Molecular Human Reproduction Vol.14, No.8 pp. 475–484, 2008
Advance Access publication on June 6, 2008 doi:10.1093/molehr/gan036

Gene expression in cultured endometrium from women with different outcomes following IVF

Nick A. Bersinger[1], Dorothea M. Wunder, Martin H. Birkhäuser and Michael D. Mueller

Department of Obstetrics and Gynaecology, Inselspital, Berne University Hospital, University of Berne, Murtenstrasse 35, Berne CH-3010, Switzerland

[1]Correspondence address. Fax: +41-31-63-20953; E-mail: nick.bersinger@dkf.unibe.ch

Estradiol and progesterone are crucial for the acquisition of receptivity and the change in transcriptional activity of target genes in the implantation window. The aim of this study was to differentiate the regulation of genes in the endometrium of patients with recurrent implantation failure (IF) versus those who became pregnant after *in vitro* fertilization (IVF) treatment. Moreover, the effect of embryo-derived factors on endometrial transcriptional activity was studied. Nine women with known IVF outcome (IF, M, miscarriage, OP, ongoing pregnancy) and undergoing hysteroscopy with endometrial biopsy were enrolled. Biopsies were taken during the midluteal phase. After culture in the presence of embryo-conditioned IVF media, total RNA was extracted and submitted to reverse transcription, target cDNA synthesis, biotin labelling, fragmentation and hybridization using the Affymetrix Human Genome U133A 2.0 Chip. Differential expression of selected genes was re-analysed by quantitative PCR, in which the results were calculated as threshold cycle differences between the groups and normalized to Glyceraldehyde phosphate dehydrogenase and β-actin. Differences were seen for several genes from endometrial tissue between the IF and the pregnancy groups, and when comparing OP with M, 1875 up- and 1807 down-regulated genes were returned. Real-time PCR analysis confirmed up-regulation for somatostatin, PLAP-2, mucin 4 and CD163, and down-regulation of glycodelin, IL-24, CD69, leukaemia inhibitory factor and prolactin receptor between Op and M. When the different embryo-conditioned media were compared, no significant differential regulation could be demonstrated. Although microarray profiling may currently not be sensitive enough for studying the effects of embryo-derived factors on the endometrium, the observed differences in gene expression between M and OP suggest that it will become an interesting tool for the identification of fertility-relevant markers produced by the endometrium.

Keywords: endometrium; gene profiling; IVF; miscarriage; implantation failure

Introduction

Two conditions have to be met in order to obtain a pregnancy: good embryo quality and high endometrial receptivity. Successful implantation is only possible during a very short phase within the luteal phase, the so-called 'implantation window' which appears to be of about 5 days in duration (Days 20–24 in a physiological menstrual cycle) (Bergh *et al*., 1992). The two hormones estradiol (E_2) and progesterone are crucial for the acquisition of receptivity and the change in transcription activity of target genes (Wilcox *et al*., 1999). Previously these genes were investigated one by one for their role in implantation. The first reports studying the implantation window globally with a large-scale genomic array (Affymetrix®) compared the expression profiles in endometrium between the late proliferative with the midluteal phase (Kao *et al*., 2002) or between days LH+2 and LH+7 in spontaneous ovulatory cycles (Riesewijk *et al*., 2003). In this latter investigation performed with the HG-U95A array, 211 genes yielded a fold change of 3 or higher between the two time points. For many of these a role in the context of endometrial receptivity or even fertility had not been previously described, but the strongest transcriptional up-regulation (107-fold) was observed for glycodelin A (GdA, synonyms PP14, PEP, PAEP). It is a secretory epithelial endometrial product (Mueller *et al*., 2000) whose serum levels are high in the late luteal phase (after the progesterone peak) of the menstrual cycle and steeply decline thereafter (Joshi *et al*., 1982). Endpoint polymerase chain reaction (PCR) and subsequent densitometric analysis yielded a 3.1-fold glycodelin gene up-regulation in the endometrium during the implantation window when compared with the late follicular phase tissue (Kao *et al*., 2002). Another microarray study, involving an almost identical protocol (day LH+8 versus LH+3 in spontaneous cycle) and the same chip (U95A), confirmed the validity of the method and identified 107 differentially expressed genes (Mirkin *et al*., 2005). At the same time the Spanish/Dutch team from the first U95A study (Riesewijk *et al*., 2003) obtained gene expression profiles from endometrium after controlled ovarian stimulation (COS) with an even larger, more recently developed array (Affymetrix HG-U133A), and differential expression was again found for more than 200 genes when compared with endometrial tissue obtained in natural cycles at similar timing (Horcajadas *et al*., 2005). This was considered a surprising observation, and it was concluded that current ovarian stimulation for treatment by *in vitro* fertilization (IVF) was far from optimal, and this issue was further strengthened by a reduction (9.8-fold) of GdA mRNA in COS (IVF) compared with spontaneous cycles.

Gene transcription rates and expression profiles in patients with implantation failure (IF) and recurrent miscarriage in comparison with ongoing, uneventful pregnancy are not well investigated to

date. It has been shown that monoamino oxidase A expression was deficient in the endometrium of women with IF (Henriquez *et al*., 2006). Moreover, the genes for C4BPA, CRABP2 and OLFM1 were found to be differentially expressed in the endometrium of patients with unexplained recurrent miscarriage when compared with normal fertile women (Lee *et al*., 2007).

The endometrium is receiving signals from the embryo floating in the uterine cavity at the time of implantation, but these have not been systematically investigated due to problems of sensitivity. A recent study with gene profiling on purified endometrial stromal cells cultured in the presence and absence of trophoblast-conditioned media found 4817 genes to be differentially regulated as a function of the presence of these media (Hess *et al*., 2007).

The first aim of our study was to investigate and differentiate the transcriptional up- and down-regulation of genes in patients with recurrent IF versus those who became pregnant after IVF treatment, and particularly between miscarried and ongoing clinical pregnancies. The second aim was to concurrently study the effect of early embryo-derived factors on the transcriptional activity in the cultured endometrium at the time of implantation, by the addition of embryo-conditioned medium from successful and unsuccessful IVF cycles as a culture supplement. To our knowledge, results from studies using such an approach have not been published to date.

