Growth hormone inhibits the JAK/STAT3 pathway by regulating SOCS1 in endometrial cells *in vitro*: A clue to enhance endometrial receptivity in recurrent implantation failure

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ABSTRACT

Recurrent implantation failure (RIF) is defined as failure to achieve clinical pregnancy after at least 3 transfers of good-quality embryos by natural or artificial means. RIF is often a complex problem with a wide variety of etiologies and mechanisms as well as treatment options. In this study, using immunohistochemistry and Western blot, we demonstrated that the expression of leukemia inhibitory factor (LIF), Janus kinase 1 (JAK1), and signal transducer and activator of transcription 3 (STAT3) was increased, while that of suppressor of cytokine signaling 1 (SOCS1) was decreased in RIF patients. Growth hormone (GH) administration proved to have positive effects on embryo implantation in RIF patients, but the action mechanism of GH has not been elucidated yet. To this aim, we studied the effects of GH on the proliferation *in vitro* of endometrial adenocarcinoma Ishikawa cells. GH stimulated the expression of LIF and SOCS1, and through SOCS1 inhibits the expression of phosphorylated STAT3, and finally inhibits the occurrence of RIF. Excessive phosphorylation of STAT can lead to decreased endometrial receptivity and abnormal embryo implantation. We also examined the effects of LIF overexpression and an LIF inhibitor (EC330) on the JAK/STAT pathway. LIF promoted cell proliferation, and the up-regulation of LIF increased the expression of SOCS1 and JAK1/STAT3 pathway-related genes in Ishikawa cells. As GH can inhibit the JAK1/STAT3 pathway through LIF, we hypothesize that upregulating SOCS1 may be a potential approach to treat RIF at the molecular level. GH can inhibit the JAK1/STAT3 pathway through LIF, up-regulating SOCS1 to treat RIF at the molecular level.

Key words: Endometrial receptivity; JAK/STAT pathway; GH; LIF; SOCS1.

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Ethics approval: The experimental protocols were approved by the Ethics Committee of the Guangzhou First People's Hospital, South China University of Technology.

Data availability: The data used to support the findings of this study are available from the corresponding author upon request.

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Introduction

Recurrent implantation failure (RIF) refers to the failure to achieve clinical pregnancy after the transfer of at least 4 embryos of good quality in fertile women.[1] RIF accounts for 10% of infertility cases in women of childbearing age.[2] The mechanism underlying this condition may be related to changes in endometrial receptivity.[3-6] Leukemia inhibitory factor (LIF) can help avoid embryo rejection by inducing endometrial receptivity, controlling trophoblast invasion, and regulating embryonic development and maternal-fetal immunity, making it a marker for endometrial receptivity.[7] Another such biomarker is integrin αvβ3, a heterodimer composed of two transmembrane α and β subunits. Being a member of a large family of cell adhesion molecules, it plays an important role in intercellular recognition and communication.[8-9]

LIF is a multifunctional cytokine in the interleukin-6 family that can activate transcription factors of the signal transducer and activator of transcription (STAT) family, especially STAT3.[10] Specifically, LIF binds to the cell surface receptor subunit GP130 and LIF receptor to induce the formation of heterodimers, which in turn activate the Janus kinase/STAT (JAK/STAT) pathway. JAK1, widely expressed in tissues, can phosphorylate all STATs.[7,11] LIF-triggered activation of the JAK1/STAT3 pathway induces phosphorylated JAK1 (P-JAK1), which subsequently induces phosphorylated STAT3 (P-STAT3). Finally, P-STAT3 enters the nucleus, binds to DNA, and regulates transcription. Like LIF, integrin αvβ3 also activates the JAK1/STAT3 pathway.

Excessive P-STAT3 is an important cause of pain and infertility, so its production must be regulated.[12] Members of the suppressor of cytokine signaling family, especially SOCS1, fulfill this function by inhibiting JAK activity. When the expression of SOCS1 was down-regulated, cell invasion and migration decreased, indicating that SOCS1 may promote cell proliferation, invasion, and migration.[13-15] During the implantation window period of RIF patients, SOCS1 expression was increased in the endometrium of the patients, and the abnormal expression of SOCS1 may be related to the failure of embryo implantation.[16] SOCS1 can be induced by a series of signal transduction pathways activated by the binding of growth hormone (GH) to its receptor on the surface of uterine epithelial cells. GH, a peptide hormone secreted by the anterior lobe of the pituitary gland, promotes growth, development, and metabolism and regulates ovarian function.[17-20] Studies have shown that growth hormone has a positive effect on embryo implantation in RIF patients.[21] GH can improve endometrial receptivity, but its mechanism of action has not been

