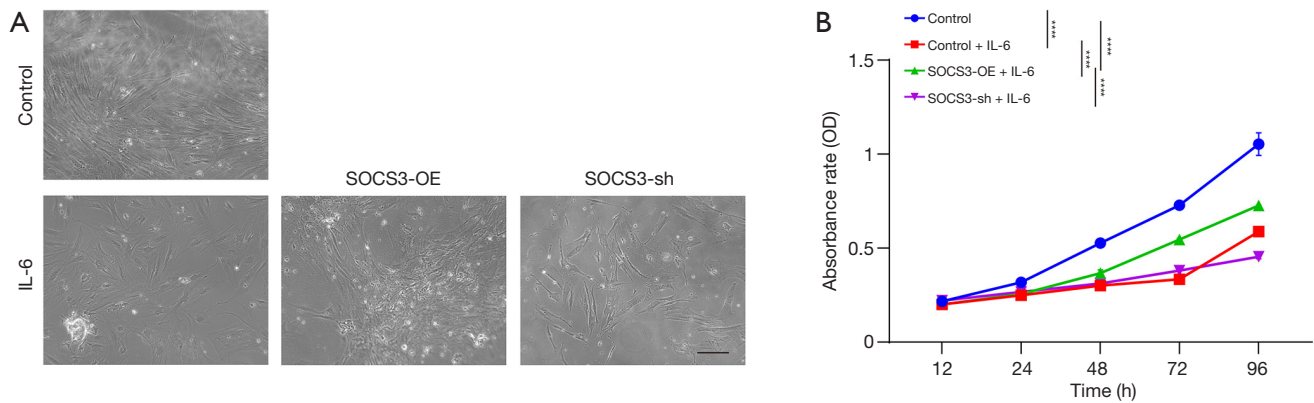
Figure 2 The role of SOCS3 in the survival of human cytotrophoblasts upon IL-6 exposure. (A) Representative photomicrographs of human cytotrophoblasts cultured in IL-6 stimuli. Bar =20 μm. (B) Cell Counting Kit-8 assay of human cytotrophoblasts following various treatments. Data are expressed as the mean ± SEM of the mean of six independent measurements. ****P<0.0001.


Figure 3 SOCS3 overexpression protected human cytotrophoblasts from IL-6-induced apoptosis. (A) The apoptosis rates of human cytotrophoblasts in the blank, IL-6 exposure, SCOS3-OE + IL-6, and SCOS3-sh + IL-6 groups were detected by flow cytometry. (B) Apoptosis cells of AnnexinV-APC/PI double staining. The data are presented as means ± SEM of triplicates from three independent tests. ****P<0.0001.

Bcl2 down-regulation were also promoted, although no statistical significance was observed. Therefore, SOCS3 knockdown had no significant additional effect on IL-6-induced apoptosis of human cytotrophoblasts. These results suggested that the feedback up-regulation of SOCS3 to a certain level might be essential to protect human cytotrophoblasts from IL-6-induced apoptosis.

SOCS3/JAK/STAT signaling in the survival of human cytotrophoblasts upon IL-6 stimuli

To comprehensively understand the mechanism of SOCS3 in regulating the apoptosis of human cytotrophoblasts during IL-6 treatment, we further examined the JAK2/STAT3 pathway. Western blot analysis showed that
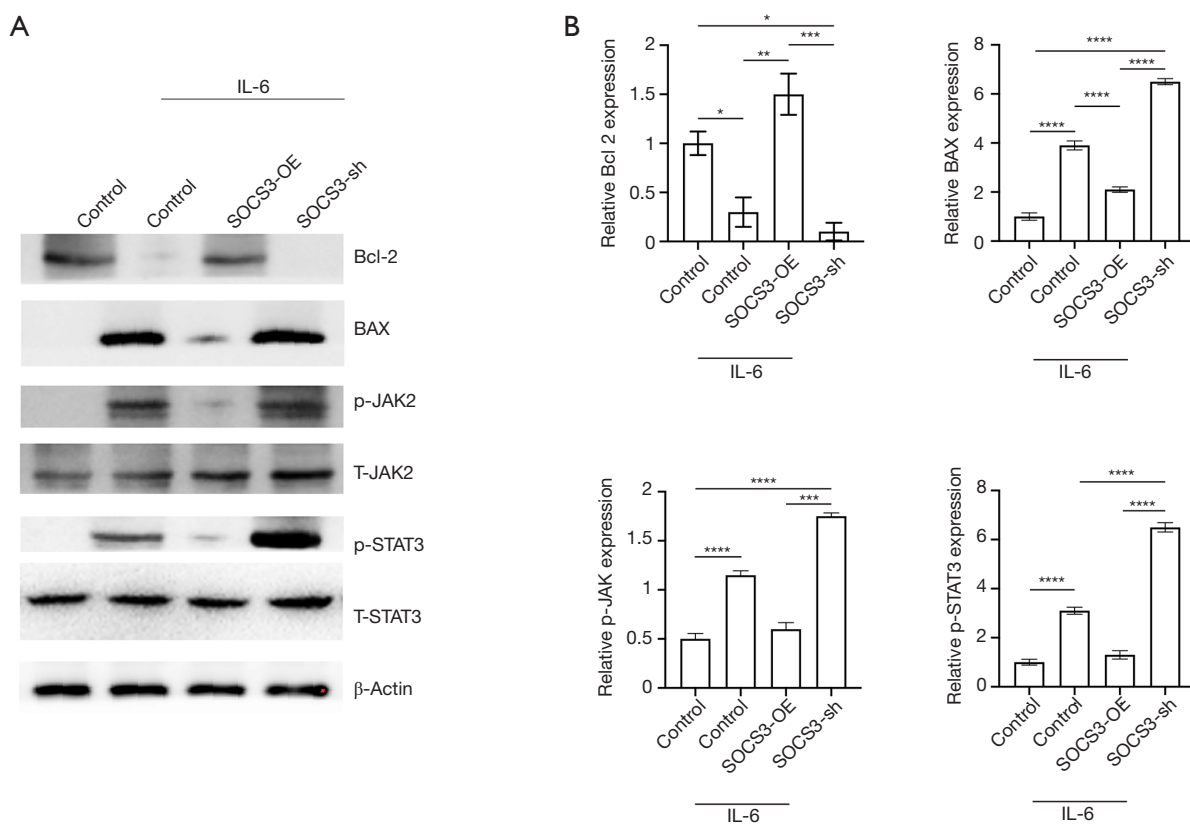
Figure 4 SOCS3/JAK/STAT signaling in the survival of human cytotrophoblasts upon IL-6 stimuli. (A) The protein expression levels of apoptotic and JAK2-STAT3 genes. The data are presented from at least three independent tests. (B) The levels of Bcl-2, BAX, p-JAK2, and p-STAT3 were measured by ImageJ software and determined by taking the ratio of the target protein band intensity against that of β-actin. Data are expressed as the mean ± SEM of the mean of three independent measurements. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