Materials and Methods

Patient selection

Using the clinical records from our Department, women fulfilling the following inclusion criteria for this study were selected. First, an endometrial tissue sample, biopsied using the Pipelle-de-Cornier suction curette and stored frozen (liquid nitrogen) in medium containing 10% (v/v) serum and 20% (v/v) dimethyl sulphoxide, must have been available in sufficient quantity from a previous hysteroscopic investigation for infertility and, second, the patient has been undergoing treatment by IVF and embryo transfer in our clinic, where the pre-embryo transfer embryo-conditioned media are routinely kept in store. The study protocol (collection of endometrium) was approved by the Ethical Committee of the University of Berne, and informed consent was obtained from the patients for both endometrial investigation as well as for the IVF treatment. Three IVF-embryo transfer outcome groups were defined: (A) IF as defined by a negative serum hCG (<2 mIU/ml) result between 14 and 17 days after embryo transfer; (B) implantation followed by early pregnancy (<12 weeks) loss; and (C) clinical, ongoing pregnancy. In order to obtain enough RNA after the explant culture in the presence of the different embryo-conditioned media supplements (see below) three or four patients were selected for each of these groups as it has been suggested elsewhere (Catalano *et al*., 2003), and their tissue was pooled at the time of setting up the suspension cultures (see below). The mean age of the women providing these three tissue pools was 33.7 ± 4.5 (SD) years and did not differ between the groups. No endometriosis was documented in these patients by laparoscopy. All tissues were taken during the implantation window in the luteal phase: the mean cycle day at biopsy was 23.6 ± 1.8 (SD) and the cycle length, again without difference between the three groups, was 30.0 ± 1.6 (SD) days.

IVF procedure

IVF was performed 3–4.5 months after hysteroscopy and the collection of the endometrial biopsy in all groups. COS using the 'long' protocol after GnRH agonist down-regulation, oocyte retrieval, intracytoplasmic sperm injection (Tesarik and Sousa, 1995) and embryo transfer were performed as described elsewhere (Wunder *et al*., 2005). Embryos were cultured in microdroplets (20 μl) of IVF G1 medium under mineral oil (both from Vitrolife, Kungsbacka, Sweden) until they were transferred on Day 3 after oocyte retrieval. Due to the legal constraints in Switzerland, embryos from the same patient were transferred. On the day of embryo transfer, 10 μl of this embryo-conditioned medium was collected, diluted 1:10 by the addition of 90 μl of the same medium to prevent evaporation and stored at -20°C until used as a cell culture supplement (see below). Embryo-conditioned media from the same patients providing endometrial tissue (see above) were pooled into the same groups A (IF), B (pregnancy loss) and C (ongoing pregnancy). Blank IVF G1 medium provided a fourth media pool.

Endometrial explant suspension culture

The endometrial tissue was thawed by immersion of the cryotube in a water bath (37°C), washed twice with 15 ml Dulbecco's MEM containing 25 mM Hepes, glutamine, 10% fetal bovine serum, penicillin, streptomycin and fungizone (all from Gibco-Invitrogen, Paisley, Scotland). We have decided to perform explant suspension cultures; the advantages are an intact three-dimensional environment during the culture experiment and the assurance to obtain sufficient amounts of RNA for hybridization, but with this system we would not be able to know the source (epithelial or stromal) of the mRNA. The tissue was coarsely dissected with scalpels on a sterile glass petri dish, and a suspension prepared by several gentle passages through a syringe fitted with an 18-gauge needle. The resulting explant size was such that central necrosis would not occur during the short duration of the culture experiment. The suspensions of each biopsied endometrium group (A, B or C) were divided into four cultures in a 24-well plate and 2 ml of complete medium without phenol red, with 25 mM Hepes, glutamine, 2% fetal bovine serum, penicillin, streptomycin and fungizone (all from Gibco-Invitrogen, Paisley, Scotland). After 24 h of culture (37°C, 5% CO_2 in air), the medium was replaced with the same but the serum was omitted, and after 24 h of such conditions of quiescence fresh medium containing embryo-conditioned supernatant was added: serum-free MEM (total volume 500 μl, containing 50 μl embryo medium in G1), sterile filtered through a 0.22 μm low protein absorption polyvinylidene fluoride membrane (Millipore, Volketswil, Switzerland). Each outcome-defined pool of embryo-conditioned medium (A, B, C and blank IVF G1 medium) was added to each endometrial tissue pool (A, B and C), yielding a total of 12 culture conditions. Due to the small amounts of tissue and particularly embryo media available, no replicate cultures were set up. After 24 h of culture, the suspension was centrifuged, the resulting supernatants frozen for further analyses and the endometrial cell pellets immediately subjected to total RNA extraction (see below).

Total RNA extraction

RNA was extracted from cellular pellets collected after suspension culture using the SV Total RNA on-column Isolation kit manufactured by Promega (Madison, WI, USA). The manufacturer's protocol was followed, and this included an on-column DNAse treatment step. The quantity and quality of the obtained RNA was analysed spectrophotometrically with a Nanodrop® photometer (Wilmington, DE, USA) and electrophoretically on an (Eukaryote Total) RNA Nano 6000 Chip (2100 Bioanalyser, Agilent® Technologies, Palo Alto, CA, USA). The total RNA yield per culture condition ranged between 6.17 and 14.19 μg, and the absorbance ratio (260–280 nm) for all extractions was 2.03 ± 0.09 (mean $\pm$ SD). Second elution fractions, as suggested in the extraction protocol to be added to increase yield, were discarded since their quality was consistently poorer (A260/280 ratio between 1.64 and 1.88). Both 18S and 28S fractions were seen as sharp peaks in the bioanalyser output. The RNA samples were stored at -80°C until the target synthesis and hybridization experiments were performed.

Gene array hybridization

Hybridizations were performed with the Human Genome U133A 2.0 Chip, obtained from Affymetrix Inc. (Santa Clara, CA, USA), covering 18 400 transcripts and variants including 14 500 characterized human genes. Three micrograms of total RNA per culture condition were used for target synthesis which includes reverse-transcription and synthesis of double-stranded cDNA according to the Affymetrix GeneChip Expression Analysis Technical Manual. Following phenol/chloroform extraction, the purified cDNA was used for an *in vitro* transcription reaction by using the IVT labelling kit (Affymetrix) to synthesize cRNA in the presence of a biotin-conjugated ribonucleotide analogue. An average of 196 μg of labelled cRNA from each reaction was purified on RNeasy Mini columns (Qiagen, Hilden, Germany) and average size of the cRNA molecules was assessed on RNA Nano 6000 Chips as above. The cRNA targets were incubated at 94°C for 35 min in Fragmentation buffer and the resulting fragments of 50–150 nucleotides were again monitored

using the Bioanalyser. All synthesis reactions were carried out using a PCR machine (T1 Thermocycler; Biometra, Göttingen, Germany) to ensure the highest possible degree of temperature control. The hybridization cocktail (130 μl) containing fragmented biotin-labelled target cRNA at a final concentration of 0.05 μg/μl was transferred into Affymetrix Human Genome U133A 2.0 Chips and incubated at 45°C on a rotator in a hybridization oven 640 (Affymetrix) for 16 h at 60 rpm. The arrays were washed and stained on a Fluidics Station 400 (Affymetrix) according to standard Affymetrix recommendations for performing the EukGE-WS2v4 protocol which includes an antibody amplification to increase the signal strength. The arrays were scanned according to the default settings using the GeneChip System confocal scanner (Hewlett-Packard, Santa Barbara, CA). Raw DAT image files were generated using MicroArray Suite (MAS 5, Affymetrix). Further data analysis was conducted using Gene-Spring® GX (Agilent Technologies) software.