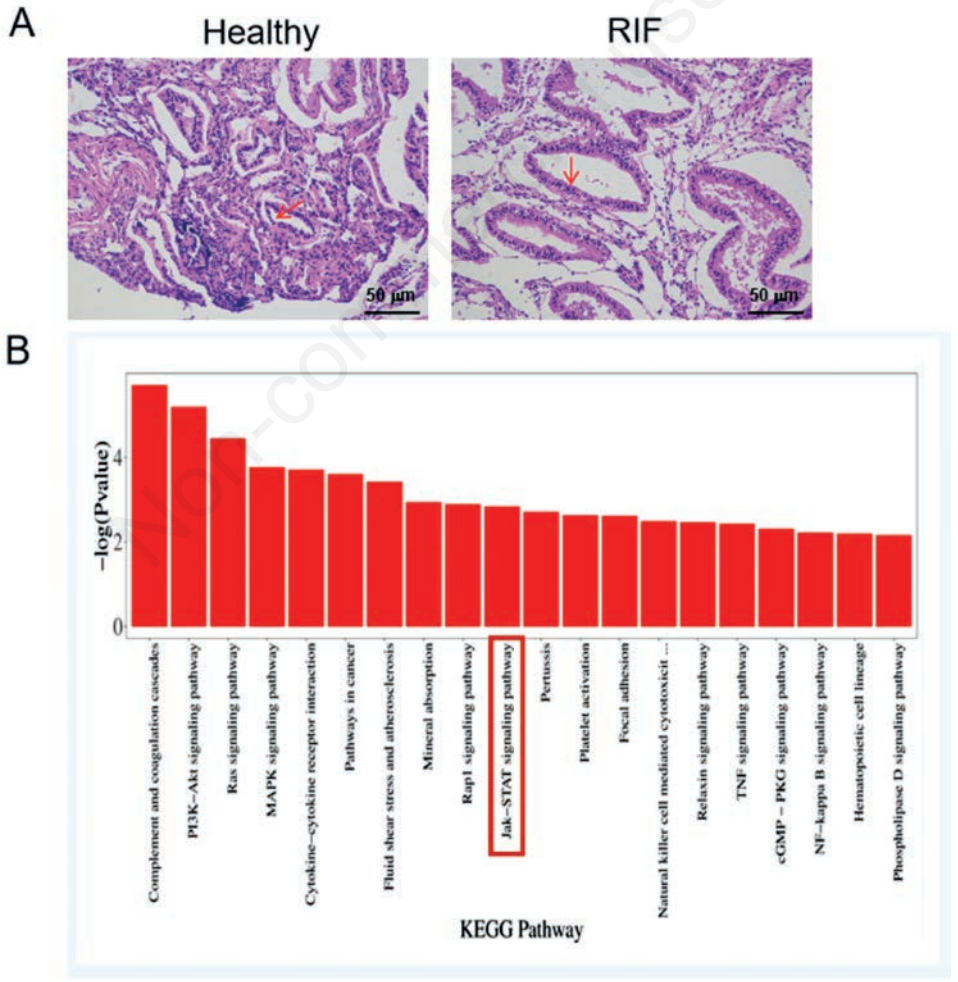

Figure 1. RIF-related histomorphological changes and related signal pathways screening. A) Hematoxylin and eosin staining was used to detect the histomorphological changes of healthy and RIF patients. B) Gene sequencing detected the expression of different signaling pathways. The red arrows point to the different forms of endometrial epithelial cells.

deciphered yet. The aim of this study was to explain the beneficial effects of GH on endometrial receptivity. We compared the transcriptomic and epigenetic changes in the eutopic endometria of healthy and RIF patients during the implantation window. Subsequently, the expressions of LIF, integrin αvβ3, and downstream proteins were determined by Western blot, after stimulating Ishikawa cells with GH. Exploring the expression changes and mechanism of GH in RIF patients can help us to obtain more influencing factors involved in the occurrence of RIF, so as to achieve the purpose of treating RIF.

Materials and Methods

Patient samples

Biopsy specimens were selected from patients with RIF in Guangzhou First People's Hospital from April to June 2022. The endometrial samples were fixed with 4% paraformaldehyde and then stored at -80°C for use. We obtained permission from the Ethics Committee of Guangzhou First People's Hospital and informed consent from the patients.

Cell cultures

HEC-1-A and Ishikawa cells were purchased from Procell Corporation and were cultured in Dulbecco's Modified Eagle Medium (Gibco, Waltham, MA, USA), containing 10% fetal bovine serum (Gibco, USA) and 1% antibiotic/antimycotic solution (Gibco), in a humidified atmosphere at 37°C and 5% CO_2. GH was diluted to the required concentration using PBS solution according to the experimental requirements.

GH treatment

Ishikawa cells were selected as the RIF group and HEC-1-A cells as the control group (healthy group). Ishikawa cells were cultured with GH (Aladdin, Biochemical Technology Co Ltd., Shanghai, China) at concentrations of 10, 20, or 50 ng/L for 4 h and used for subsequent experiments.[22]

Transfection

A plasmid overexpressing LIF (LIF-over), negative control (NC), and LIF silenced expression vector (LIF shRNA) were obtained from Boster Biological Technology Co., Ltd. (Pleasanton, CA, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), according to the manufacturer's protocol.

