STAT3 enhances intracellular Fas-mediated apoptotic signals in HHUA human endometrial epithelial cells

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Abstract. Several endometrial signal transducer and activator of transcription 3 (STAT3)-activating cytokines are reported to be essential for blastocyst implantation, with inhibition of STAT3 activation in the endometrium also reported to prevent implantation. To investigate STAT3 signals in endometrial epithelial cells, the activation and inactivation effects of STAT3 signals were examined in the human endometrial epithelial cell line HHUA, which is thought to retain many of the intracellular signaling pathways found in normal human endometrial epithelial cells. Five STAT3-activating cytokines, IL-11, IL-10, LIF, oncostatin M and leptin, enhanced the Fas-mediated apoptosis of the HHUA cells without any increase in cell surface Fas antigen expression. STAT3 siRNA transfection suppressed STAT3 expression in HHUA cells and significantly inhibited Fas-mediated cell death. These results indicate that intracellular apoptotic signals in HHUA cells are constitutively activated and regulated by STAT3-mediated signals. This apoptosis-promoting effect of STAT3 in HHUA cells is completely different from many previous reports demonstrating anti-apoptotic effects by STAT3 activation. The STAT3 signals in HHUA cells may be specific to the human endometrial epithelial cell lineage in the regulation of blastocyst implantation.

Introduction

Unlike most normal adult tissues, cyclic growth and tissue remodeling occurs within the uterine endometrium throughout the reproductive years. Tissue remodeling is regulated by a balance between cell growth and selective cell death (apoptosis). Apoptotic cells are found in normal human endometrial epithelium, and the number of endometrial apoptotic cells is reported to increase during the secretory phase of the menstrual cycle (1-3), the blastocyst implantation period. Fas-mediated apoptosis is thought to affect blastocyst implantation, since the Fas antigen, a cell surface apoptotic death receptor, is expressed in the pre-implantation human endometrial epithelium (4-6), and endometrial epithelial apoptosis occurs during blastocyst implantation (7,8). Moreover, specific stimulation of Fas antigen induces the apoptosis of normal human endometrial epithelial cells (6,9,10).

Recent studies in cytokine-null or receptor-null mice have revealed that several interleukin (IL)-6 family cytokines may play essential roles in blastocyst implantation and/or endometrial stromal decidualization. Leukemia inhibitory factor (LIF)-null female mice exhibited impaired blastocyst implantation that can be partially restored by intraperitoneal administration of LIF (11). Additionally, transfer of recovered embryos from LIF-null mice to wild-type pseudopregnant female mice resulted in implantation and pregnancy. Female mice with a null mutation in the IL-11 receptor α chain are also reported to be infertile due to defective decidualization (12). Oncostatin M (OSM) binds competitively to the LIF receptor to regulate LIF functions on targeted cells, although the physiological functions of OSM in the human endometrium are not entirely understood (13). Both IL-11 and LIF bind to each specific receptor and activate signal transducer and activator of transcription 3 (STAT3), while specific inhibition of STAT3 activation in the endometrium prevents implantation (14). These results indicate that STAT3-activating cytokines may play important roles in human blastocyst implantation. The first step of blastocyst implantation is endometrial epithelial cell apoptosis following blastocyst attachment to the epithelium. Therefore, we examined the effects of STAT3-activating cytokines on the Fas-mediated apoptosis of human endometrial epithelial cells.

In the present experiments, five STAT3-activating cytokines, LIF, IL-11, OSM, leptin and IL-10, were used. The leptin/leptin receptor system has been reported to be a regulator of the blastocyst implantation process (15). IL-10 is an anti-inflammatory cytokine expressed in the endometrium and placenta, although IL-10-null mice do not have impaired embryo implantation (16). In the present study, the HHUA

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human endometrial epithelial cell line was selected because the cells express high levels of Fas antigen as well as functional estrogen receptors and progesterone receptors, similar to the normal human endometrial epithelium during the implantation period (17), and form glandular luminal structures in collagen gel cultures, similar to the structures of normal glandular epithelial cells (18). Karyotyping analysis performed on 20 HHUA cells revealed normal 46XX karyotypes (19). HHUA cells are known to express functional Fas antigens on their cell surface that mediate specific apoptotic signals (9). Based on these characteristics, HHUA cells are considered to retain many of the intracellular signaling pathways found in normal human endometrial epithelial cells. Hence, in the present study, HHUA cells were used to examine the effects of five STAT3-activating cytokines on basic cellular functions, such as proliferation, viability and apoptosis.

