Running title: The LIF pathway in unexplained infertility

Disturbances in the LIF pathway in the endometrium among women with unexplained infertility

Lusine Aghajanova MD, PhD[a, b], Signe Altmäe MSc[a], Kerstin Bjuresten[a], Outi Hovatta MD, PhD[a], Britt-Marie Landgren MD, PhD[a], Anneli Stavreus-Evers PhD[a,c].

[a]*Department of Clinical Science Intervention and Technology, Division of Obstetrics and Gynecology, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden,* [b]*Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, USA,*[c3]*Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden*

Corresponding author: Lusine Aghajanova, M.D., Ph.D.
Current address: 513 Parnassus Avenue, HSW 1671
UCSF, Department of Obstetrics, Gynecology and Reproductive Sciences
San Francisco, 94143-0556, CA, USA
Email: aghajanoval@obgyn.ucsf.edu

Acknowledgment of financial support:
This work was supported by The Swedish Research Council, The Swedish Society of Medicine, Uppsala University, Karolinska Institutet, the Goljes Foundation, the Åke Wibergs Foundation and R&D grants from Stockholm County Council and Karolinska Institutet (ALF).

Capsule: Leukemia inhibitory factor (LIF) is important in the implantation process. We studied LIF, LIF receptors LIFR and gp130, and LIF inhibitory factor SOCS1 in endometria of women with unexplained infertility *vs.* fertile controls. We report here that some women with unexplained infertility had disturbances in the LIF pathway in mid-secretory phase endometrium.

Abstract

Objective: To study the expression of Leukemia Inhibitory Factor (LIF), its receptors LIFR and gp130 and its inhibitor SOCS1 in endometria from fertile women and infertile women with unexplained infertility. Signaling through the LIF pathway is involved in maintenance of a receptive state of human endometrium. Impaired endometrial receptivity might be a cause of female infertility.

Design: Prospective clinical study.

Setting: Hospital-based IVF unit and university-affiliated reproductive research laboratories.

Patients: Twenty-six healthy fertile women and 14 women with unexplained infertility.

Interventions: Endometrial biopsy.

Main outcome measure(s): pinopode formation, expression of LIF, LIFR, gp130 and SOCS1 protein and mRNA in endometrial biopsies.

Results: The expression of LIFR in the endometrium was negatively correlated to the expression of SOCS1 and positively correlated to the formation of pinopodes. In control fertile women, simultaneous intense apical staining of LIFR and gp130 together with faint SOCS1 staining was observed in epithelial cells, while the opposite was seen in most women with unexplained infertility.

Conclusion: Unexplained infertility in some women might be explained by disturbances in the LIF pathway in mid-secretory phase endometrium.

Key words: *LIF, LIFR, gp130, SOCS1, human endometrium, unexplained infertility*

Introduction

Approximately 30% of infertile couples worldwide suffer from infertility of an unknown cause, thus being diagnosed as cases of unexplained infertility (1). Females in such couples usually have normal ovulatory cycles, normal hormonal profiles and no organic pathology, and males have normal semen. It can be suspected that some of these women have aberrant endometrial function that may result in impaired implantation.

The endometrium is receptive for implantation of a blastocyst during a well-defined period in the secretory phase of the menstrual cycle. Both the endometrium and the blastocyst produce factors which may be involved in the cross-talk necessary for successful implantation (2-5). One of the most important and studied substances related to implantation is leukemia inhibitory factor (LIF). The implantation process has been found to be defective in LIF knockout mice, but it could be restored by exogenous LIF. LIF-/- embryos implanted normally in the uteri of wild-type recipients (6), indicating the importance of the cytokine for mouse implantation.

Leukemia inhibitory factor acts on cells through binding to the heterodimeric LIF receptor, which consists of two transmembrane proteins, LIF receptor (LIFR) and gp130 (7). The former binds specifically to LIF and the latter binds several cytokines that share a signal-transducing subunit in their receptor-transducer mechanism (7, 8).

The SOCS1 (suppressor of cytokine signaling-1) gene encodes for a protein that negatively regulates JAK/STAT pathways by interacting with JAKs (9). SOCS1 inhibits LIF-mediated signaling and is expressed during early pregnancy in ovine endometrium (10), rat decidual cells (11) and human villous placenta (12).

In a previous study, we showed that LIFR expression, especially in the luminal epithelium of human endometrium, was highest during the mid-secretory phase, the suggested time of implantation (13). On the embryonic side, human blastocysts have been found to express both LIFR and gp130 transcripts (14-17). Expression of gp130 has been reported in the inner cell mass of the blastocyst, while LIFR was also expressed in the trophoblast (17). Leukemia inhibitory factor produced by the embryo may exert its action on the inner cell mass or on the endometrium, hence participating in embryo-maternal cross-talk (17).

Pinopodes are ectoplasmic protrusions of endometrial epithelial cells that have been suggested to be ultrastructural markers of receptive endometrium (13). However, opposite opinions regarding the use of pinopodes as markers of endometrial receptivity have also been presented (18-21). We have previously shown strong correlation between the expression of LIF and LIFR in the endometrial epithelia of healthy fertile women, and the appearance of pinopodes. (13). Secretion of LIF from human pinopodes has been shown, using immuno-scanning electron microscopy (22).

Both LIF transcripts and protein are present in human endometrium (14, 23, 24). The protein has also been detected in uterine flushings during the time of expected implantation in fertile women, with gradually increasing concentrations from days LH+7 to LH+12 (25). However, the concentration of LIF in flushings from women with unexplained infertility was significantly lower than in those obtained from fertile women. Cultured endometrial explants from infertile women (women with repeated implantation failures or unexplained primary infertility) produced less LIF than explants from fertile women (26, 27).

It may be possible that impaired endometrial development causes infertility in some women (28, 29). Here, we studied the possible role of LIF signaling in the pathogenesis of unexplained infertility by studying the patterns of expression of LIF, LIFR, gp130 and SOCS1 proteins and mRNA in endometrial biopsy samples from infertile women undergoing IVF treatment in our clinic, in comparison with the expression patterns in endometrial samples from healthy volunteer donors. The expression patterns were correlated to the formation of pinopodes on the endometrial surface.