Pathway expression analysis

Datasets related to RIF were downloaded from The Cancer Genome Atlas database to retrieve the expression of each pathway gene. Data were collated and mapped to select abnormal expression pathways in RIF.

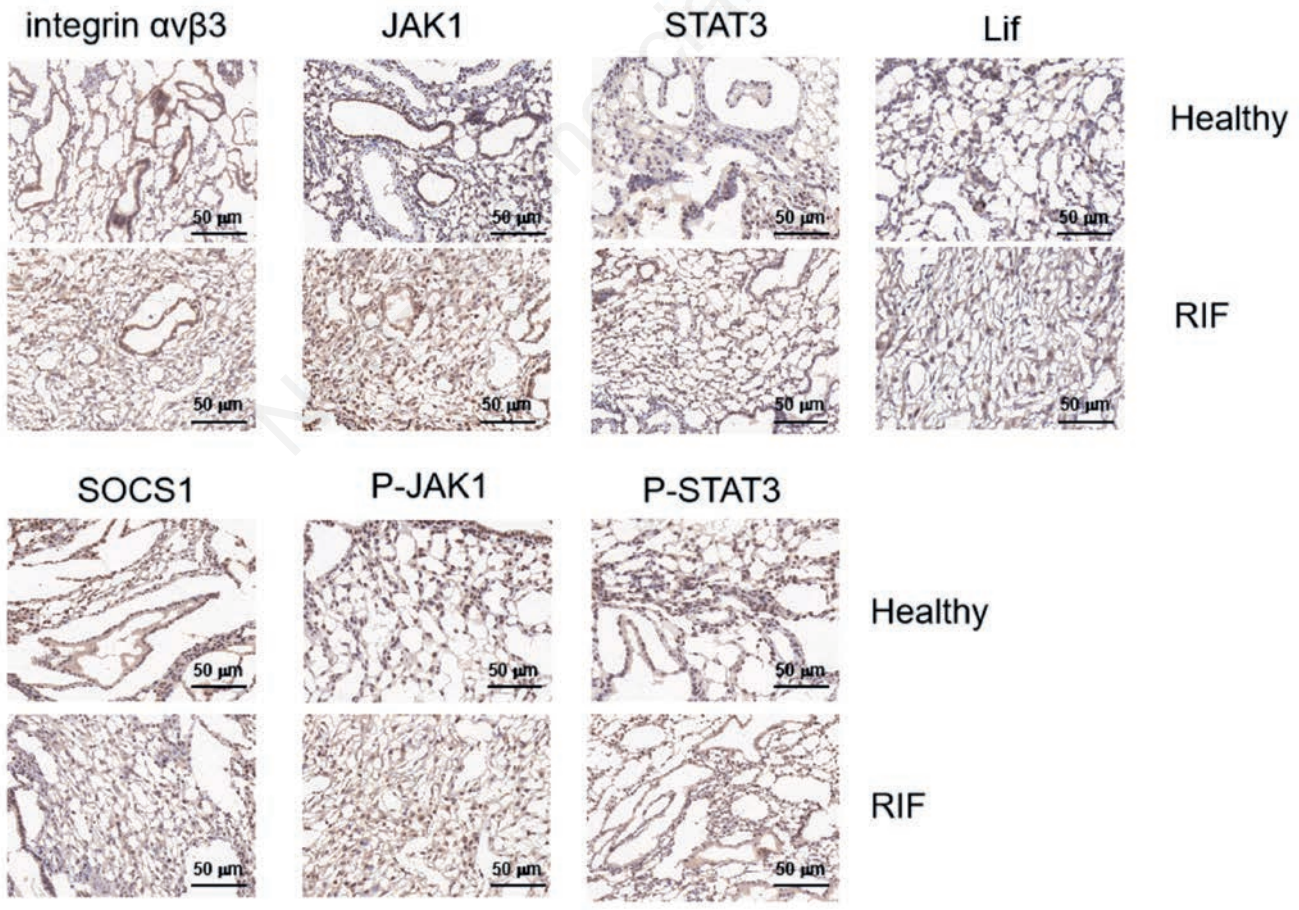

Figure 2. Immunohistochemical staining of healthy and RIF patients under an optical microscope.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from Ishikawa cells using TRIzol reagent (Invitrogen AB, Stockholm, Sweden), according to the manufacturer's instructions, then cDNA was synthesized by reverse transcription. Using cDNA as template, premix, primers, and the template were added according to instructions for qPCR. The thermal cycling program was as follows: pre-denaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 34 s. β-actin served as the internal control to normalize the gene expression results.