RT and quantitative PCR of selected transcripts

From the results obtained by the automatic ranking with the Gene-Spring® software (Tables I and II), the transcripts shown in Tables III and IV were selected for quantitative investigation of the amplification either for their extent of up- or down-regulation, or for their relevance in the context of reproductive function. The total RNA samples extracted from the pelleted endometrial explant tissue after culture (0.5 μg, see above) were reverse transcribed using the Moloney murine leukaemia virus enzyme (M-MLV, Gibco-Invitrogen, Paisley, Scotland, final concentration 10 U/μl, total sample volume 20 μl). Quantitative PCR was performed with cDNA from 10 ng RNA per well, in duplicate, using the Taqman® system and Assays-on-Demand® with the primers/probe samples (single-tube system) listed in Tables III and IV on a Model ABI-7900 sequence detector (all from Applied Biosystems, Foster City, CA, USA). Threshold cycle (Ct) plot data were subsequently normalized for each marker and each cluster comparing the different endometrial tissues or the different embryo-conditioned media used in culture. Glyceraldehyde phosphate dehydrogenase (GAPDH, Hs0099999905_m1) and β-actin (Hs00242273_m1) were used as a housekeeping gene control.

Results

Automatic screening of the raw data identified the genes which were found to be up- or down-regulated by a factor of 2 or more depending on the arrays that were compared (endometria or medium supplement; ongoing pregnancy, miscarriage or IF). These genes, with their factor of up- or down-regulation, are shown in Table I. The data were then analysed by the Genespring SX software; this returned the clustering for the comparison of the different endometrial tissues such as IF (group A), miscarriage (group B) and healthy ongoing pregnancy (group C) on one hand, and of the different embryo media added to the culture (blank G1 medium, groups A, B and C as above) on the other. The clusters for the endometria are shown in Fig. 1. The system identified 12 different sets of patterns with moderate or strong up- or down-regulation. These differences were seen either for all pregnancies when compared with IFs (groups B+C versus A, e.g. sets 3, 5 7 and 11) or, more importantly for the aim of this project which is the identification of women with recurrent miscarriage, between groups C versus B (sets 2, 4, 8, 9, 10 and 12). Sets with only small variations (sets 1, 2 and 6) were discarded from the analysis. Similarly, those with an up- or down-regulation for both groups A and C versus group B (sets 10 and 11) were removed. The most interesting clusters were thus identified in sets 4 (702 genes up-regulated but not further analysed due to a marginally lower expression level in group B compared with A), 8 (1807 genes down-regulated), 9 (983 genes up-regulated) and particularly 12 with fewer genes (190) than in the others but with the strongest up-regulation.

Real-time PCR analysis of genes selected from the array results (Tables I and II) and listed in the corresponding Tables III and IV confirmed significant up-regulation in endometrial tissue from implantation cycles in comparison with IFs for CD163 ($P = 0.002$) and, to a lesser extent, PLAP-2 ($P = 0.049$, Fig. 2). More interestingly, when comparing the difference in transcriptional activity between ongoing pregnancies and miscarriages (groups C versus B, Fig. 3), strong differences were observed for somatostatin, PLAP-2, mucin 4 and CD163 were found (all increased in group C, $P < 0.001$). Many of the other studied molecules were down-regulated between pregnancy and IF endometrium (Fig. 2). For collagen IV, CEACAM-1, LIF and fibronectin no difference was observed between groups with successful and failed implantation (Fig. 2). IL-24, CD69, LIF, prolactin receptor (PRL-R) and glycodelin were strongly down-regulated between endometrial tissue from women with ongoing and miscarried pregnancies (all $P < 0.001$, Fig. 3). Laminin and fetuin B, on the other hand, distinguished between implantation and failure groups (Fig. 2) but did not depend on the outcome of an initially established pregnancy (Fig. 3). For Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) the findings were inconsistent; a limited up-regulation of this gene was observed in the ongoing pregnancy in comparison with the miscarried group, but this was not the case in the combined implantation (B+C) versus the non-implantation group (A). When the different embryo-conditioned media were compared between these groups, no significant up- or down-regulation could be confirmed by real-time PCR (data not shown).

Discussion

The up- or down-regulation of the genes selected from our microarray experiment results (Tables III and IV) could be largely confirmed by our real-time RT-PCR experiments, though the extent of the variation did not necessarily reflect the factor that was returned by the automatic (Gene-Spring®) analysis of the array hybridization. In particular, highly significant ($P = 0.0001$ or lower) up- or down-regulations between endometrial tissues from women with ongoing compared with miscarried pregnancies were confirmed in RT-PCR for somatostatin (up), PLAP-2 (up), Mucin-4 (up), glycodelin (down), IL-24 (down), CD69 (down) and LIF (down).

Leukaemia inhibitory factor (LIF) and glycodelin (PP14, PAEP) are of particular interest in the context of implantation. LIF had been suggested to play a role in the embryo implantation process in mice (Stewart *et al*., 1992), but such a function had not been directly confirmed in the human, and the information in the literature is not consistent. LIF production by cultured endometrial epithelial cells has been demonstrated (Laird *et al*., 1997), and uterine flushings at the time of implantation (LH+7 to LH+10) contained more LIF protein in fertile women than in those with unexplained infertility (Laird *et al*., 1997). In this study we could not confirm, using quantitative PCR, the differential gene expression for LIF observed in the array hybridization experiment between implantation versus non-implantation cycles (Fig. 2). Similarly, in cultured endometrial explants (thus also taking the full tissue into account) no differences in LIF protein production between fertile and infertile women were found in one study (Delage *et al*., 1995), while in another this was the case in the midluteal (but not in the proliferative) phase (Hambartsoumian E, 1998). On the other hand, a more recent study on uterine flushings towards the end of the cycle (Day 26) reported lower LIF concentrations in subsequently pregnant women (Lédée *et al*., 2002), which would be in better agreement with our observations made in this study and the reduced gene expression observed previously in the endometrium (Horcajadas *et al*., 2005). The contradictory observations with immuno-analytical LIF protein determinations in uterine flushings could be explained by cycle-dependent secretion variations between fertile and infertile women, or simply by the

Table I. List of genes, up- or down-regulated by a factor of at least 2.0 between different endometrial tissues (in alphabetical order).