Materials and methods

Subjects

Endometrial biopsy samples were obtained from 26 healthy women with proven fertility. Fourteen of the women were healthy volunteers and twelve women were attending Karolinska University Hospital Huddinge for tubal sterilization. The mean age of these women was 32 years (25 to 40) and their BMI was between 18 and 25 kg/m^2. None of them had taken any steroid hormones for at least three months prior to the study or used an intrauterine device for at least six months prior to the study. The concentration of luteinizing hormone (LH) in morning urine (Clearplan; Unipath Ltd., Bedford, United Kingdom) was used to determine the day of the surge.

Endometrial biopsy samples were also obtained from 14 women with unexplained infertility, attending the hospital for treatment of infertility. Patient characteristics are shown in Table 1. Unexplained infertility in couples was diagnosed by means of a set of tests that included hormonal analyses and at least two semen analyses. All women had normal ovarian function. The mean age of the women was 33 ± 4.0 (± SEM) years, their cycle length was 28.9 ± 2.0 (± SEM) days and the duration of menses was 4.8 ± 0.8 (± SEM) days. All women had serum concentrations of follicle-stimulating hormone (FSH) below 11 IU/L during the early follicular phase (day 2–5). All women had serum prolactin concentrations below 20 μg/L, and normal thyroid stimulating hormone (TSH) and thyroid hormone serum concentrations. All women had patent Fallopian tubes as demonstrated by hysterosonosalpingography, normal weight, with BMI between 18 and 25 kg/m^2, and no recognizable endometriotic symptoms in clinical examination or ultrasonography. Their partners had normal semen analysis results.

The Ethics Committees of the Karolinska Institute approved this study, and informed consent was obtained from all participating subjects.

Dating of biopsy samples

The endometrial biopsy samples were obtained during the secretory phase. They were precisely timed according to the LH surge, with the day of the surge being day LH+0. All samples included in the study corresponded histologically to the assumed time in the cycle according to the criteria published by Noyes et al. (30).

Preparation of biopsy samples

Endometrial biopsy samples obtained by Randall curette were divided into two or three pieces depending on their size. They were prepared for analysis by scanning electron microscopy (SEM), histology, immunohistochemistry and real-time PCR.

The samples for SEM were fixed in a solution containing 2.5% (wt/vol) glutaraldehyde, 0.5% paraformaldehyde, 0.1 M sucrose, 0.1 M sodium cacodylate and 3 mM calcium chloride (pH 7.4). Pieces for immunostaining were fixed immediately in 4% formaldehyde for a maximum of 24 hours, transferred to 70% ethanol and stored until embedding. The part of endometrial tissue saved for real-time PCR analysis was immediately snap-frozen and stored in liquid nitrogen.

Scanning electron microscopy

Endometrial biopsy pieces from all women were evaluated using SEM. The samples were washed twice in buffer containing sodium cacodylate and calcium chloride (pH 7.4) and once in distilled water. After this, they were dehydrated, first in increasing concentrations of ethanol (70%, 95% and 99.5%), then in acetone, and dried in a critical point drier with carbon dioxide. The specimens were mounted on a specimen holder, coated with platinum and examined using a Jeol 820 scanning electron microscope (Jeol Ltd., Tokyo, Japan).

Immunohistochemistry

All samples were analyzed for LIF, LIFR, gp130 and SOCS1 protein expression in the endometrium by immunohistochemistry. Paraffin-embedded samples from all women were cut (4 μm), deparaffinized and washed. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 10 min. Slides to be stained for LIF, LIFR, gp130 antibodies (R&D Systems, Oxford, UK) and SOCS1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were incubated with 10% normal rabbit (blocking) serum in PBS (Vector Laboratories, Burlingame, CA, USA) for 1 hour (LIF and gp130) or 30 minutes (LIFR and SOCS1). Polyclonal goat antibody against human LIF was used at a final concentration of 2 μg/mL overnight at room temperature (RT), goat polyclonal IgG

against human LIFR was used at 2.5 μg/mL for 1 hour at RT, goat polyclonal antibody against human gp130 was applied at 2.5 μg/mL for 1.5 hours, and goat polyclonal antibody against human SOCS1 was used at 1 μg/mL, overnight at RT.

The specificity of these antibodies has been described previously (13, 31-33). Negative control slides for LIF, LIFR, gp130 and SOCS1 were incubated with non-immune goat IgG (SDS, Falkenberg, Sweden). The cells were then incubated for 1 hour with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). The bound antibodies were visualized by means of formation of an avidin-biotin complex with peroxidase (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA, USA), for 30 min, following application of 3,3-diaminobenzidine in H_2O_2 (DAB-kit, Vector Laboratories, Burlingame, CA, USA). All slides were counterstained with hematoxylin, dehydrated in ascending concentrations of alcohol, mounted with Pertex (Histolab, Gothenburg, Sweden) and viewed under a light microscope.

Evaluation of immunohistochemistry

Two observers, unaware of the identity of the slides, evaluated the staining intensity of each sample. The staining intensity was graded on a scale of 0 = no staining, 0/1 = a few stained cells, 1 = faint staining, 2 = moderate staining, and 3 = strong staining. Each sample was analyzed two or three times, and the average value from both observers was presented.

Preparation of RNA and cDNA

The following endometrial biopsy sample types underwent QPCR analysis: normal proliferative phase (*n*=2), normal early secretory phase (*n*=3), normal mid-secretory phase (*n*=3), normal late secretory phase (*n*=2), and unexplained infertility mid-secretory phase biopsy samples (*n*=3).

The frozen tissue was homogenized in lysis buffer. RNA was prepared according to the Rneasy Mini-kit (Qiagen, Venlo, The Netherlands) protocol for animal tissues. The concentration and purity of the RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

Synthesis of cDNA from endometrial tissue samples was performed using Superscript III First Strand Synthesis for RT-PCR reagents (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA from tissue samples per reaction were reverse-transcribed using oligo(dT) as a primer. From 50 to 800 ng of total mRNA from endometrial cells per reaction were used. Random hexamers were used as primers. The samples were then stored at -20 °C until real-time PCR was performed.