Western blot

Western blot was performed as previously described.[23] First, Ishikawa cells were collected and mixed with RIPA lysis buffer, and the cell supernatant lysed by RIPA lysis buffer (Abcam, Cambridge, UK) was obtained by centrifugation. The protein concentration was determined using a protein BCA kit (Abcam) to ensure that the concentrations were consistent between the control and the experimental groups. The RIPA lysis buffer was added, followed by boiling water for 10 min, after which the sample was quickly put on ice. A total 40 μL of cell lysate was loaded on to the prepared 10% gel and the proteins were separated by electrophoresis at 80 V for 2 h. The proteins were transferred to a polyvinylidene fluoride membrane at a constant current of 300 A. The membrane was blocked for 2 h in phosphate-buffered saline (PBS)-Tween 20, containing skimmed milk powder, followed by an overnight incubation with primary antibodies (anti-LIF, -integrin αvβ3, -JAK1, -STAT3, -SOCS1, -P-JAK1, -P-STAT and -beta-actin; purchased from Abcam; dilution concentration: 1:2000) at 4°C. After washing, the membrane was incubated with secondary horseradish peroxidase-conjugated IgG antibody (1:1000, Merck, Rahway, NJ, USA) and incubated at room temperature for 1 h, and the protein bands were stained with an enhanced chemiluminescence kit (Millipore, Burlington, MA, USA), and the protein bands were detected with a CCD chemiluminescence imager. Using β-actin as the internal reference, ImageJ software was used to calculate the gray values of protein bands.

Hematoxylin and eosin staining

Endometrial tissue samples were embedded in paraffin and then sectioned. The sections were dewaxed with xylene for 5min, and then washed with ethanol for 3 times. The slices were placed in hematoxylin and stained for 10 min, differentiated by 1% hydrochloric acid ethanol for 3 s, cleaned with tap water and return to blue for 10 s, stained with eosin for 1 min, and dehydrated with

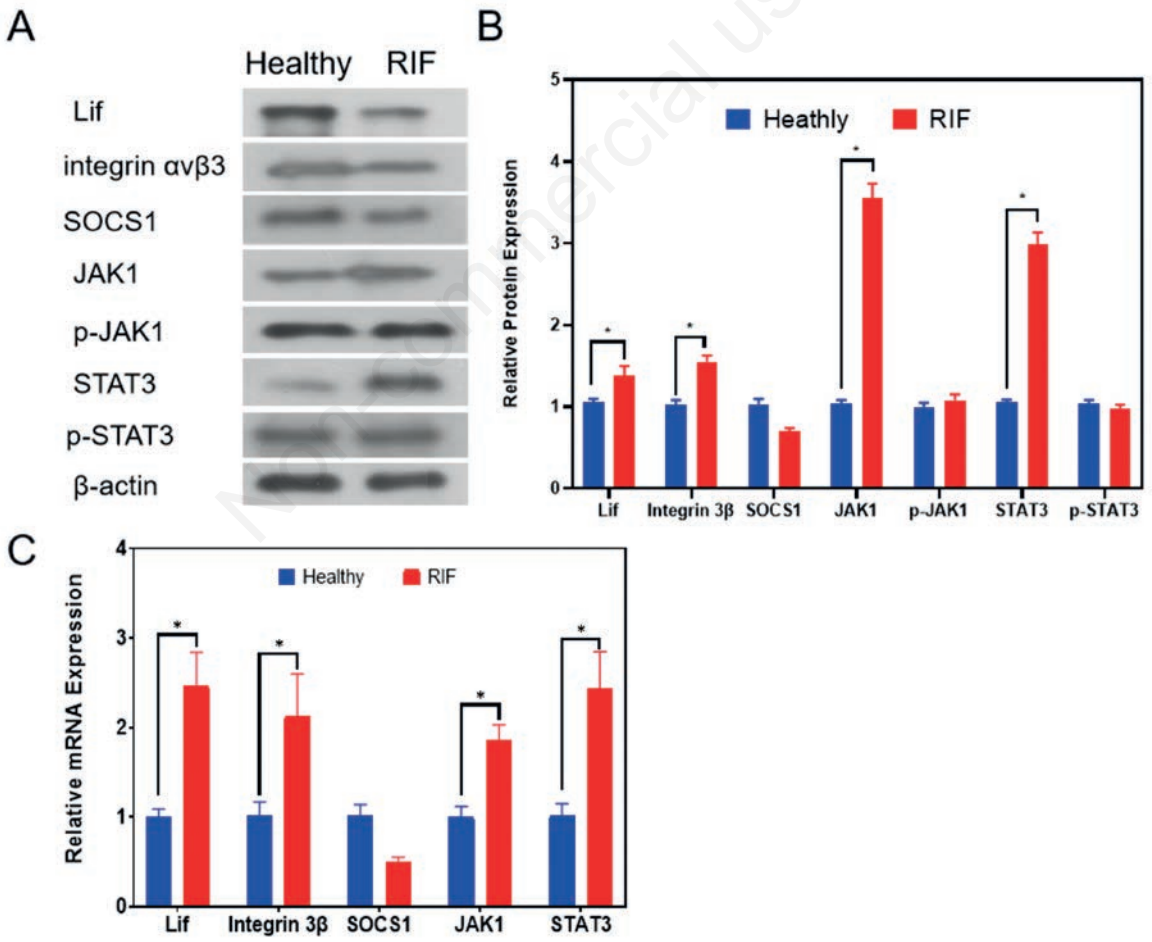

Figure 3. Western blot and QRT-PCR were used to evaluate the expression of related proteins and mRNA in healthy and RIF patients. A,B) The expression of related proteins was detected by Western blot. C) The expression of related mRNA was detected by QRT-PCR. *p<0.05.