Materials and methods

Cell line and culture. The HHUA cell line (17) was obtained from the Riken Cell Bank (Tsukuba, Japan). All cells in these experiments were cultured in OPTI-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Equitech Bio Inc., Ingram, TX, USA), penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (0.25 μg/ml; Invitrogen) in the presence of 5% CO_2 and 95% air at 37˚C.

Cell proliferation assay. The cell proliferation effects of human recombinant cytokines or mouse anti-human Fas monoclonal IgM (clone CH-11; MBL, Nagoya, Japan) on HHUA cells were measured. HHUA cells were seeded onto 96-well plates at $5x10^3$ cells/well (n=6) and incubated with various concentrations of recombinant human cytokines or anti-Fas IgM for 48 h. At the end of the treatments, viable cell numbers were determined using a cell counting kit (Dojin Chemical Laboratory Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader. A viability of 100% was defined as the absorbance obtained for cells without any treatment. All five STAT3-activating cytokines used in the cultures were purchased from PeproTech EC Ltd. (London, UK).

Cell viability assay. Cell viability was examined using the cell counting kit. The stimulatory effects of anti-Fas IgM (clone CH-11) and one of the STAT3-activating cytokines on the cell viability of HHUA cells were assayed as follows: on day 1, HHUA cells in the log phase were detached using 0.25% trypsin/1 mM EDTA (Invitrogen) and cultured overnight in 96-well plates (5,000 cells/well). On day 2, combinations of anti-Fas IgM and one of the five STAT3-activating cytokines were added to the cells. On day 4, the viable cell numbers were counted using the kit. The final concentration of recombinant human cytokines used in the culture media were 25 ng/ml for IL-11, IL-10, LIF and OSM, and 50 ng/ml for leptin.

DNA fragmentation assay. HHUA cells in the log phase were detached with 0.25% trypsin/1 mM EDTA and cultured overnight ($3x10^6$ cells/dish). On day 2, one of the five recombinant human cytokines was added to the cells for 1 h, followed by the addition of anti-Fas IgM (final concentration, 75 ng/ml). On day 4, genomic DNA was extracted from all cells, including dead cells, using a SepaGene DNA extraction kit (Sankyo-Junyaku Co. Ltd., Tokyo, Japan) and treated with 100 μg/ml RNase A (Sigma, St. Louis, MO, USA) in TE buffer (10 mM Tris, pH 8.0, 2 mM EDTA) for 90 min at 37˚C to remove any contaminating RNA. Approximately 20 μg of the genomic DNA was electrophoresed on a 1.2% (w/v) agarose gel at 50 V for approximately 2 h, stained with 5 μg/ml ethidium bromide and visualized by UV illumination. The final concentrations of recombinant human cytokines in the culture media were 100 ng/ml for IL-11, IL-10, LIF and OSM, and 200 ng/ml for leptin.

Semi-quantitative flow cytometry. HHUA cells were detached and re-cultured in dishes as described above. Untreated HHUA cells and HHUA cells treated with each cytokine for 2 days were detached from the dishes using 3 mM EDTA in phosphate-buffered saline (PBS) and stained. Cells ($3x10^5$) were incubated with an excess of mouse anti-human Fas (CD95) monoclonal antibody (clone UB2; MBL) for 20 min at 4˚C, then washed twice with washing buffer (PBS containing 2% fetal calf serum and 0.1% NaN_3) and incubated with a secondary antibody [FITC-conjugated goat anti-mouse IgG (H+L); Dako-Japan, Kyoto, Japan] for 20 min at 4˚C. After two washes, the cells were suspended in 200 μl of washing buffer and analyzed with a FACSCalibur™ (Becton Dickinson, Mountain View, CA, USA). The final concentrations of recombinant human cytokines in culture media were 100 ng/ml for IL-11, IL-10, LIF and OSM, and 200 ng/ml for leptin.

Transfection of STAT3 siRNAs. The STAT3 siRNA and the negative control siRNA were obtained from a SureSilencing™ Human STAT3 siRNA kit (SuperArray Biosci Corp., Frederick, MD, USA). Lipofectamine 2000 (Invitrogen) was used as the transfection reagent according to the manufacturer's instructions. For the experiments, cells were seeded onto 6-well plates ($2.5x10^5$ cells/well) or 10-cm dishes ($2x10^6$ cells/dish), cultured for 24 h and then transfected with the STAT3 siRNAs or control siRNA. Subsequently, the cells were cultured for 72-120 h for protein assays before being harvested as indicated.