Real-time PCR

Real-time PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Sybrgreen was used to detect the amplification. The reaction mixture consisted of 1× Sybrgreen PCR mastermix, 4 μL of the RT reaction mixture, corresponding to 4 ng cDNA, and the primer concentrations were 250 nM. The final reaction volume was 15 μL. The cDNA was heated to 50 °C for 2 minutes and denatured at 95 °C for 10 minutes. This was followed by 45 cycles of denaturation at 95 °C for 15 seconds, and combined primer annealing/extension at 60 °C for one minute. Fluorescence data was acquired by measurements taken at every extension step and presented as a plot of fluorescence intensity versus cycle number.

Standard curves were constructed using duplicates of seven serial dilutions of cDNA prepared from RNA of known concentration. Standard curves were created and run in parallel with the unknown samples in each experiment. Fluorescence was measured and the threshold cycle (C_t) values at each point in the standard curve were plotted against the log (ng) of the initial concentration (Higuchi et al., 1993). The standard curves displayed linear correlation for all genes. The relative concentrations of unknown samples were calculated against the standard. Each sample was run in duplicate and the target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control.

Primer sequences and the sizes of the PCR products are presented in Table 2. Differences in expression levels between different groups of women were analyzed by means of the Mann–Whitney rank sum test. Significance was accepted at a p-value of ≤ 0.05.

Results

Presence of pinopodes on the endometrial surface

Pinopode formation was observed in 88% of samples obtained during days LH+6 to LH+9 from fertile women, while pinopodes in endometria from women with unexplained infertility were seen in 57% of samples (Table 3). The presence of pinopodes was correlated to LIFR expression in the luminal epithelium (r^2=0.421, p=0.01).

Expression of LIF protein and mRNA during normal menstrual cycles and in mid-secretory endometria of infertile women

In endometrial samples from fertile women, there were no significant differences in LIF mRNA levels throughout the cycle (Figure 1A). LIF immunostaining in mid-luteal phase endometrium is not presented since it was reported in a previous study (13).

Immunostaining of LIF, and mRNA expression, were seen in the endometria of all women with unexplained infertility (Figures 2A and 3A–C). However, low staining intensity was observed in luminal epithelial cells in five of fourteen women with unexplained infertility compared with two of fourteen fertile women (Table 4). In the glandular epithelial cells, three of fourteen biopsy samples from infertile women showed low staining intensity compared with none in the fertile group.

Expression of LIFR protein and mRNA during normal menstrual cycles and in mid-secretory endometria of infertile women

No significant difference in LIFR mRNA levels throughout the cycle was observed in endometrial samples from fertile women (Figure 1B). LIFR protein expression throughout the luteal phase has been described previously (13).

During the mid-secretory phase, all fertile women showed intense staining of LIFR in the luminal and glandular epithelium, while only four of fourteen women with unexplained infertility exhibited moderate to strong staining intensity of LIFR in luminal epithelial cells and five of the fourteen women showed moderate to strong staining in the glandular epithelial cells (Table 4). Endometrial stromal cells demonstrated very weak immunostaining (Figure 3D–F). No significant difference in LIFR mRNA expression was found between the fertile and infertile groups (Figure 2B).

Expression of gp130 protein and mRNA during normal menstrual cycles and in mid-secretory endometria of infertile women

Immunostaining of gp130 in endometrial samples from fertile women was more intense in luminal epithelium than in glandular epithelium. Little staining was seen in the stroma. The most intense immunostaining of gp130 was seen during the mid-secretory phase (Figure 3G, Table 5), where cytoplasmic staining of gp130 in glandular and luminal epithelium was increased significantly compared with early secretory phase samples ($p<0.05$) (Table 5). None of these differences were reflected in the mRNA levels (Figure 1C).

During the mid-secretory phase, gp130 was localized predominantly on the apical surfaces of glandular and luminal epithelial cells, whereas in the late secretory phase the protein was mainly seen in the cytoplasm (Table 5, Figure 3G). Surface expression of gp130 in glandular epithelium was significantly less in the late secretory phase compared with the early secretory phase ($p<0.01$) (Table 5).

Immunostaining of gp130, and gp130 mRNA in endometria from women with unexplained infertility is shown in Figures 2C and 3H. Two out of fourteen women with unexplained infertility showed moderate to strong staining on the apical side of the luminal epithelium, while all fertile women had moderate to strong staining in the apical surface of the luminal epithelium (Table 4). In the glandular epithelium, all biopsy samples from infertile women showed low staining intensity, while moderate to strong staining was seen in 81% of samples from fertile women (Table 4). A similar tendency was seen as regards cytoplasmic staining (Table 4).

Expression of SOCS1 protein and mRNA during normal menstrual cycles and in mid-secretory endometria of infertile women

Immunostaining of SOCS1 was most intense in the apical part of the luminal and glandular epithelial cells in endometrial samples from fertile women (Figure 3J). The immunostaining did not vary significantly throughout normal menstrual cycles (Table 5).

SOCS1 mRNA was abundant in normal endometrial tissue. The highest gene expression

was observed in the proliferative phase, which corresponded to the lowest expression of LIF and LIFR in the menstrual cycle (Figure 1A, B and D). There was a negative correlation between LIFR and SOCS1 protein expression in luminal epithelium (r^2=-0.393, p=0.04).

Immunostaining and mRNA levels of SOCS1 in mid-secretory endometria from infertile women are presented in Figures 2D and 3K. Most women, both fertile and infertile, showed strong apical SOCS1 staining in epithelial cells (Table 4). We noticed a tendency for redistribution of apical SOCS1 expression from luminal epithelium in fertile women to glandular epithelium in infertile women. All fertile women showed almost no cytoplasmic staining of SOCS1 in glandular and luminal epithelium, while four of fourteen women with unexplained infertility showed strong cytoplasmic staining in the luminal epithelium and thirteen of fourteen infertile women showed strong cytoplasmic staining in the glandular epithelium (Table 4).