ethanol for 3 times. Finally, the sections were treated with xylene for 1 min, sealed with neutral gum, and observed under microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[24] The tissue paraffin sections were dewaxed and washed thrice with PBS, fixed in methanol for 20 min, incubated with PBS containing 5% bovine serum albumin and 0.2% Triton X-100 for 2 h followed by primary antibodies overnight, washed with PBS, incubated with a secondary antibody (Goat anti-rabbit antibody, 1:2000, Abcam) at 37°C for 1 h, stained with 4′,6-diamidino-2-phenylindole, dihydrochloride for 5 min, and observed under a biological microscope. Five visual fields were randomly selected in the microscope field, and the positive staining area in the visual field was evaluated by naked eye observation, and the average value was calculated. Finally, the average value of each group was selected for comparison. In the negative control group, the primary antibody solution was replaced by pre-immunization serum, and other steps were the same. Primary antibodies against LIF, integrin αvβ3, JAK1, STAT3, SOCS1, P-JAK1, and P-STAT were raised in rabbits (1:200, Boster Biological Technology Co., Ltd.).

Cell counting kit-8 (CCK8) proliferation

Ishikawa cell suspension (100 μL, approximately 10,000 cells) was added to wells of a 96-well plate and cultured with various concentrations of GH in a CO_2 incubator for 24 h. Then, 100 μL of DMEM medium containing 10 μL of CCK8 solution (Abcam) was added to each well and incubated for 1-4 h. The optical density of the sample was measured at 450 nm by an Enzyme-linked immunoassay (Thermo Fisher, Waltham, MA, USA).

Statistical analysis

SPSS 17.0 was used for statistical analysis. All experimental results were expressed as means ± SD. One-way analysis of variance was used for inter-group comparisons. A p-value <0.05 indicated statistically significant differences.

Results

Immunohistochemistry of endometrial tissue from patients with RIF

Endometrial tissue from healthy subjects was compared with that from RIF patients using hematoxylin and eosin staining (Figure 1A). The healthy endometrial cells were arranged neatly and the muscular layer was thick. The epithelial cells of ectopic endometrium are flat. Abnormal activation of JAK/STAT signaling was observed in tissues from RIF patients (Figure 1B). The expres-

