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Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy

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BACKGROUND: Gene expression profiling of normal receptive endometrium has been characterized, but intrinsic defects in endometrial gene expression associated with implantation failure have not been reported. METHODS: Women who had previously participated as recipients in oocyte donation cycles and repeatedly exhibited implantation failure (Group A, study group) or had at least one successful cycle (Group B, control group) and spontaneously fertile women (Group C, normal fertility group) were recruited. All were treated with exogenous estradiol and progesterone to induce an endometrial cycle, and an endometrial biopsy was taken on the seventh day of progesterone administration. RNA from each sample was analysed by cDNA microarrays to identify differentially expressed genes between groups. RESULTS: 63 transcripts were differentially expressed (≥2-fold) between Groups A and B, of which 16 were subjected to real time RT–PCR. Eleven of these were significantly decreased in Group A with regard to Groups B and C. Among the dysregulated genes were MMP-7, CXCR4, PAEP and C4BPA. CONCLUSIONS: Repeated implantation failure in some oocyte recipients is associated with an intrinsic defect in the expression of multiple genes in their endometrium. Significantly decreased levels of several transcripts in endometria without manifest abnormalities is demonstrated for the first time and shown to be associated with implantation failure.

Keywords: endometrium; implantation; microarrays; oocyte donation; uterine receptivity

Introduction

The success of embryo implantation depends on blastocyst quality and endometrial receptivity (Giudice, 1995). It has been shown that, in most mammals, there is only a restricted time during the uterine cycle during which implantation can occur (Psychoyos, 1986). In women, the maternally directed receptive phase or 'window' for embryo implantation appears to be of ~5 day duration, from Days 20 to 24 of the cycle (Bergh and Navot, 1992). Acquisition of receptivity is driven by estradiol (E_2) and progesterone, which acting through their receptors, changes the transcription rate of target genes (O'Malley and Tsai, 1992). Endometrial receptivity has been shown to be associated with a certain repertoire of genes whose expression is either enhanced or decreased in comparison with pre-receptive stages. Consequently, it follows that abnormal gene expression in the endometrium could result in implantation failure and infertility. Conversely, one likely deficiency to be found among primary female infertility of unknown origin may be an intrinsic defect in the expression of crucial genes for implantation (Tabibzadeh, 1998).

Microarray technology has been used to identify transcripts whose level change significantly throughout the endometrial cycle (Ponnampalam *et al*., 2004; Talbi *et al*., 2006) or during the transition from the late proliferative (Kao *et al*., 2002; Borthwick *et al*., 2003) or from the early secretory phase (Carson *et al*., 2002; Riesewijk *et al*., 2003; Mirkin *et al*., 2005) to the receptive phase. However, in the human, progesterone not only drives the acquisition of receptivity in

preparation for an embryo that may reach the uterine cavity, but also sets up the machinery to carry on menstruation in the absence of embryonic signaling. Hence, not all transcripts whose level changes throughout the luteal phase are necessarily involved in endometrial receptivity. Another approach utilized to identify genes potentially relevant to endometrial receptivity has been to characterize the endometrial gene expression profile under conditions of diminished fertility such as endometriosis (Kao *et al*., 2003) or intrauterine device (IUD) (Horcajadas *et al*., 2006).

The strategy reported here was to identify genes whose disturbed expression is consistently associated with implantation failure, in the absence of recognizable genital tract, embryonic and endocrine factors. We hypothesized that the pattern of gene expression in the endometrium during the receptive period may differ between women who have had successful embryo implantation, and those who have not, after repeated embryo transfer. Thus, we investigated whether the pattern of gene expression in the human endometrium during a receptive period induced with exogenous E_2 and progesterone has a defined relationship with previous outcomes of repeated oocyte donation cycles. Microarrays were used to assess levels of multiple transcripts in endometrial biopsies taken during the implantation window induced by exogenous hormones in women who were previously recipients in oocyte donation cycles. These women had either no evidence of implantation in more than one cycle of embryo transfer or had become pregnant.

Materials and Methods

Subjects

All volunteers were enrolled after giving informed consent, under a protocol conducted in accordance with the guidelines in The Declaration of Helsinki, independently approved by the Ethics and Scientific Review Committees of Instituto Chileno de Medicina Reproductiva, Universidad de Chile and Clínica Las Condes.