Discussion

The main finding in the present study is that high levels of LIFR and gp130 immunostaining were correlated to low SOCS1 immunostaining in the endometrium during the luteal phase of normal menstrual cycles, while this regulation seems to be disturbed in women with unexplained infertility. We hence identified a group of women

with unexplained infertility who had expression patterns of LIFR and SOCS1 that deviated from those seen in fertile women.

The endometrium undergoes both morphological and biochemical changes at the time of implantation. However, the precise characteristics of a receptive endometrium are still not established. The concept of an endometrial factor as a cause of female infertility has been suggested, but still needs to be defined.

The nature and purpose of pinopodes in the endometrium is currently being investigated, and so far there is no clear consensus on the structure of pinopodes and the time of their appearance. This is probably the reason why some groups find pinopodes throughout the luteal phase, while others show pinopodes only in relation to the putative window of implantation (13, 18, 34). In our study, pinopodes were more abundant in endometria from fertile women than in endometria from infertile women. We found that 57% of the women with unexplained infertility presented with pinopode-forming endometrium. It has been shown that pinopodes have the ability to secrete LIF in the human endometrium (22). Thus, the role of these structures as indicators of endometrial function still needs to be clarified. In the present study, the presence of pinopodes was significantly correlated to LIFR expression in the luminal epithelium, but not to LIF or gp130 expression.

We showed, for the first time, the presence of SOCS1 mRNA and protein in human endometrium. The expression of SOCS1 in endometrial epithelial cells appeared to be more pronounced in the apical part of the cell. This is the same localization as is seen for LIFR and gp130, suggesting a possible regulatory function of SOCS1 on LIF action. However, immunostaining of SOCS1 on the endometrial cell surface and in the cytoplasm did not vary significantly throughout the normal menstrual cycle, which might be a result of inter-individual variation.

High levels of LIFR were correlated with high expression of gp130 both in glandular and luminal epithelium. This could be important for communication with embryo signaling at the time of implantation, as it has been shown that the embryo produces LIF (17, 35).

In the present study, mRNA levels did not vary to the same extent as the protein levels. This may be a result of the fact that not all samples were used for real time PCR. Luminal epithelial cells do not form the majority of cells in the endometrium, which may explain the lack of variation. In preparation for real-time PCR, the whole tissue is homogenized

and analyzed at the same time, and it is more difficult to observe changes in single cell types.

We found low staining intensity of LIF in mid-secretory endometrial biopsy samples from approximately 30% of women with unexplained infertility, while all fertile women had moderate to strong LIF immunostaining in the mid-secretory phase (13). These data correlate with indications from previous studies that LIF expression is aberrantly expressed in endometria from infertile women (25-27). Further, infertile women with endometriosis have been observed to show lower immunostaining of LIF compared with fertile controls (25, 35).

In our study, strong apical staining of LIFR was seen in glandular and luminal epithelium in approximately 30% of the women with unexplained infertility, compared with 100% of the fertile women. In most women with unexplained infertility, apical staining of gp130 was weak or absent, while it was strong in all endometria from fertile women. This is in line with the results of a previous study, where patients with unexplained infertility showed reduced secretion of soluble gp130 compared with fertile controls during the implantation window (31). These data point to less efficient LIF action in some women with unexplained infertility.

In conclusion, we found that several women with unexplained infertility had high endometrial levels of the inhibitor of LIF action, SOCS1, combined with low levels of LIFR and gp130, in contrast to the situation in all other women. It is likely that the function of the LIF signaling pathway is affected in these infertile women, and this could possibly offer an explanation for their infertility. Hence, we identified a small group of women with unexplained infertility, whose condition could be explained by disturbances in the LIF pathway in the endometrium. Better understanding of molecular changes in the endometrium in cases of infertility could provide the possibility of pathogenetic treatment of women with unexplained infertility.

References

1. Smith S, Pfeifer SM, Collins JA. Diagnosis and management of female infertility. Jama 2003; 290:1767-70
2. Giudice LC. Potential biochemical markers of uterine receptivity. Hum Reprod 1999; 14 Suppl 2:3-16
3. Giudice LC. Genes associated with embryonic attachment and implantation and the role of progesterone. J Reprod Med 1999; 44:165-71

4. Horcajadas JA, Riesewijk A, Martin J, Cervero A, Mosselman S, Pellicer A, Simon C. Global gene expression profiling of human endometrial receptivity. J Reprod Immunol 2004; 63:41-9

5. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC. Global gene profiling in human endometrium during the window of implantation. Endocrinology 2002;143:2119-38

6. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992; 359:76-9

7. Classen-Linke I, Muller-Newen G, Heinrich PC, Beier HM, von Rango U. The cytokine receptor gp130 and its soluble form are under hormonal control in human endometrium and decidua. Mol Hum Reprod 2004; 10:495-504

8. Sanchez-Cuenca J, Martin JC, Pellicer A, Simon C. Cytokine pleiotropy and redundancy – gp130 cytokines in human implantation. Immunol Today 1999; 20:57-9

9. Truchet S, Chebrout M, Djediat C, Wietzerbin J, Debey P. Presence of permanently activated signal transducers and activators of transcription in nuclear interchromatin granules of unstimulated mouse oocytes and preimplantation embryos. Biol Reprod 2004; 71:1330-9

10. Sandra O, Bataillon I, Roux P, Martal J, Charpigny G, Reinaud P, Bolifraud P, Germain G, Al-Gubory KH. Suppressor of cytokine signalling (SOCS) genes are expressed in the endometrium and regulated by conceptus signals during early pregnancy in the ewe. J Mol Endocrinol 2005; 34:637-44