Western blotting. For Western blot analysis, the cells were collected at 72-120 h post-transfection with the STAT3 siRNAs or control siRNA, and lysed with PBS containing 1% NP-40, 0.1% sodium dodecyl sulfate, complete protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN, USA) and 1 mM phenylmethyl sulfonyl fluoride. The protein concentrations of the cell lysates were quantified by Coomassie Plus Protein assays (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of the total proteins were separated by SDS-PAGE using a 7.5% gel, and then transferred to a polyvinylidene fluoride membrane (ATTO Corp., Tokyo, Japan). After sequential incubations with primary and secondary antibodies, the immunocomplexes on the membranes were detected using enhanced chemiluminescence (ECL) or ECL plus kits (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were mouse anti-human STAT3 monoclonal antibody (clone STAAD22A; Cosmo Bio Co., Ltd., Tokyo, Japan) and mouse monoclonal anti-β-actin antibody

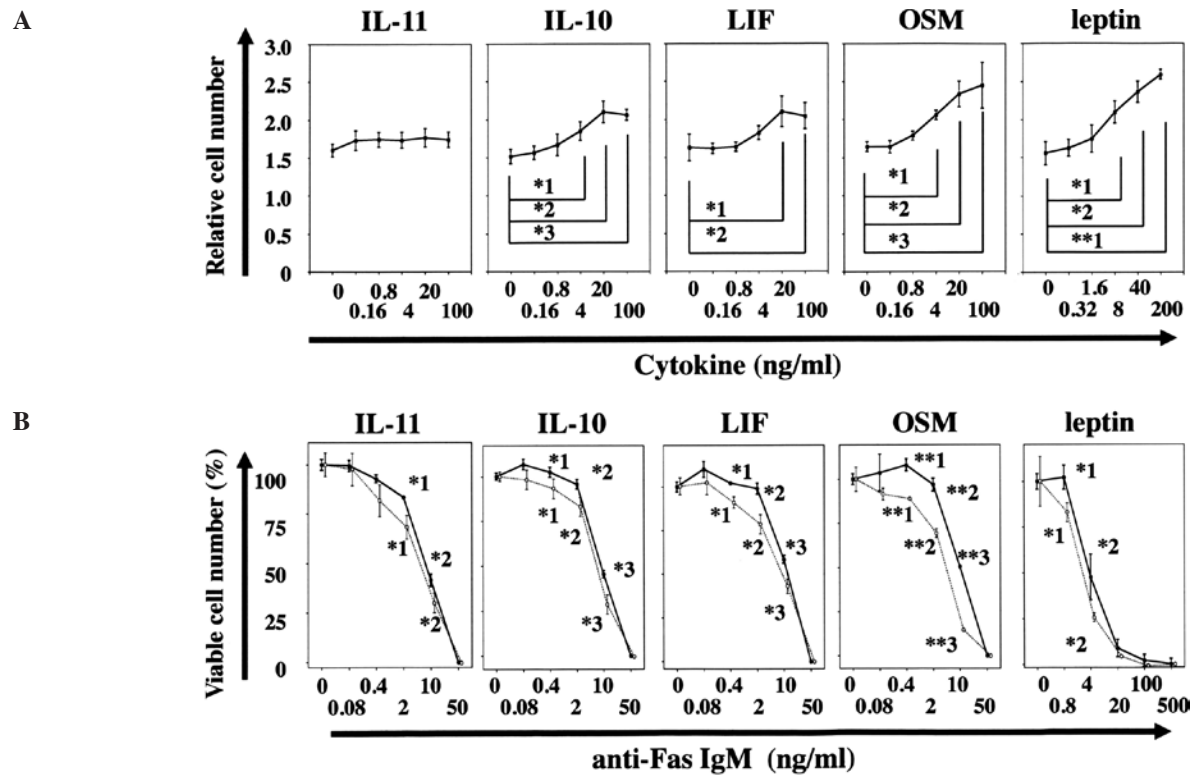

Figure 1. Effects of STAT3-activating cytokines on the cell proliferation and Fas-stimulated cell death of HHUA cells. (A) Effects of STAT3-activating cytokines on the cell proliferation of HHUA cells. $^{*}1$, $^{*}2$, $^{*}3$, $p<0.05$; $^{**}1$, $p<0.01$. (B) Effects of STAT3-activating cytokines on the Fas-stimulated cell death of HHUA cells. Cells were co-treated with anti-Fas IgM and a cytokine for 2 days. The final concentrations of the recombinant human cytokines were 25 ng/ml for IL-11, IL-10, LIF and OSM, and 50 ng/ml for leptin. $^{*}1$, $^{*}2$, $^{*}3$, $p<0.05$; $^{**}1$, $p<0.01$.