ORIGINAL SOURCE FILE DATA		Outcome Group		
Gene	Gene Name	C versus B	C versus A	B versus A
Up-regulation, >2				
210431_at	alkaline phosphatase, placental-like 2	14.53		
208588_at	apoptosis inhibitor		4.73	2.70
210828_s_at	aryl hydrocarbon receptor nuclear translocator		2.36	
206576_s_at	carcinoembryonic antigen-related cell adhesion molecule 1	5.07		
211883_x_at	carcinoembryonic antigen-related cell adhesion molecule 1	5.47	8.91	
201884_at	carcinoembryonic antigen-related cell adhesion molecule 5	9.59		
215049_x_at	CD163 antigen		3.01	
208653_s_at	CD164 antigen, sialomucin		2.94	
207315_at	CD226 antigen		4.25	2.43
203507_at	CD68 antigen		2.07	
214049_x_at	CD7 antigen (p41)		2.19	
211189_x_at	CD84 antigen (leukocyte antigen)		2.48	
203757_s_at	CEA-related cell adhesion molecule 6		3.67	
202310_s_at	collagen, type I, alpha 1		2.80	
202311_s_at	collagen, type I, alpha 1		2.94	
202403_s_at	collagen, type I, alpha 2		3.32	2.07
202404_s_at	collagen, type I, alpha 2		4.16	
213992_at	collagen, type IV, alpha 6		2.06	
213622_at	collagen, type IX, alpha 2		3.53	2.50
212488_at	collagen, type V, alpha 1		2.18	
212489_at	collagen, type V, alpha 1		2.79	
221729_at	collagen, type V, alpha 2		4.20	
221730_at	collagen, type V, alpha 2		2.93	2.23
212091_s_at	collagen, type VI, alpha 1		2.43	
212940_at	collagen, type VI, alpha 1		2.45	
213428_s_at	collagen, type VI, alpha 1		2.20	
201438_at	collagen, type VI, alpha 3		4.06	2.99
211343_s_at	collagen, type XIII, alpha 1		3.25	2.11
211809_x_at	collagen, type XIII, alpha 1		2.36	
207802_at	cysteine-rich secretory protein 3		7.60	
201325_s_at	epithelial membrane protein 1		5.32	
209365_s_at	extracellular matrix protein 1		4.18	
210521_s_at	fetuin B	5.34		
216377_x_at	human placental alkaline phosphatase-like gene 5′ region	4.61		
210141_s_at	inhibin, alpha	2.25	4.52	2.01
209540_at	insulin-like growth factor 1 (somatomedin C)			2.06
203851_at	insulin-like growth factor binding protein 6		3.97	2.24
204989_s_at	integrin, beta 4		2.13	
209827_s_at	interleukin 16 (lymphocyte chemoattractant factor)		2.58	
209792_s_at	kallikrein 10		4.03	
210089_s_at	laminin, alpha 4		2.43	
210990_s_at	laminin, alpha 4		13.50	7.47
208949_s_at	lectin, galactoside-binding, soluble, 3 (galectin 3)		2.76	
202291_s_at	matrix Gla protein		7.76	
203876_s_at	matrix metalloproteinase 11 (stromelysin 3)		4.39	2.26
203878_s_at	matrix metalloproteinase 11 (stromelysin 3)		13.06	
204580_at	matrix metalloproteinase 12 (macrophage elastase)		9.42	6.30
220541_at	matrix metalloproteinase 26	9.13	7.01	
210015_s_at	microtubule-associated protein 2	8.68	6.92	
204895_x_at	mucin 4, tracheobronchial	17.42		
220158_at	placental protein 13-like protein		3.39	
208131_s_at	prostaglandin I2 (prostacyclin) synthase		7.40	
213921_at	somatostatin	43.93	13.04	
203145_at	Sperm-associated antigen 5		4.02	2.58
203167_at	tissue inhibitor of metalloproteinase 2		3.45	
201147_s_at	tissue inhibitor of metalloproteinase 3		2.25	
201148_s_at	tissue inhibitor of metalloproteinase 3		4.76	3.42
203085_s_at	transforming growth factor, beta 1 (Camurati-Engelmann disease)		4.66	2.71
201506_at	transforming growth factor, beta-induced, 68kDa		7.53	
Down-regulation, <0.5				
205357_s_at	angiotensin II receptor, type 1		0.421	
209795_at	CD69 antigen (p60, early T-cell activation antigen)	0.388	0.349	
204533_at	chemokine (C-X-C motif) ligand 10		0.382	0.215
207442_at	colony stimulating factor 3 (granulocyte)			0.473
203591_s_at	colony stimulating factor 3 receptor (granulocyte)		0.274	0.434
206504_at	cytochrome P450, family 24, subfamily A, polypeptide 1		0.109	
217245_at	early lymphoid activation protein		0.194	

Continued

Table I. *Continued*

ORIGINAL SOURCE FILE DATA		Outcome Group		
Gene	Gene Name	C versus B	C versus A	B versus A
206101_at	extracellular matrix protein 2, female organ and adipocyte specific		0.420	
205782_at	fibroblast growth factor 7 (keratinocyte growth factor)	0.335	0.297	
219250_s_at	fibronectin leucine rich transmembrane protein 3	0.329		
207345_at	follistatin		0.423	
206859_s_at	glycodeln / PP14	0.146	0.148	
206010_at	hyaluronan binding protein 2	0.464		
202410_x_at	insulin-like growth factor 2 (somatomedin A)	0.208	0.143	
205302_at	insulin-like growth factor binding protein 1		0.172	0.408
212143_s_at	insulin-like growth factor binding protein 3		0.430	
208084_at	integrin, beta 6		0.447	
208261_x_at	interferon, alpha 10		0.335	0.405
204415_at	interferon, alpha-inducible protein (clone IFI-6–16)		0.425	0.335
209417_s_at	interferon-induced protein 35		0.325	
214453_s_at	interferon-induced protein 44		0.372	0.46
205067_at	interleukin 1, beta		0.447	
206926_s_at	interleukin 11	0.336	0.282	
219115_s_at	interleukin 20 receptor, alpha		0.158	0.480
206569_at	interleukin 24	0.387	0.320	
205207_at	interleukin 6 (interferon, beta 2) 0.425			
208193_at	interleukin 9 0.145 0.231			
205266_at	leukaemia inhibitory factor (cholinergic differentiation factor)	0.291	0.260	
203101_s_at	mannosyl (alpha-1,6-)-glycoprotein beta-1,2-GlcNAc-transferase			0.361
203365_s_at	matrix metalloproteinase 15 (membrane-inserted)	0.325	0.254	
204574_s_at	Matrix metalloproteinase 19		0.500	
204259_at	matrix metalloproteinase 7 (matrilysin, uterine)		0.366	
214952_at	neural cell adhesion molecule 1			0.210
209652_s_at	placental growth factor, VEGF-related protein		0.437	
201981_at	pregnancy-associated plasma protein A		0.413	
205220_at	putative chemokine receptor		0.320	
219140_s_at	Retinol binding protein 4, plasma		0.118	0.179
209719_x_at	serine (or cysteine) proteinase inhibitor B (ovalbumin), member 3		0.076	
210413_x_at	serine (or cysteine) proteinase inhibitor B (ovalbumin), member 4		0.118	
211906_s_at	serine (or cysteine) proteinase inhibitor B (ovalbumin), member 4		0.054	
218681_s_at	stromal cell-derived factor 2-like 1	0.478		
206990_at	tenascin R (restrictin, janusin)			0.483
220462_at	TGF-beta induced apotosis protein 2			0.145
205599_at	TNF receptor-associated factor 1		0.269	
207332_s_at	transferrin receptor (p90, CD71)		0.415	
206943_at	TGF, beta receptor I (activin A receptor type II-like kinase, 53kDa)		0.378	
202688_at	tumour necrosis factor (ligand) superfamily, member 10		0.492	
209499_x_at	tumour necrosis factor (ligand) superfamily, member 13	0.438	0.316	
210314_x_at	tumour necrosis factor (ligand) superfamily, member 13		0.405	
211495_x_at	tumour necrosis factor (ligand) superfamily, member 13		0.449	