11. Deb S, Tessier C, Prigent-Tessier A, Barkai U, Ferguson-Gottschall S, Srivastava RK, Faliszek J, Gibori G. The expression of interleukin-6 (IL-6), IL-6 receptor, and gp130-kilodalton glycoprotein in the rat decidua and a decidual cell line: regulation by 17beta-estradiol and prolactin. Endocrinology 1999; 140:4442-50

12. Blumenstein M, Bowen-Shauver JM, Keelan JA, Mitchell MD. Identification of suppressors of cytokine signaling (SOCS) proteins in human gestational tissues: differential regulation is associated with the onset of labor. J Clin Endocrinol Metab 2002; 87:1094-7

13. Aghajanova L, Stavreus-Evers A, Nikas Y, Hovatta O, Landgren BM. Coexpression of pinopodes and leukemia inhibitory factor, as well as its receptor, in human endometrium. Fertil Steril 2003; 79 Suppl 1:808-14

14. Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. J Reprod Fertil 1994; 101:421-6

15. Chen HF, Shew JY, Ho HN, Hsu WL, Yang YS. Expression of leukemia inhibitory factor and its receptor in preimplantation embryos. Fertil Steril 1999; 72:713-9

16. van Eijk MJ, Mandelbaum J, Salat-Baroux J, Belaisch-Allart J, Plachot M, Junca AM, Mummery CL. Expression of leukaemia inhibitory factor receptor subunits LIFR beta and gp130 in human oocytes and preimplantation embryos. Mol Hum Reprod 1996; 2:355-60

17. Wanggren K, Lalitkumar PG, Hambiliki F, Stabi B, Gemzell-Danielsson K, Stavreus-Evers A. Leukaemia inhibitory factor receptor and gp130 in the human Fallopian tube and endometrium before and after mifepristone treatment and in the human preimplantation embryo. Mol Hum Reprod 2007; 13:391-7

18. Acosta AA, Elberger L, Borghi M, Calamera JC, Chemes H, Doncel GF, Kliman H, Lema B, Lustig L, Papier S. Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil Steril 2000; 73:788-98

19. Usadi RS, Murray MJ, Bagnell RC, Fritz MA, Kowalik AI, Meyer WR, Lessey BA. Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility. Fertil Steril 2003; 79:970-4

20. Quinn C, Ryan E, Claessens EA, Greenblatt E, Hawrylyshyn P, Cruickshank B, Hannam T, Dunk C, Casper RF. The presence of pinopodes in the human endometrium does not delineate the implantation window. Fertil Steril 2007; 87:1015-21

21. Quinn CE, Detmar J, Casper RF. Pinopodes are present in Lif null and Hoxa10 null mice. Fertil Steril 2007; 88:1021-8

22. Kabir-Salmani M, Nikzad H, Shiokawa S, Akimoto Y, Iwashita M. Secretory role for human uterodomes (pinopods): secretion of LIF. Mol Hum Reprod 2005; 11:553-9

23. Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Stewart CL. Leukemia inhibitory factor (LIF) and LIF receptor expression in human

endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci U S A 1996; 93:3115-20

24. Vogiagis D, Marsh MM, Fry RC, Salamonsen LA. Leukaemia inhibitory factor in human endometrium throughout the menstrual cycle. J Endocrinol 1996; 148:95-102

25. Laird SM, Tuckerman EM, Dalton CF, Dunphy BC, Li TC, Zhang X. The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture. Hum Reprod 1997; 12:569-74

26. Delage G, Moreau JF, Taupin JL, Freitas S, Hambartsoumian E, Olivennes F, Fanchin R, Letur-Konirsch H, Frydman R, Chaouat G. In-vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. Hum Reprod 1995; 10:2483-8

27. Hambartsoumian E. Endometrial leukemia inhibitory factor (LIF) as a possible cause of unexplained infertility and multiple failures of implantation. Am J Reprod Immunol 1998; 39:137-43

28. Aghajanova L. Leukemia inhibitory factor and human embryo implantation. Ann N Y Acad Sci 2004; 1034:176-83

29. Kelley RL, Kind KL, Lane M, Robker RL, Thompson JG, Edwards LJ. Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. Am J Physiol Endocrinol Metab 2006; 291:E761-70

30. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1951; 1:3-25

31. Sherwin JR, Smith SK, Wilson A, Sharkey AM. Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained infertility. J Clin Endocrinol Metab 2002; 87:3953-60

32. Stavreus-Evers A, Mandelin E, Koistinen R, Aghajanova L, Hovatta O, Seppala M. Glycodelin is present in pinopodes of receptive-phase human endometrium and is associated with down-regulation of progesterone receptor B. Fertil Steril 2006; 85:1803-11

33. Egan PJ, Lawlor KE, Alexander WS, Wicks IP. Suppressor of cytokine signaling-1 regulates acute inflammatory arthritis and T cell activation. J Clin Invest 2003; 111:915-24

34. Nikas G, Makrigiannakis A. Endometrial pinopodes and uterine receptivity. Ann N Y Acad Sci 2003; 997:120-3

35. Dimitriadis E, Stoikos C, Stafford-Bell M, Clark I, Paiva P, Kovacs G, Salamonsen LA. Interleukin-11, IL-11 receptor alpha and leukemia inhibitory factor are dysregulated in endometrium of infertile women with endometriosis during the implantation window. J Reprod Immunol 2006; 69:53-64

Figure 1. Relative expression of LIF (A), LIFR (B), gp130 (C) and SOCS1 (D) mRNA in human endometrium as revealed by real-time RT-PCR. P – proliferative phase (*n*=2), E – early secretory phase (*n*=3), M – mid-secretory phase (*n*=3), L – late secretory phase (*n*=2).

Figure 2. Relative expression of LIF (A), LIFR (B), gp130 (C) and SOCS1 (D) mRNA in mid-secretory human endometria from fertile (Control) women compared with women with unexplained infertility (Infertile); *n*=3 in each group.