the phosphorylation of JAK2 and STAT3 in human cytotrophoblasts was significantly inhibited by SOCS3 overexpression during IL-6 treatment (*Figure 4*). On the other hand, the knockdown of SOCS3 marginally promoted these phosphorylated modifications. These results indicated that the JAK/STAT signaling pathway was involved in IL-6-induced apoptosis of human cytotrophoblasts and that negative feedback regulation by SOCS3 expression was required to promote the survival of human cytotrophoblasts.

Discussion

SOCS3 is a negative regulator of the JAK/STAT signaling transduction pathway. In this study, our results showed that IL-6 exposure induced apoptosis of human cytotrophoblasts. However, apoptosis tended to be reduced after SOCS3 overexpression and was significantly increased after SOCS3 knockdown. Further analyses showed that

the overexpression of SOCS3 decreased the levels of phosphorylated JAK and STAT3, which were up-regulated by IL-6 stimulation.

IL-6 is a crucial modulator of mammalian responses to tissue damage caused by infection and inflammation, which are among the major causes of human preterm birth. Consistent with this, our previous study showed that the expression of IL-6 was notably increased in preterm placental tissues (20). As SOCS3 played a negative feedback role in IL-6-induced activation of JAK/STAT3 signaling (20,29), the overexpression of SOCS3 in preterm placental tissue-derived cytotrophoblasts also inhibited IL-6-induced p-STAT3, while SOCS3-knockdown had the opposite effect.

Given the high expression of SOCS3 and low expression of p-STAT3 in preterm placental tissues (20), it remains to be determined whether cytotrophoblast apoptosis is inhibited *in vivo*. Furthermore, it has been reported that SOCS3 can regulate the quantity and type of STAT signal

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generated from the IL-6 receptor (IL-6R), and the absence of SOCS3 significantly induces the phosphorylation of both STAT3 and STAT1 (29,30). Thus, the phosphorylation status and exact roles of other STAT proteins in preterm placental tissues and cultured cytotrophoblasts remains to be determined.

On the other hand, both the fetal and the maternal immune systems were actively involved in maintaining normal pregnancy (31-33). It has been reported that IL-6 stimulation promotes STAT3 phosphorylation in both fetal and adult monocytes (34). Although fetal and adult monocytes express similar levels of SOCS3, the expression of IL-6R in adult monocytes was significantly lower than that in fetal monocytes, which resulted in markedly higher STAT3 phosphorylation in fetal monocytes exposed to IL-6. Illustration of the mechanism underlying IL-6/STAT3/SOCS3 signaling regulation in both fetal and adult monocytes would provide new insights into inflammation-associated preterm birth.

Infection during pregnancy leads to increased expression of IL-6 and ultimately to a high expression level of SOCS3, which negatively regulates the JAK/STAT3 signaling pathway. During minor infections, SCOS3 can protect trophoblast cells and prevent premature delivery. However, during severe infection, the negative regulatory effect of SOCS3 is insufficient, and trophoblast cell apoptosis leads to premature birth. The expression of SOCS3 in patients with recurrent abortion is decreased (35); however, the specific mechanism remains unclear. It may be related to DNA methylation and other appearance modifications. Clarifying the mechanism of abnormally low SOCS3 expression may serve as a molecular marker for predicting recurrent abortion.

Conclusions

In summary, IL-6 induced apoptosis of human cytotrophoblasts, while the overexpression of SOCS3 exhibited protective effects. Further analyses showed that the overexpression of SOCS3 decreased the levels of phosphorylated JAK2 and STAT3, which were up-regulated by IL-6 stimulation. Our results suggest that SOCS3 is a potential therapeutic target for preterm births.

Acknowledgments

Footnote

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