Outcome group A, implantation failure; B, implantation occurred but pregnancy miscarried before 12 weeks of gestation; C, successful ongoing pregnancy.

different antibodies that had been used in the two studies (Laird *et al*., 1997; Lédée *et al*., 2002). LIF is highly glycosylated and comes in three independently regulated transcripts; these may have been recognized to varying degrees by the different antibodies due to epitope masking. Gene expression studies, like the one presented here, overcome this problem but do not give us information on the biological function of the protein in fertility and implantation regulation. What is new and has not been reported to date is the significant difference in LIF gene expression between ongoing and miscarried pregnancies (Fig. 3), with levels more than eightfold ($=2^{3.03}$) lower in the women whose gestations went successfully to term. It is therefore necessary not only to determine LIF gene expression quantitatively with PCR in a larger group of women, but also to measure the different LIF protein transcripts with a high sensitivity immunoassay as a function of the menstrual cycle.

For glycodelin, not only an immunosuppressive (Vigne *et al*., 2001) but also a contraceptive role has been proposed (Bolton *et al*., 1987; Oehninger *et al*., 1995). While the former role favours embryo implantation, the latter one is acting against. These two functions were suggested to depend on the glycosylation pattern (Dell *et al*., 1995), and it is thus possible that these two are produced in a sequential manner, i.e. the immunosuppresive component acting at the moment of embryo implantation and the contraceptive action functioning outside the implantation window (Seppala *et al*., 1997). Our glycodelin findings in this study (reduced transcription levels in successful pregnancies), the proposed contraceptive action of this molecule under defined conditions, and the up-regulation of glycodelin in the presence of 2,3,7,8-TCDD (dioxin) which we have previously demonstrated (Mueller *et al*., 2005), are thus in agreement. Reports on serum glycodelin levels in the literature are not easy to interpret, and no normal day-to-day cycle levels obtained with recent immunoanalytical methodology are available. In one study (Wood *et al*., 1990), serum glycodelin concentrations did not differ during the implantation phase between successful and unsuccessful outcome in assisted conception cycles. In another one (Suzuki *et al*., 2000) which used a new enzyme immunoassay method, increased levels in implantation IVF cycles were observed 8 days after embryo transfer when compared with IF cycles. On the other hand, serum glycodelin

Table II. List of genes, up- or down-regulated by a factor of at least 2.0 between different supplemented embryo-conditioned media or medium blank (G1).

ORIGINAL SOURCE FILE DATA		Outcome Group			
Gene	Gene Name	C versus B	C versus G1	B versus G1	A versus G1
Up-regulation, >2					
210081_at	advanced glycosylation endproduct-specific receptor			4.96	
222257_s_at	angiotensin I CE (peptidyl-dipeptidase A) 2	3.76			
203645_s_at	CD163 antigen		4.06		
205758_at	CD8 antigen, alpha polypeptide (p32)	2.27			
205114_s_at	chemokine (C-C motif) ligand 3			2.02	2.00
220351_at	chemokine (C-C motif) receptor-like 1	3.29			
218975_at	collagen, type V, alpha 3				2.13
202533_s_at	dihydrofolate reductase	3.27			
220630_s_at	eosinophil chemotactic cytokine	4.83			
222112_at	EGF receptor substrate EPS15R			7.78	
214701_s_at	fibronectin 1	2.30			
211414_at	Glutaminase	3.79			
211372_s_at	Human soluble type II IL-1 receptor mRNA		2.216	2.96	2.21
209540_at	insulin-like growth factor 1 (somatomedin C)			3.03	2.67
204949_at	intercellular adhesion molecule 3			2.14	2.58
214569_at	interferon, alpha 5		3.323	3.17	3.07
207901_at	interleukin 12B (natural killer cell stimulatory factor 2)	2.18			
211516_at	interleukin 5 receptor, alpha	2.07		12.25	
204584_at	LC1 adhesion molecule			5.17	2.32
203876_s_at	matrix metalloproteinase 11 (stromelysin 3)			4.62	
203930_s_at	microtubule-associated protein tau			8.41	8.21
210289_at	N-acetyltransferase 8 (camello like)			18.27	
210355_at	parathyroid hormone-like hormone	2.26			
216638_s_at	prolactin receptor	3.34			
208205_at	protocadherin alpha 3	2.90			
206664_at	sucrase-isomaltase		8.675	6.11	8.51
214034_at	type 1 TNF receptor shedding aminopeptidase regulator			4.44	2.41
Down-regulation, <0.5					
208218_s_at	activin A receptor, type IB			0.353	0.138
206112_at	ankyrin repeat domain 7				0.125
205467_at	caspase 10, apoptosis-related cysteine protease				0.440
203645_s_at	CD163 antigen	0.475			
211189_x_at	CD84 antigen (leukocyte antigen)				0.331
210945_at	collagen, type IV, alpha 6	0.464			
214587_at	collagen, type VIII, alpha 1		0.245	0.236	0.373
208399_s_at	endothelin 3		0.222	0.160	0.362
203193_at	estrogen-related receptor alpha				0.396
205829_at	hydroxysteroid (17-beta) dehydrogenase 1	0.226			
203820_s_at	IGF-II mRNA-binding protein 3				0.465
208402_at	IL-17 (cytotoxic T-lympho-assoc'd serine esterase 8)		0.094		0.124
216190_x_at	integrin beta 1 (fibronectin receptor, antigen CD29)				0.272
205718_at	integrin, beta 7			0.353	
221165_s_at	interleukin 22				0.494
207538_at	interleukin 4				0.417
207952_at	interleukin 5 (colony-stimulating factor, eosinophil)		0.405		0.346
214270_s_at	MT-associated protein, RP/EB family, member 3	0.184			

Outcome group A, implantation failure; B, implantation occurred but pregnancy miscarried before 12 weeks of gestation; C, successful ongoing pregnancy; G1, blank medium control (Vitrolife G1 medium).

concentrations were found to be lower in IVF cycles leading to pregnancy before the initiation of the ovarian stimulation (Day 2 of the treatment cycle) (Andersen *et al*., 1992). In the study presented here, we are observing reduced glycodelin transcription levels even in a different cycle than the one in which the women achieved a successful IVF pregnancy; this is in agreement with the study by Andersen *et al*. (1992) and will increase the value of an endometrial biopsy for fertility investigations. It is not possible, however, to distinguish between glycosylation isoforms in transcription profiling for obvious reasons.