Figure 3. Immunostaining of LIF, LIFR, gp130 and SOCS1 in human endometrium. All pictures are from endometria obtained during the mid-secretory phase. A. Staining of LIF in endometrium from a fertile woman. B. Staining of LIF in endometrium from a woman with unexplained infertility. C. Negative control.
D. Staining of LIFR in endometrium from a fertile woman. E. Staining of LIFR in endometrium from a woman with unexplained infertility. F. Negative control.
G. Staining of gp130 in endometrium from a fertile woman. H. Staining of gp130 in endometrium from a woman with unexplained infertility. I. Negative control.

J. Staining of SOCS1 in endometrium from a fertile woman. K. Staining of SOCS1 in endometrium from a woman with unexplained infertility. L. Negative control. *Magnification ×40*.

Table 1. Characterization of patients undergoing assisted reproduction treatment. Data are presented as the mean ± standard error of the mean (SEM). Significance was accepted at the 0.05 level.

Type of infertility	n	Age	BMI	Pregnancy	Para	Menses duration	Cycle length	IVF trials	ICSI trials	% IVF cycles	% ICSI cycles
Male factor	17	34.7±3.1	23.0±3.0	0.7±0.9	0.2±0.4	5.1±1.2	28.5±2.1	2.3±1.9	3.1±1.7	19.4	80.6
Unexplained	10	33.6±4.0	21.6±2.5	0.4±0.6	0.6±0.3	4.8±0.8	28.9±2.0	1.7±1.3	1.3±1.5	90.3	9.7

Table 2. Primer sequences used in real-time PCR

Gene	Sense primer 5‘ – 3‘	Antisense primer 5‘ – 3‘	size (bp)
GAPDH	GAA GGT GAA GGT CGG AGT CAA C	CAG AGT TAA AAG CAG CCC TGG T	71
LIF	TGA ACC AGA TCA GGA GCC AAC T	CCA CAT AGC TTG TCC AGG TTG TT	115
LIFR	GTG GCA GTG GCT GTC ATT GTT GGA GTG GT	TCA TCT GCG GCT GGG TTT GGT ATT TCT TC	356
SOCS1	GTG GCA GCC GAC AAT GCA GT	CGA GGC CAT CTT CAC GCT AAG G	345
Gp130	CTG TAT CAC AGA CTG GCA ACA AG	GCA TTT GCT CTC TGC TAA GTT CC	79

Table 3. Presence of pinopodes, visualized using scanning electron microscopy, on the endometrial surface in control and infertile women.

Group of women	Total no. of samples	No. of samples with pinopodes	Percentage with pinopodes
Fertile	*n*=17	*n*=15	88%
Unexplained infertility	*n*=14	*n*=8	57%

Table 4. Numbers of mid-secretory phase biopsy samples from fertile women and from women with unexplained infertility showing strong immunostaining of LIF, LIFR, gp130 and SOCS1. LE – luminal epithelium; GE – glandular epithelium.

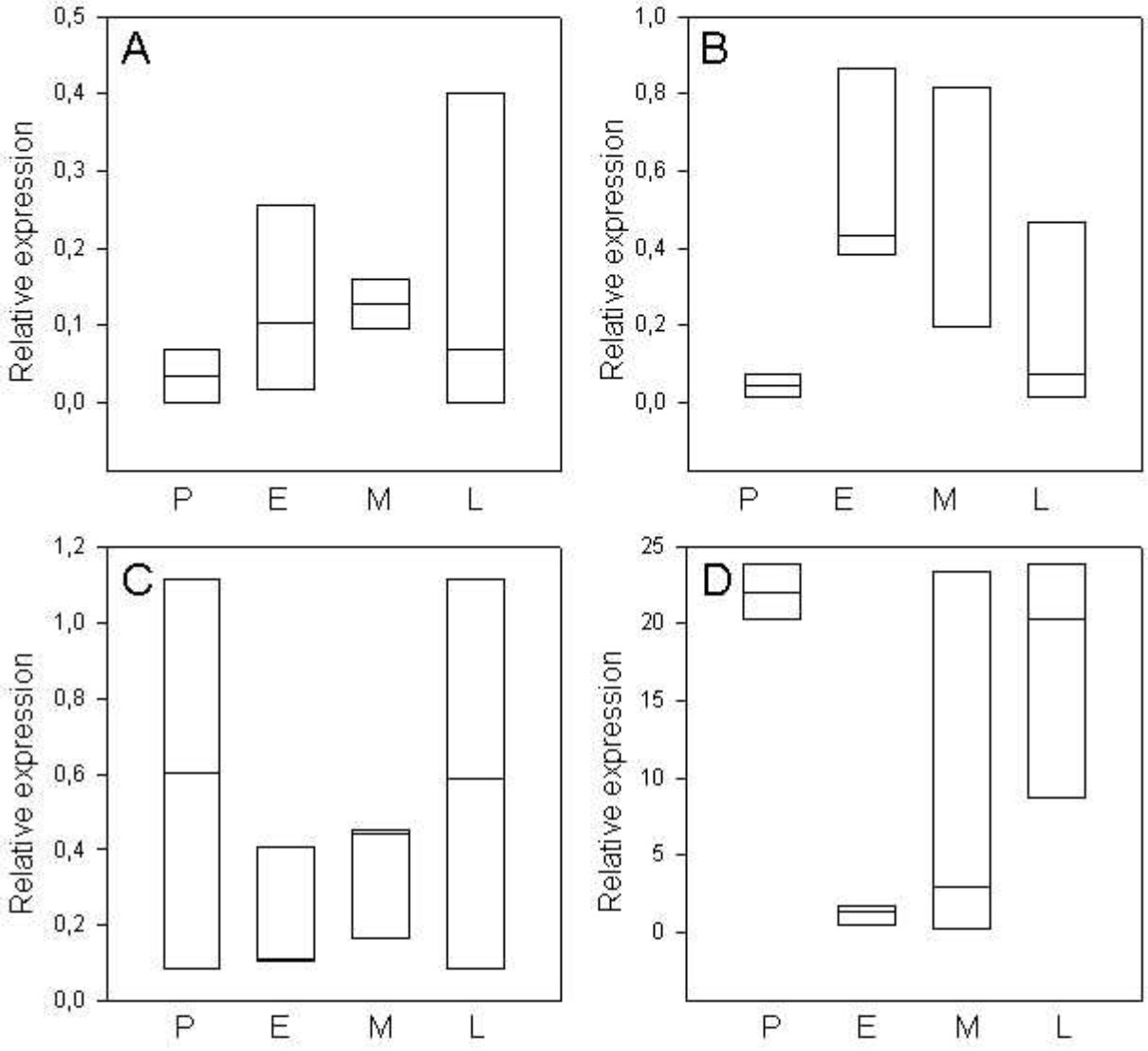
Group	No. of samples	No. of samples with strong staining in LE	No. of samples with strong staining in GE
		LIF	LIF
Fertile	*n*=14	*n*=12 (85%)	*n*=14 (100%)
Infertile	*n*=14	*n*=9 (64%)	*n*=11 (78%)
		LIFR	LIFR
Fertile	*n*=12	*n*=12 (100%)	*n*=12 (100%)
Infertile	*n*=14	*n*=4 (28%)	*n*=5 (35 %)
		gp130 apical staining	gp130 apical staining
Fertile	*n*=11	*n*=11 (100%)	*n*=9 (81%)
Infertile	*n*=14	*n*=2 (14%)	*n*=0 (0%)
		gp130 cytoplasmic staining	gp130 cytoplasmic staining
Fertile	*n*=11	*n*=10 (91%)	*n*=5 (45%)
Infertile	*n*=14	*n*=5 (35%)	*n*=4 (28%)
		SOCS1 apical staining	SOCS1 apical staining
Fertile	*n*=14	*n*=14 (100%)	*n*=12 (85%)
Infertile	*n*=14	*n*=11 (78%)	*n*=12 (85%)
		SOCS1 cytoplasmic staining	SOCS1 cytoplasmic staining
Fertile	*n*=13	*n*=0 (0%)	*n*=0 (0%)
Infertile	*n*=14	*n*=4 (28%)	*n*=13 (93%)