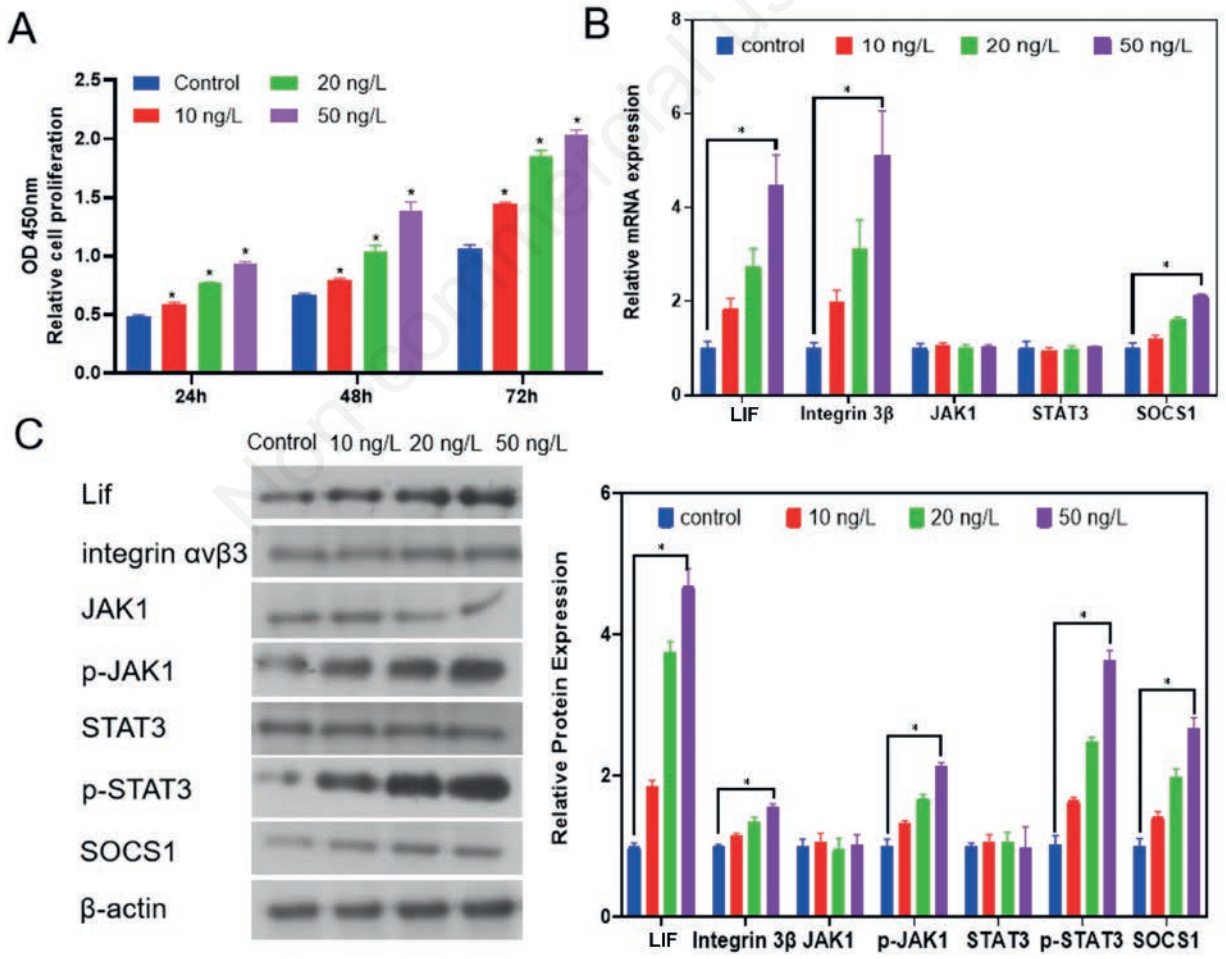

Figure 4. Effect of different growth hormone concentrations in Ishikawa cells. A) Effects on cell proliferation. B) Effect on JAK/STAT pathway mRNA expression and (C) related protein expression. *p<0.05.

sion of JAK1 and STAT3 in the RIF group was significantly higher than that in the healthy group, as evidenced by immunohistochemical analysis. The expressions of P-JAK1, P-STAT3, SOCS1, LIF, and integrin αvβ3 did not significantly differ between the two groups (Figure 2).

We used Western blot to evaluate the expression of RIF-related proteins and their downstream regulators. The expressions of LIF, integrin αvβ3, JAK1, and STAT3 in the RIF group significantly differed from those in healthy endometrial tissues (Figure 3A, 3B); on the other hand, the expression of P-JAK1 and P-STAT3 did not. SOCS1 expression significantly decreased in the RIF group compared with the healthy group. We also used qPCR to confirm the expression patterns of the corresponding genes. The gene expression of LIF, integrin αvβ3, JAK1, and STAT3 was significantly higher in the RIF group compared with the healthy group (Figure 3C), whereas that of SOCS1 was significantly lower than in the healthy group.

Impact of GH on the expression of biomarkers and the JAK1/STAT3 pathway in Ishikawa cells

Different concentrations of GH (10, 20, 50 ng/L) could promote the proliferation of Ishikawa cells (Figure 4A). The mRNA levels of LIF, integrin αvβ3, and SOCS1 increased significantly with GH concentration (Figure 4B). Using Western blot, we showed that upon culturing Ishikawa cells with increasing concentrations of GH, the expression of LIF and integrin αvβ3 increased significantly, while that of JAK1 and STAT3 did not. JAK1 and STAT3 expression did not differ significantly at the mRNA level as well. However, the downstream regulators P-JAK1 and P-STAT3 were significantly increased. SOCS1 expression was also positively correlated with GH concentration. Overall, GH stimulated the JAK1/STAT3 pathway in Ishikawa cells and enhanced the expression of LIF.

LIF regulates the JAK1/STAT3 pathway in Ishikawa cells

To further study the effect of LIF on the uterine receptivity-related JAK1/STAT3 pathway, we constructed an LIF overexpression vector (LIF-over) and LIF short hairpin RNA (shRNA; LIF-sh1, -sh2, -sh3) (Figure 5 A,B). The effect of LIF on cell proliferation was studied using the CCK8 assay. Overexpression of LIF boosted cell proliferation (Figure 5C), while introducing LIF inhibitors EC330 significantly inhibited cell proliferation. We verified the effects of LIF overexpression and the LIF inhibitor EC330 on the expressions of integrin αvβ3, JAK1, STAT3, P-JAK1, P-STAT3, and SOCS1 (Figure 6). The protein levels of integrin αvβ3, SOCS1, P-JAK1, and P-STAT3 significantly increased with LIF expression, clearly indicating that LIF can regulate the JAK/STAT pathway and promotes the production of P-JAK1 and P-STAT3 by inducing the expression of SOCS1.