(Sigma Chemical Co.). The membranes were stripped and reprobed with an anti-β-actin antibody.

Statistical analysis. Data were expressed as the means ± SD. Comparisons between experimental groups were performed by analysis of variance (ANOVA). If the ANOVA was significant, *post-hoc* comparisons were conducted using Scheffe's test. The level of statistical significance was set at $p<0.05$.

Results

Effects of STAT3-activating cytokines on cell proliferation and Fas-stimulated cell death of HHUA cells. First, the effects of the five STAT3-activating cytokines on the cell proliferation of HHUA cells were examined. As shown in Fig. 1A, four cytokines, with the exception of IL-11, stimulated the cell proliferation of HHUA cells in a dose-dependent manner. IL-11 did not demonstrate any effects on cell proliferation. Co-stimulatory effects with the cytokines and anti-Fas IgM on the cell viability of the HHUA cells were also examined (Fig. 1B). All five of the STAT3-activating cytokines, including IL-11, significantly enhanced Fas-stimulated cell death in HHUA cells.

Effects of STAT3-activating cytokines on Fas-mediated DNA fragmentation and cell surface Fas antigen expression in HHUA cells. To confirm that the five STAT3-activating cytokines enhanced Fas-stimulated apoptosis in HHUA cells, DNA fragmentation assays were performed. As shown in Fig. 2A, the addition of the STAT3-activating cytokines apparently enhanced anti-Fas IgM-stimulated DNA fragmentation in HHUA cells. To examine whether the five STAT3-activating cytokines stimulated cell surface Fas antigen expression to enhance apoptosis in HHUA cells, semi-quantitative flow cytometric assays were carried out on the cytokine-treated HHUA cells. Flow cytometric experiments showed that five STAT3-activating cytokines did not apparently stimulate cell surface Fas antigen expression on HHUA cells (Fig. 2B).

Effects of STAT3 siRNA transfection on Fas-mediated cell death of HHUA cells. To clarify whether the enhancement of Fas-mediated apoptotic signals by five cytokines was due to activation of the STAT3 signaling pathway, targeted knockdown of STAT3 in HHUA cells was performed. STAT3 siRNA transfection into HHUA cells suppressed STAT3 protein expression (Fig. 3A); however, no changes in proliferation and cell viability were apparent by microscopic observations (Fig. 3B). STAT3 siRNA transfection, however, significantly enhanced the cell viability of HHUA cells treated with anti-Fas IgM (Fig. 3C).

Discussion

The present study demonstrates that five STAT3-activating cytokines enhance Fas-stimulated apoptotic susceptibility in HHUA human endometrial epithelial cells. HHUA cells do not express any Fas ligand mRNA (20), and the five STAT3-activating cytokines do not stimulate cell surface Fas antigen