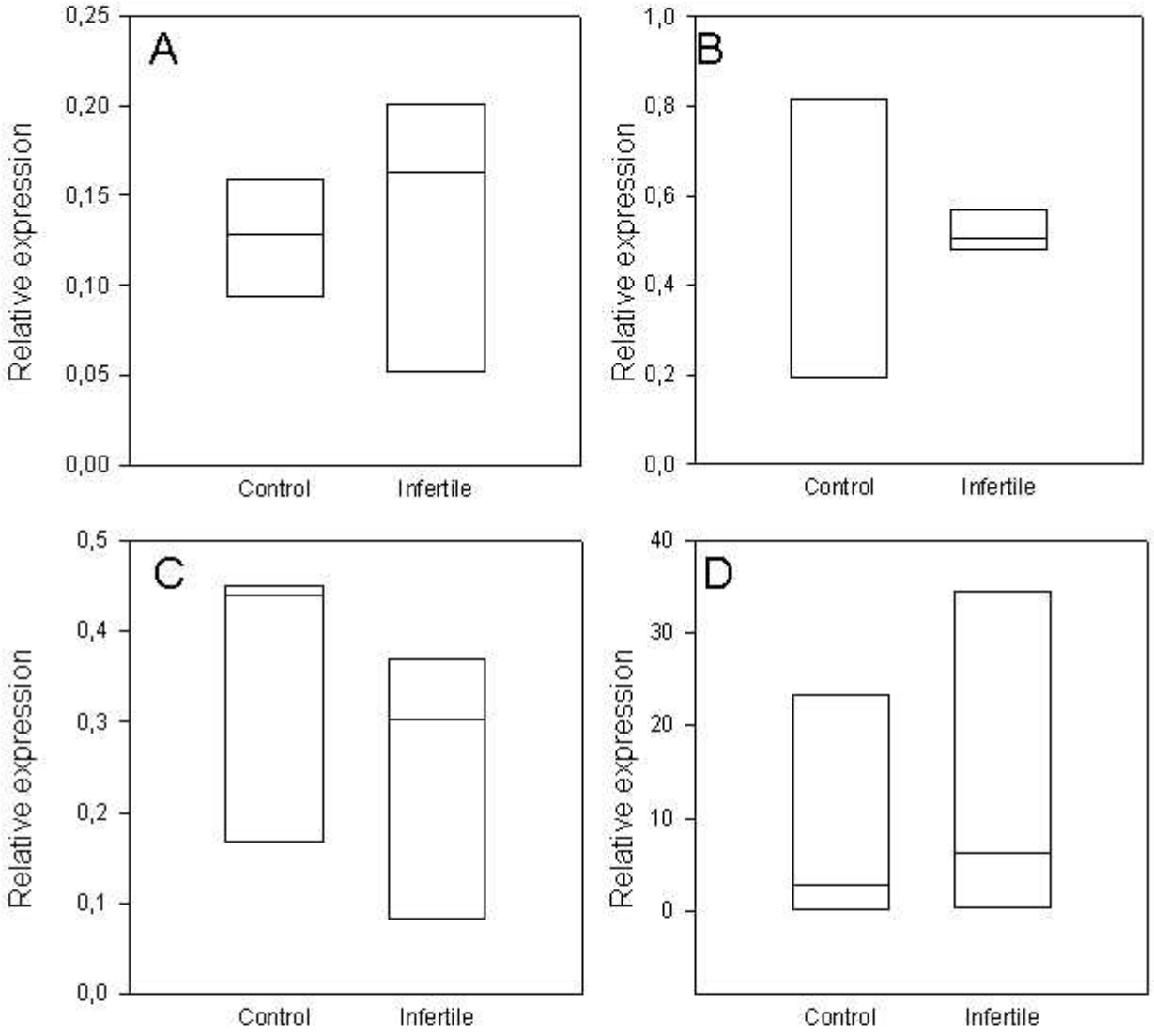
Table 5. Semi-quantitative evaluation of immunostaining of healthy human endometrium with anti-human gp130 and SOCS1 antibodies.