The structural, matrix constituent proteins yielded less consistent results. For laminin (α4), discordant observations between the array hybridization (up 13.5-fold) and real-time PCR (significantly down by $2^{1.04}$ fold, $P = 0.003$) were made for ongoing pregnancy endometrium versus IF. We have also observed, in the microarray, up-regulations between 2.1- and 4.2-fold for various forms of collagen between implantation and non-implantation endometria. Mirkin *et al*. (2005) also reported a 2.4-fold up-regulation for collagen IV between the midluteal and the early luteal phase in a spontaneous cycle. In quantitative PCR, however, we were unable to demonstrate an expression difference between implantation and non-implantation endometrial tissue and, when comparing endometrium from ongoing with miscarried pregnancy patients, we found a significantly decreased expression level.

For somatostatin no biological function in relation to infertility and the endometrium is known to date. Somatostatin is present in pre-ovulatory follicular fluid, but no association with follicular size, fertility parameters or embryo morphology was noted (Holst *et al*., 1994), and

Table III. Selected markers characterized for their up- or down-regulation between different endometrial tissues, as a function of subsequent pregnancy, in explant culture.

Marker name	Outcome Group	Factor	AoD
Up-regulated		Fold up	
Somatostatin	C versus B	43.92	Hs00356144_m1
Mucin-4	C versus B	17.42	Hs00366414_m1
PLAP-2a	C versus B	14.53	Hs00741068_g1
Fetuin B	C versus B	5.35	Hs00608480_m1
CEACAM-1b	C versus B	5.07	Hs00236077_m1
Laminin, _4	C versus A	13.50	Hs00158588_m1
CD163 antigen	C versus A	3.01	Hs00174705_m1
Down-regulated		Fold down	
Glycodelin / PP14	C versus B	−6.74	Hs00171462_m1
LIFc	C versus B	−3.44	Hs00171455_m1
Interleukin-24	C versus B	−2.59	Hs00169533_m1
CD69 antigen	C versus B	−2.58	Hs00156399_m1

Outcome group A, implantation failure; B, implantation occurred but pregnancy miscarried before 12 weeks of gestation; C, successful ongoing pregnancy.
[a]Alkaline phosphatase, placental-like 2.
[b]Carcinoembryonic antigen-related cell adhesion molecule-1.
[c]Leukaemia inhibitory factor.

Table IV. Selected markers characterized for their up- or down-regulation between different embryo-conditioned media, depending on outcome, added in explant culture.

Marker name	Outcome Group	Factor	AoD
Up-regulated		Fold up	
Prolactin receptor	C versus B	3.34	Hs00168739_m1
Fibronectin 1	C versus B	2.30	Hs00277509_m1
CD8 antigen (p32)	C versus B	2.27	Hs00233520_m1
Down-regulated		Fold down	
CD163 antigen	C versus B	−2.11	Hs00174705_m1
Collagen IV _6	C versus B	−2.16	Hs00361494_m1

Outcome group A, implantation failure; B, implantation occurred but pregnancy miscarried before 12 weeks of gestation; C, successful ongoing pregnancy.

there are no recent publications in this context in the literature. It is difficult to speculate on a pregnancy-promoting function of somatostatin at this stage. The hormone has recently been found to inhibit the phosphatidyl 3-kinase (PI3K) signalling pathway via its receptor-2 (sst2, Bousquet *et al*., 2006), and this receptor has previously been identified in the endometrium throughout the menstrual cycle (Green *et al*., 2002). The ability of endometrial cells to migrate on collagen IV substrate is PI3K mediated (Gentilini *et al*., 2007) and growth factor dependent (Cao *et al*., 2007). However, it is not possible to assess the relevance of endometrial somatostatin on circulating, growth hormone-dependent IGF-I which is produced in the liver.

Fetuin B is a variant of fetuin A, similarly produced in the liver and the human placenta and playing a role in fetal life and calcium metabolism. In contrast to fetuin A, it is present in the serum at concentrations that are higher in women than in men (Denecke *et al*., 2003). No information on fetuins in the endometrium could be found in the context of infertility. Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) is expressed in epithelial tissues and was suggested to play a role in trophoblast–endometrium interaction (Bamberger *et al*., 2006). This would be in agreement with the up-regulated expression that we have observed here between ongoing and miscarrying pregnancy groups in both gene array and quantitative PCR. CD163 is only expressed in monocytes and macrophages, which are present in the endometrium and play a role as mediators in inflammation processes. CD163 protein production was shown *in vitro* to be inducible by anti-inflammatory agents such as interleukin-10 and glucocorticoids (Buechler *et al*., 2000). In our context it is interesting to note the correlation between up-regulated CD163 gene expression in the successful pregnancy group and the anti-inflammatory environment to which this antigen is associated.

While we have confirmed, in real-time PCR, clear up- or down-regulations for several markers between pregnancy and non-pregnancy endometrium, and even more interestingly between ongoing and miscarried pregnancies, this was not the case between the different embryo-conditioned media. Array hybridization returned an up-regulation, between embryo media from ongoing and aborted pregnancies, for prolactin receptor (PRL-R), fibronectin 1 and CD8/p32 antigen, and a down-regulation for CD163 antigen and collagen IVα6 (Tables III and IV). None of these findings could be confirmed by real-time PCR between these different embryo-conditioned cultures. However, between the different endometrial tissues significant differences were found for most of these: pRL-R, CD8, fibronectin and collagen IV were down, whereas CD163 was up (Figs. 2 and 3). These discrepancies could be explained by the presence of implanting and non-implanting embryos in the same culture medium which had subsequently been used as a supplement in our endometrial cultures, or by an insufficient sensitivity of our 'bio-assay' system (the culture). Embryo-conditioned media came in 10 μl samples and thus ended up in a 1:100 dilution due to the distribution into different patient groups and the suspension culture volume of 0.5 ml. One large investigation with gene profiling, using the Affymetrix HG-U133A microarray on purified endometrial stromal cells cultured