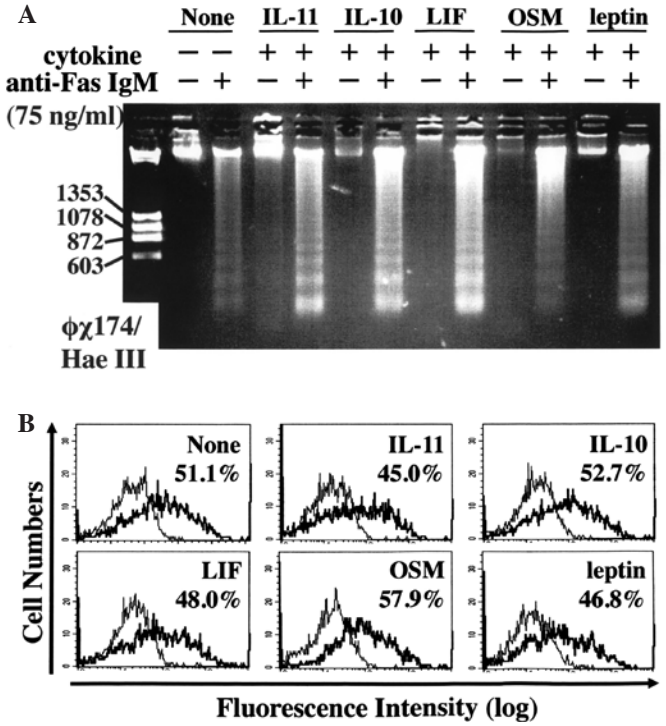

Figure 2. Effects of STAT3-activating cytokines on Fas-mediated DNA fragmentation and cell surface Fas antigen expression in HHUA cells. (A) Effects of STAT3-activating cytokines on Fas-mediated DNA fragmentation in HHUA cells. DNA extracted from HHUA cells co-stimulated with 75 ng/ml of anti-Fas IgM and one of the STAT3-activating cytokines were analyzed by DNA fragmentation assays. The final concentrations of the recombinant human cytokines in culture media were 100 ng/ml for IL-11, IL-10, LIF and OSM, and 200 ng/ml for leptin. (B) Quantitative flow cytometric analyses of cell surface Fas-antigen expression levels on HHUA cells treated with five STAT3-activating cytokines. HHUA cells were pre-treated for 48 h with each STAT3-activating cytokine. The final concentrations of the recombinant human cytokines in culture media were 100 ng/ml for IL-11, IL-10, LIF and OSM, and 200 ng/ml for leptin. The thick lines indicate Fas expression and the thin lines indicate negative controls. Positivity was calculated as the positive area (%) of Fas expression against a control area.

expression on HHUA cells. These results indicate that STAT3 activation enhances intracellular signals of Fas-mediated apoptosis. In the absence of STAT3-activating cytokines, specific knockdown of STAT3 expression by STAT3 siRNA transfection significantly inhibits the Fas-mediated apoptosis of HHUA cells, suggesting that STAT3-activating signals constitutively enhance Fas-mediated intracellular apoptotic signals in HHUA cells. In conclusion, the intracellular apoptotic signals in HHUA cells can be constitutively activated and regulated by STAT3-mediated signals.

There are many reports demonstrating that STAT3 activation plays anti-apoptotic roles in various cancer cells, including glioblastoma stem cells (21), glioma cells (22), prostate cancer cells (23), laryngeal cancer cells (24), cholangiocarcinoma cells (25), esophageal adenocarcinoma cells (26), gastric cancer cells (27), myeloid leukemia cells (28) and T-cell lymphoma cells (29). The anti-apoptotic functions of STAT3 signals are also reported in non-tumor cells, such as proximal renal tubular epithelial cells (30) and bronchial epithelial cells (31). Inhibition of STAT3 signals is reported to enhance radiosensitivity in squamous cell carcinoma cells (32), laryngeal cancer cells (33) and glioma cells (34), suggesting that STAT3 acti-