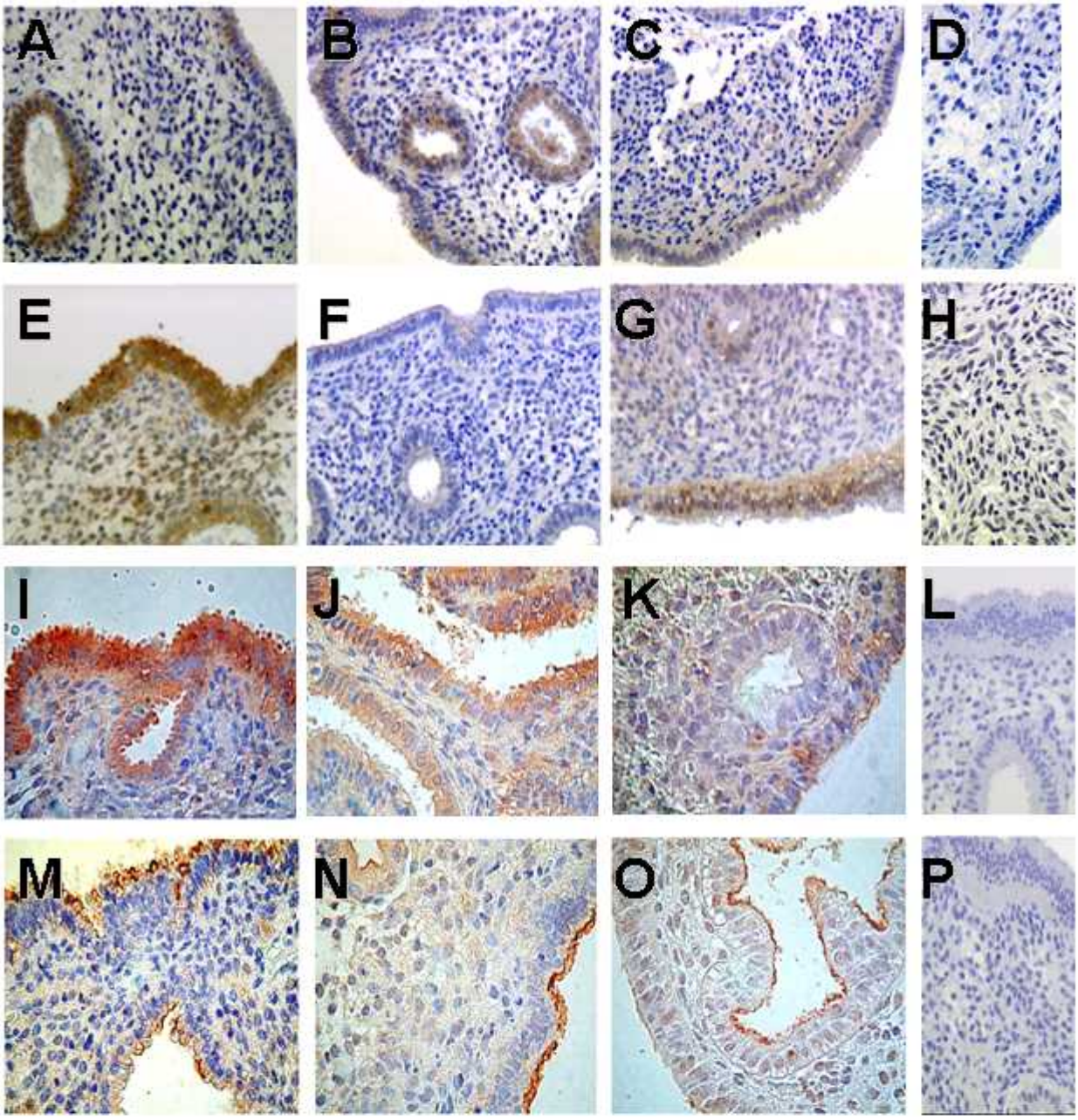
Antibody		Glandular epithelium				Luminal epithelium			
		P	E	M	L	P	E	M	L
gp130	surface	0.5 (0.0-1.5)	0.5 (0.0-1.5)	2.0 (1.0-2.5) [a]	1.0 (0.5-2.0)	0.5 (0.0-2.0)	0.5 (0.0-2.0) [ab]	2.0 (1.5-3.0)	1.0 (0.0-3.0)
	cytoplasm	0.5 (0.0-2.0)	0.5 (0.0-2.0) [a]	1.0 (1.0-2.0)	2.0 (2.0)	0.5 (0.0-2.0)	0.5 (0.0-2.0)	2.0 (1.0-3.0)	2.5 (2.5-3.0)
SOCS1	surface	2.0 (1.5-3.0)	2.0 (0.5-2.5)	2.0 (1.0-2.5)	2.0 (1.5-3.0)	2.0 (1.0-3.0)	2.0 (0.0-3.0)	3.0 (1.0-3.0)	2.0 (1.0-3.0)
	cytoplasm	0.5 (0.0-1.0)	1.0 (0.0-2.0)	0.5 (0.0-2.0)	1.0 (0.0-2.5)	1.0 (0.0-1.5)	1.0 (0.0-2.5)	0.5 (0.0-1.5)	1.0 (0.0-1.5)

[a] - statistically significant difference compared with late secretory phase (p<0.04); [b] - statistically significant difference compared with mid-secretory phase (p<0.05).

P – proliferative phase, E – early secretory phase, M – mid-secretory phase, L – late secretory phase.


A
B
C
D
Relative expression
0,5
0,4
0,3
0,2
0,1
0,0
1,0
0,8
0,6
0,4
0,2
0,0
1,2
1,0
0,8
0,6
0,4
0,2
0,0
25
20
15
10
5
0
P
E
M
L


A
B
C
D
Relative expression
0,25
0,20
0,15
0,10
0,05
0,00
1,0
0,8
0,6
0,4
0,2
0,0
0,5
0,4
0,3
0,2
0,1
0,0
40
30
20
10
0
Control
Infertile


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P