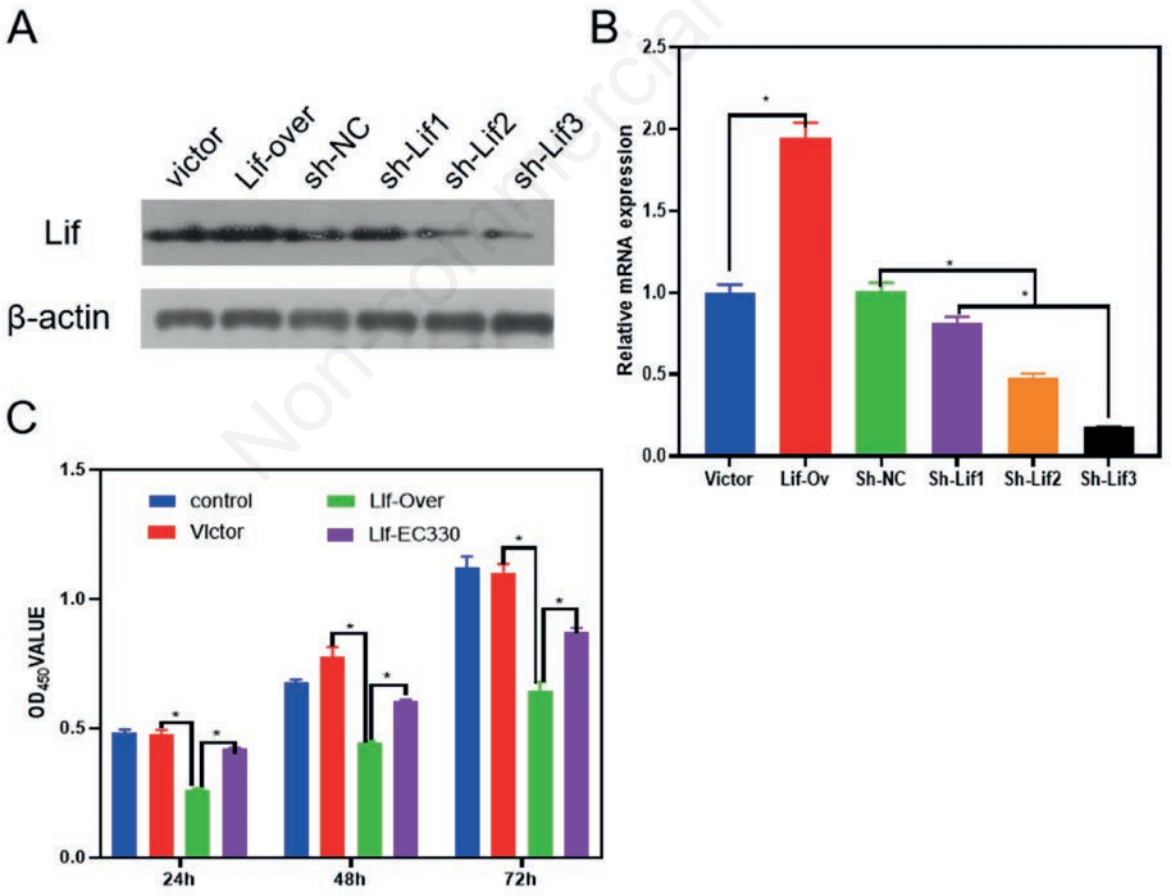

Figure 5. Expression efficiency of leukemia inhibitory factor (LIF), overexpressed (LIF-over) vector and LIF shRNA and its effect on cell proliferation. A) Western blot and QRT-PCR (B) were used to detect the expression of LIF protein and mRNA. C) Effect of LIF expression on cell proliferation. *p<0.05.