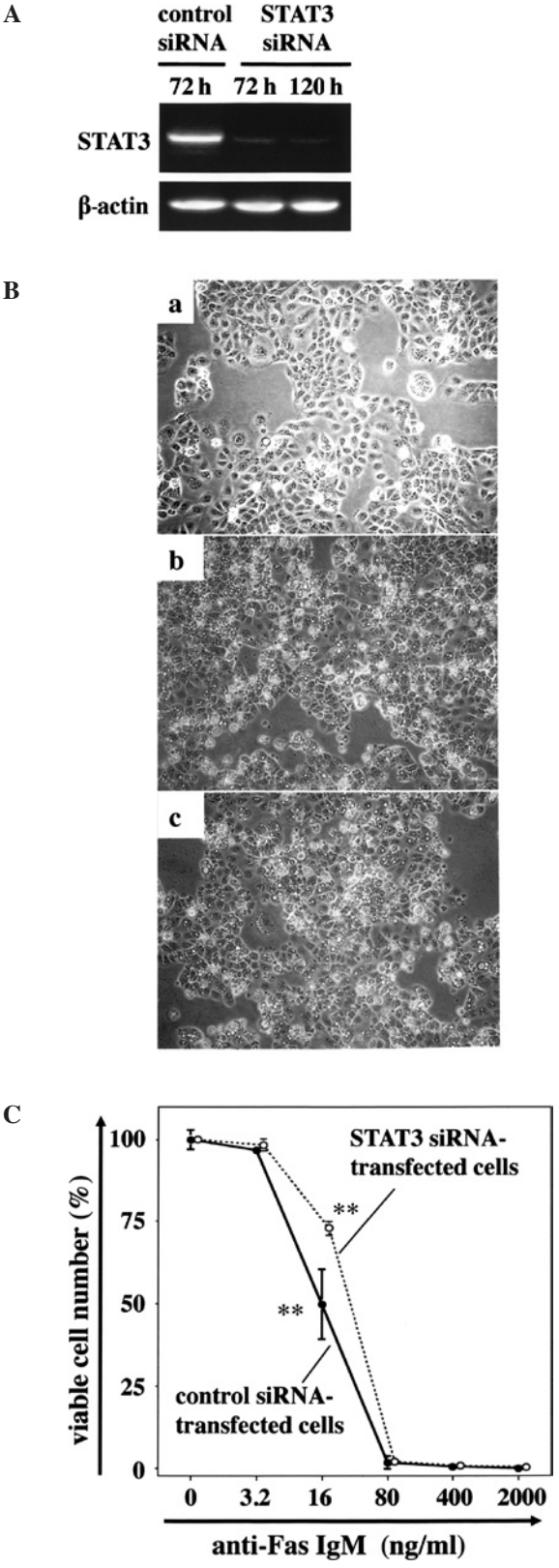

Figure 3. The effects of targeted knockdown of STAT3 expression by STAT3 siRNA transfection into HHUA cells on cell proliferation and Fas-mediated apoptosis. (A) Western blot analysis of HHUA cells transfected with siRNAs. STAT3 protein expression was strongly inhibited 72 h after siRNA transfection, while β-actin expression was not affected at all. (B) Phase contrast microscope views of the HHUA cells. a, untreated HHUA cells; b, HHUA cells treated with control siRNA for 48 h; c, HHUA cells treated with STAT3 siRNA for 48 h. Targeted knockdown of STAT3 in the HHUA cells did not alter the proliferation of the cells compared to cells transfected with the control siRNA. (C) Fas-mediated apoptosis was significantly inhibited in the STAT3 siRNA-transfected HHUA cells. $^{**}p<0.01$.

vation enhances cell survival against radiation cell death. As for Fas-mediated apoptosis, it has been reported that STAT3-siRNA induces Fas-mediated apoptosis *in vitro* and *in vivo* in breast cancer cells (35). To the best of our knowledge, there have been no previous reports stating that STAT3 activation enhances apoptosis in any cells. Our present study may be the first to report that STAT3 activation enhances intracellular apoptotic signals. This exceptional phenomenon in HHUA cells may be caused by the specific lineage of HHUA cells.

Five STAT3-activating cytokines were used in the present experiments. Although IL-10, leptin, LIF and OSM dose-dependently simulated the cell proliferation of the HHUA cells, IL-11 did not affect cell proliferation at all. IL-11 enhanced the Fas-mediated apoptosis of the HHUA cells. Targeted knockdown of STAT3 expression by STAT3-siRNA treatment has previously been reported to inhibit the cell proliferation of various cells, including prostate cancer cells (23), laryngeal cancer cells (24), cutaneous squamous cell carcinomas (36), multiple myeloma cells (37), hepatocellular carcinoma cells (38) and glioma cells (39). However, there have been no reports indicating that STAT3 activation inhibits the proliferation of any cells. The growth-promoting effects by four STAT3-activating cytokines in HHUA cells coincided with previous reports that STAT3-activated signals enhanced cell proliferation. It is unknown why IL-11 did not stimulate the proliferation of HHUA cells. Our previous study demonstrated that epidermal growth factor (EGF), a STAT3-activating cytokine, enhanced Fas-mediated apoptosis, while EGF did not enhance cell proliferation or cell surface Fas expression in HHUA cells (40). The effects of IL-11 in the present study coincide with the effects of the EGF on HHUA cells.

Normal human endometrial epithelial cells play cell lineage-specific physiological roles in blastocyst implantation. When the blastocyst is implanted into the endometrium, endometrial epithelial cells become apoptotic to accept blastocyst invasion, and then endometrial epithelial cells proliferate to cover the invaded blastocyst. Increased apoptotic susceptibility and growth-promoting activity of endometrial epithelial cells during blastocyst implantation may reflect the results that apoptotic susceptibility and cell proliferation were enhanced by STAT3-activating cytokines in HHUA cells. Both IL-11 and LIF are essential STAT3-activating cytokines for blastocyst implantation (11,12). Since HHUA are not normal cells, even though the cells have several characteristics of normal human endometrial epithelial cells, further investigations using normal endometrial cells are required to conclude that the STAT3-mediated effects observed on HHUA cell function during blastocyst implantation in the normal endometrium are indeed genuine.

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