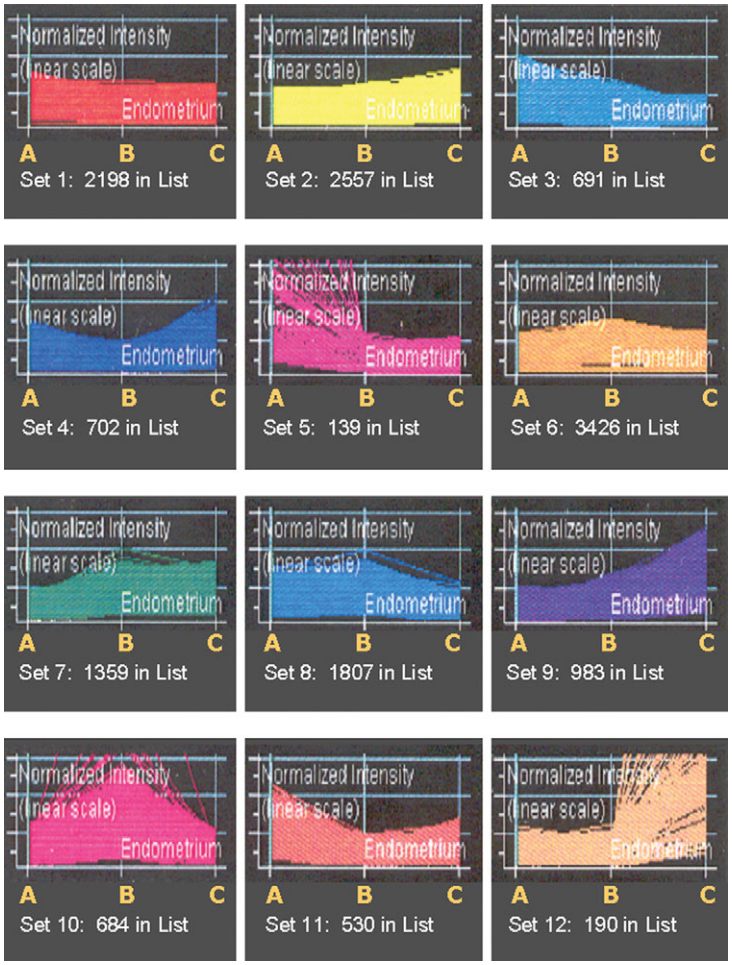

Figure 1: Genespring® expression analysis comparing the three groups of endometrial tissue; the 12 sets were generated automatically.
Each coloured line corresponds to a gene; and the number of lines making up each of the 12 sets is given under each graph. A, endometrium from patients never getting pregnant (implantation failure); B, endometrium from women with miscarriage (pregnancies lost before 12 weeks of gestation); C, endometrium from women who later succeeded in a healthy pregnancy proceeding to term.

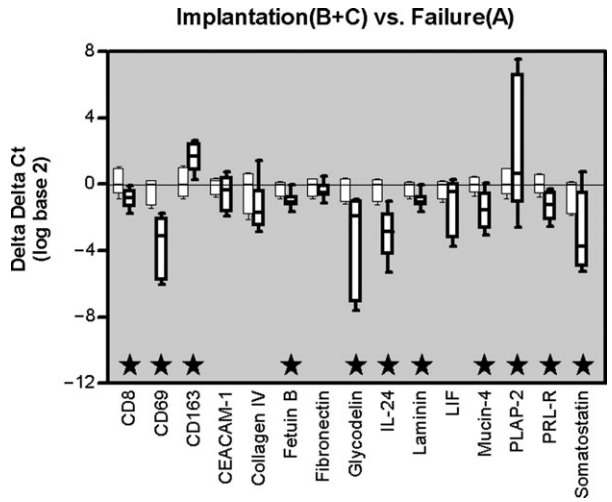

Figure 2: Quantitative PCR analysis of selected genes (in alphabetical order) in cultured endometrial tissue: comparison between implantation failures and successful implantations (comprising both ongoing pregnancies and miscarriages).
Values are expressed in ΔΔCt, i.e. in logarithms on base 2, normalized first against both β-actin and GAPDH reference gene expression, and then against implantation failure ($\Delta Ct = 2^0 = 1$, faint horizontal line). Thin lined boxes and whiskers represent group A (implantation failure) and thick line boxes/whiskers groups B and C (pregnancy). Significantly ($P < 0.05$) up- or down-regulated markers are labelled with an asterisk.

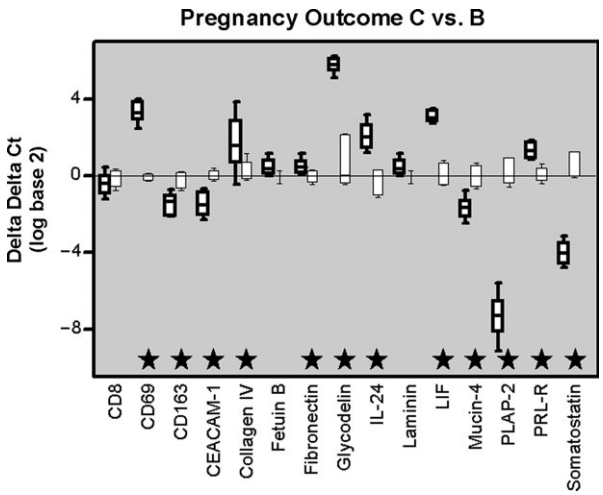

Figure 3: Quantitative PCR analysis of selected genes (in alphabetical order) in successful implantations: comparison between cultured endometrial tissue from women with healthy, ongoing pregnancies and women with pregnancies which terminated in miscarriage.
Values are expressed in ΔΔCt, i.e. in logarithms on base 2, normalized first against both β-actin and GAPDH reference gene expression, and then against successful, ongoing pregnancy ($\Delta Ct = 2^0 = 1$, faint horizontal line). Thick lined boxes and whiskers represent group B (miscarriage) and thin lined boxes/whiskers group C (ongoing pregnancy). Significantly ($P < 0.05$) up- or down-regulated markers are labelled with an asterisk.

in the presence and the absence of trophoblast-conditioned media, was recently published (Hess *et al*., 2007). A total of 4817 genes were found to be differentially regulated as a function of the presence of signals from the trophoblast, so we believe that this line of investigation, i.e. embryo-derived factors and signals, should nevertheless be continued. It also has to be noted that preimplantation genetic testing is illegal in Switzerland. For this reason we were unable to ascertain the genetic integrity of the transferred embryos by fluorescent *in situ* hybridization techniques and to conclude that IF (in our group A) was solely due to inadequate endometrial function.

In conclusion, while this microarray approach (at least with the high-density arrays) may not be currently sensitive enough for the study of the effects of embryo-derived factors on the endometrium *in vitro*, gene expression profiling will become an interesting tool for the identification of new fertility-related and obstetrically relevant markers produced by the endometrium in natural and stimulated cycles. The findings from the few previous profiling studies have stimulated quantitative investigations on LIF and glycodelin, two proteins whose roles in implantation modulation are far from being understood. Our findings here indicate that microarray technology will, by the identification of new markers, provide useful information towards the understanding of unexplained IF and pregnancy loss in the future.

Acknowledgements

Our thanks go to Dr Philippe Demougin, Life Science Training Facility of the Biocentrum (University of Basel, Switzerland) for tuition during the gene expression array experiments, to Susanne von Wyl and her team in the IVF unit, to Anne Vaucher in the research laboratory for technical assistance and to the Department of Clinical Research (DKF, University of Berne) for making their equipment (real-time PCR) available.

Funding

University of Berne: Department of Obstetrics and Gynaecology and Department of Clinical Research.

References

Andersen CY, Westergaard LG, Teisner B, Byskov AG, Ziebe S, Helledie L, Petersen K, Westergaard JG. Changes induced in serum protein profiles by ovarian stimulation during in-vitro fertilisation - embryo transfer treatment: a comparison between conception and non-conception cycles. *Hum Reprod* 1992;**7**:585–591.

Bamberger AM, Minas V, Kalantaridou SN, Radde J, Sadeghian H, Loning T, Charalampopoulos I, Brummer J, Wagener C, Bamberger CM *et al.* Corticotropin-releasing hormone modulates human trophoblast invasion through carcinoembryonic antigen-related cell adhesion molecule-1 regulation. *Am J Pathol* 2006;**168**:141–150.