Discussion

RIF is a hormone-dependent, chronic gynecological disease characterized by endometrial tissue infiltration and growth outside the endometrial layer.[25] Ectopic endometrium can invade any part of the body, such as the kidney, ureter, and the lungs, but it is mostly limited to the pelvic cavity. Ovarian and sacrococcygeal ligaments are the most common sites of invasion, followed by uterine, rectal uterine concave, visceral peritoneum, and others. RIF usually includes dysmenorrhea, non-menstrual pelvic pain, and infertility. Epidemiological studies have shown that the incidence of RIF in women of childbearing age is 5-10%. About 50% of women with RIF are infertile. It is a common cause of infertility, which can be treated with hormones. Modern studies have shown that the deletion of SMAD1/5 can lead to endometrial defects in mice, thus affecting embryo implantation and leading to the occurrence of RIF.[26] As a regulator of goat endometrial receptivity, BCL2L15 can also regulate endometrial receptivity through STAT1 pathway and affect embryo implantation.[27] Compared with healthy women, the levels of LIF, integrin αvβ3, JAK1, and STAT3 were significantly increased in women with RIF, while SOCS1 expression was significantly reduced. SOCS1 inhibits the JAK1/STAT3 pathway. The decrease of SOCS1 expression leads to the over-activation of P-STAT3 *via* the JAK1/STAT3 pathway, which can cause decreased endometrial receptivity, dysmenorrhea, and even abnormal embryo implantation. As can be seen from Figure 1, there are many pathways with abnormal expression in RIF, but there are more clinical studies on JAK1/STAT3, which can prove that JAK1/STAT3 is related to changes in endometrial receptivity. Therefore, this study continued to explore the mechanism of JAK1/STAT3 affecting endometrial receptivity. This study found that GH could increase the expression of LIF, integrin αvβ3, and SOCS1 while decreasing the production of P-STAT3 in Ishikawa cells, confirming that GH regulates the JAK1/STAT3 pathway and enhances endometrial receptivity. The expression of LIF and SOCS1 increased with increasing GH concentrations, while P-STAT3 showed the opposite trend. GH improves uterine content tolerance, perhaps, by affecting the expression of LIF, promoting the expression of SOCS1, and inhibiting the JAK1/STAT3 pathway to reduce P-STAT3 expression. To verify that the effect of LIF on the JAK1/STAT3 pathway is mediated by SOCS1, we overexpressed LIF and detected its effect on the relevant proteins. Cell proliferation was significantly affected when LIF expression decreased. After LIF expression was inhibited, the expression of JAK1/STAT3 pathway-related proteins was affected. Overexpression of LIF significantly increased SOCS1 expression and significantly decreased P-STAT3 expression, indicating that the effect of GH on the JAK1/STAT3 pathway is regulated by LIF, resulting in increased endometrial receptivity.

We confirmed using *in vitro* Ishikawa cells experiments that GH can inhibit the JAK/STAT pathway by regulating SOCS1 expression through LIF in the event of RIF, thereby improving endometrial receptivity. However, this study focuses on the regulation of endometrial receptivity, and there is a lack of *in vivo* animal experiments to further verify the effects of growth hormone and JAK1/STAT3 pathway on embryo implantation. In the future, we can continue to conduct experiments to further verify the therapeutic effects of GH and JAK1/STAT3 pathways on RIF.

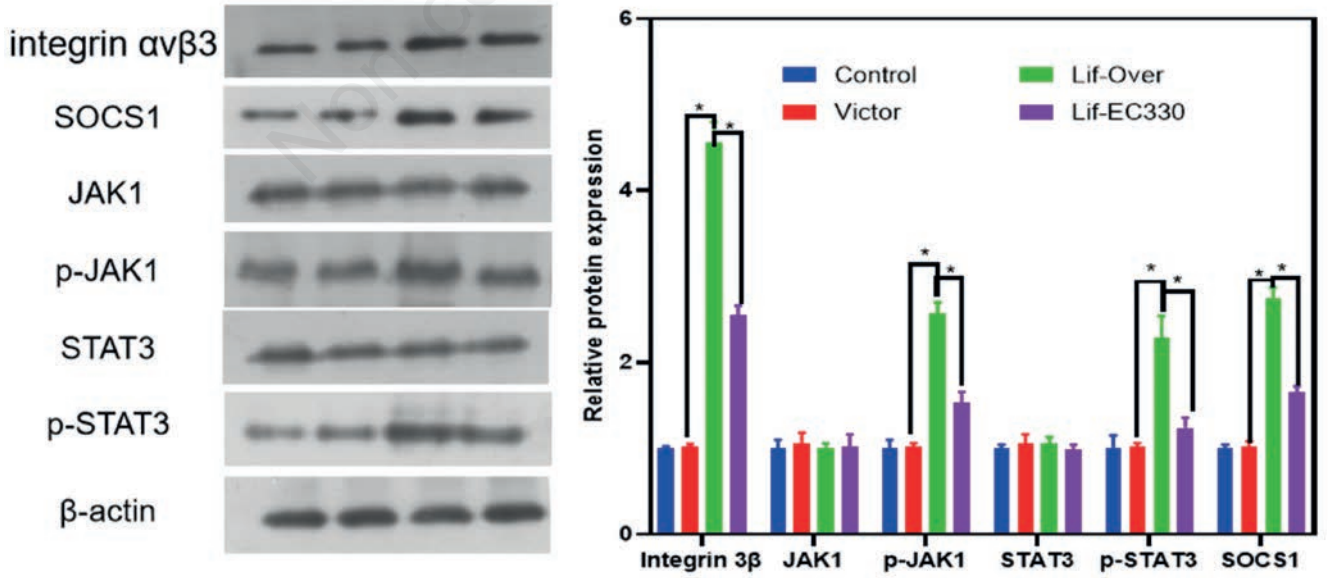

Figure 6. Overexpression of the leukemia inhibitory factor (LIF) and inhibition of LIF rescue the effect of related protein expression in Ishikawa cells. *p<0.05.

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