Bergh PA, Navot D. The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertil Steril* 1992;**58**:537–542.

Bolton AE, Clough KJ, Stoker RJ, Pockley AG, Mowles EA, Westwood OM, Chapman MG. Identification of placental protein 14 as an immunosuppressive factor in human reproduction. *Lancet* 1987;**14**:593–595.

Bousquet C, Guillermet J, Saint-Laurent N, Archer E, Lopez F, Fanjul M, Ferrand A, Fourmy D, Pichereaux C, Monsarrat B *et al.* Direct binding of p85 to sst2 somatostatin receptor reveals a novel mechanism for inhibiting PI3K pathway. *EMBO J* 2006;**25**:3943–3954.

Buechler C, Ritter M, Orso E, Langmann T, Klucken J, Schmitz G. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* 2000;**67**:97–103.

Cao Z, Liu LZ, Dixon DA, Zheng JZ, Chandran B, Jiang BH. Insulin-like growth factor-I induces cyclooxygenase-2 expression via PI3K, MAPK and PKC signaling pathways in human ovarian cancer cells. *Cell Signal* 2007;**19**:1542–1553.

Catalano RD, Yanaihara A, Evans AL, Rocha D, Prentice A, Saldi S, Print CG, Charnock DS, Sharkey AM, Smith SK. The effect of RU-486 on the gene expression profile in an endometrial explant model. *Mol Hum Reprod* 2003;**9**:465–473.

Delage G, Moreau JF, Taupin JL, Freitas S, Hambartsoumian E, Olivennes F, Fanchin R, Letur 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**:2483–2488.

Dell A, Morris HR, Easton RL, Panico M, Patankar M, Oehninger S, Koistinen R, Koistinen H, Seppala M, Clark GF. Structural analysis of the oligosaccharides derived from glycodelin, a human glycoprotein with potent immunosuppressive and contraceptive activities. *J Biol Chem* 1995;**270**:24116–24126.

Denecke B, Graber S, Schafer C, Heiss A, Woltje M, Jahnen W. Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochem J* 2003;**376**:135–145.

Gentilini D, Busacca M, Di Francesco S, Vignali M, Viganò P, Di Blasio AM. PI3K/Akt and ERK1/2 signalling pathways are involved in endometrial cell migration induced by 17beta-estradiol and growth factors. *Mol Hum Reprod* 2007;**13**:317–322.

Green VL, Richmond I, Maguiness S, Robinson J, Helboe L, Adams IP, Drummond NS, Atkin SL. Somatostatin receptor 2 expression in the human endometrium through the menstrual cycle. *Clin Endocrinol (Oxf)* 2002;**56**:609–614.

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**:137–143.

Henriquez S, Tapia A, Quezada M, Vargas M, Cardenas H, Rios M, Salvatierra AM, Croxatto H, Orihuela P, Zegers F *et al.* Deficient expression of monoamine oxidase A in the endometrium is associated with implantation failure in women participating as recipients in oocate donation. *Mol Hum Reprod* 2006;**12**:749–754.

Hess AP, Hamilton AE, Talbi S, Dosiou C, Nyegaard M, Nayak N, Genbecev O, Mavrogianis P, Ferrer K, Kruessel J *et al.* Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. *Biol Reprod* 2007;**76**:102–117.

Holst N, Haug E, Tanbo T, Abyholm T, Jacobsen MB. Somatostatin in human follicular fluid. *Hum Reprod* 1994;**9**:1448–1451.

Horcajadas JA, Riesewijk A, Polman J, Van Os R, Pellicer A, Mosselman S, Simon C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod* 2005;**11**:195–205.

Joshi SG, Bank JF, Henriques ES, Makarachi A, Matties G. Serum levels of a progestagen-associated endometrial protein during the menstrual cycle and pregnancy. *J Clin Endocrinol Metab* 1982;**55**:642–648.

Kao LC, Tulac S, Lobo S, Imani B, Yang YP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC. Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 2002;**143**:2119–2138.

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**:569–574.

Lédée N, Laprée G, Taupin JL, Dubanchet S, Frydman R, Chaouat G. Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. *Hum Reprod* 2002;**17**:213–218.

Lee J, Oh J, Choi E, Park I, Han C, Kim DH, Choi BC, Kim JW, Cho C. Differentially expressed genes implicated in unexplained recurrent spontaneous abortion. *Int J Biochem Cell Biol* 2007;**39**:2265–2277.

Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S. In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod* 2005;**20**:2104–2117.

Mueller MD, Vigne JL, Vaisse C, Taylor RN. Glycodelin: a pane in the implantation window. *Semin Reprod Med* 2000;**18**:289–298.

Mueller MD, Vigne JL, Streit M, Tee MK, Raio L, Dreher E, Bersinger NA, Taylor RN. 2,3,7,8-tetrachlorodibenzo-p-dioxin increases glycodelin gene and protein expression in human endometrium. *J Clin Endocrinol Metab* 2005;**90**:4809–4815.

Oehninger S, Coddington CC, Hodgen GD, Seppala M. Factors affecting fertilisation: endometrial placental protein 14 reduces the capacity of human spermatozoa to bind to the human zonal pellucida. *Fertil Steril* 1995;**63**:377–383.

Riesewijk A, Martin J, Van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S, Simon C. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol Hum Reprod* 2003;**9**:253–264.

Seppala M, Koistinen H, Koistinen R, Dell A, Morris HR, Oehninger S, Clark GF. Glycodelins as regulators of early events of reproduction. *Clin Endocrinol* 1997;**46**:381–386.

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, Abbondanzo SJ. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 1992;**359**:76–79.

Suzuki Y, Sugiyama R, Fukumine N, Usuda S, Itoh H, Isaka K, Takayama M, Teisner B. Clinical applications of serum placental protein 14 (PP14) measurement in the IVF-ET cycle. *J Obstet Gynecol Res* 2000;**26**:295–302.

Tesarik J, Sousa M. Key elements of highly efficient intracytoplasmatic sperm injection technique: Ca+ fluxes and oocyte cytoplasmic dislocation. *Fertil Steril* 1995;**64**:770–776.

Vigne JL, Hornung D, Mueller MD, Taylor RN. Purification and characterization of an immunomodulatory endometrial protein, glycodelin. *J Biol Chem* 2001;**276**:17101–17105.

Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *New Engl J Med* 1999;**340**:1769–1799.

Wood PL, Iffland CA, Allen E, Bentick B, Burton P, Shaw RW, Bell SC. Serum levels of pregnancy-associated endometrial α2-globulin (α2-PEG), a glycosylated α-lactoglobulin homologue, in successful and unsuccessful assisted conception. *Hum Reprod* 1990;**5**:421–426.

Wunder DM, Kretschmer R, Bersinger NA. Concentrations of leptin and C-reactive protein in serum and follicular fluid during assisted reproductive cycles. *Hum Reprod* 2005;**20**:1266–1271.

Submitted on February 23, 2008; resubmitted on May 30, 2008; accepted on June 2, 2008