Three groups of women were recruited. Group A comprised 5 women whose endometrial biopsies were used for microarray analysis ($n = 3$) and real time RT–PCR confirmation ($n = 5$). Women in this group had never been pregnant and had previously participated more than once as recipients in an oocyte donation program. At no time had they born evidence of embryo implantation after transfer of embryos of good morphology, at least equivalent to embryos transferred to the oocyte donor who became pregnant. Since good quality embryos with the ability to implant and develop normally derive from good quality oocytes, it was required that the oocyte donor had become pregnant from the same oocyte pool. Group B comprised 6 women who had previously become pregnant as recipients in oocyte donation cycles and delivered live infants. Group C comprised six women with a history of normal fertility in natural cycles (three or more live births). Women of Group C were surgically sterilized at least 1 year prior to their participation for reasons unrelated to this study and had regular menstrual cycles (26–35 days). Women of Groups A and B underwent a comprehensive evaluation. The general exclusion criteria for all volunteers included: metabolic or endocrine diseases other than those leading to ovarian failure, chronic use of medication other than HRT, drug abuse, obesity, endometriosis, pelvic inflammatory disease and current genital tract infection. None of the participants had polycystic ovary syndrome. In Group A, all the standard clinical investigations were done including laparoscopy and known causes of implantation failure attributable to endocrine, endometrial, tubal or pelvic pathologies, as well as to any male factor potentially relevant for IVF were ruled out.

Induction of endometrial cycle

All subjects from Groups A, B and C underwent the induction of an artificial endometrial cycle with exogenous E_2 and progesterone. The pattern of gene expression in the human endometrium during the receptive period induced by replacement therapy with E_2 and progesterone has not been established, but it is known to be compatible with implantation.

Women having spontaneous menstrual cycles were treated with an oral contraceptive (levonorgestrel 0.25 mg and ethinyl E_2 0.05 mg) for 10–21 days as convenient, to suppress spontaneous cyclicity. Down-regulation of ovarian function with the gonadotrophin-releasing hormone (GnRH) agonist, leuprolide acetate (Lupron; TAP pharmaceuticals, Deerfield, IL, USA), was initiated on the last day of contraceptive administration at a dose of 0.5 mg s.c. daily for 7 days. Women with no ovarian function did not receive GnRH agonist therapy. Before proceeding with the hormonal replacement therapy, all volunteers with spontaneous menstrual cycles had serum $E_2 < 100$ pmol/l, serum luteinizing hormone ≤ 3 international units and no ovarian cysts detected by ultrasound on the seventh day of leuprolide acetate administration.

In order to induce endometrial proliferation and differentiation, cycling and non-cycling women underwent the same hormonal replacement therapy. The protocol used was the same as in their oocyte donation cycles. For estrogen replacement, micronized E_2 was given at a dose of 4 mg/day on Days 1–7 and 6 mg/day on Days 8–20 (Day 1 = first day of E_2 administration). The endometrial response was assessed on Day 14 measuring endometrial thickness by ultrasound, and values ≥ 11 mm were considered adequate. Micronized progesterone, 600 mg/day, was administered from Days 14 to 20 inclusive as follows: 400 mg/day orally and 200 mg/day vaginally. On Day 20, endometrial thickness was documented by ultrasound and an endometrial biopsy was taken from the uterine fundus.

Biopsies from all groups were performed using a standard endometrial suction curette (Pipelle de Cornier; Laboratoire C.C.D., Paris, France) under sterile conditions. A portion of each sample was fixed in 4% paraformaldehyde in phosphate-buffered saline for histological evaluation and the remainder was snap frozen in liquid nitrogen and stored at -80°C until use. All biopsies were classified as normal secretory endometrium with no differences between the groups. No sign of inflammatory process was found in any of them.

cDNA microarrays

cDNA microarrays were produced at the National Cancer Institute, LMT microarray core facility (NCI-Frederick, Frederick, MD, USA). The cDNA set from the human UniGEM 2.0 library, comprised 9128 PCR products (Incyte Genomics Inc., Palo Alto, CA, USA), spotted on poly-L-lysine (Sigma; St Louis, MO, USA) coated glass slides with a MicroGrid II microarrayer (Biorobotics; Cambridgeshire, UK). The gene list is available at http://nciarray.nci.nih.gov.

RNA isolation, amplification and target labeling

Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) as directed by the manufacturer, using Phase lock tubes (Eppendorf, Westbury, NY, USA) to maximize RNA recovery. The quality of the RNA was checked with the Lab-on-a-Chip total RNA nano biosizing assay (Agilent Technologies, Inc., Palo Alto, CA, USA) by examining the 18s and 28s

ribosomal bands. Purity of isolated RNAs was quantified spectrophotometrically by the A260/A280 ratio.

Three of five endometrial biopsies from Group A were individually used for microarray analysis, whereas RNA samples from control groups (B, $n = 6$ and C, $n = 6$) were pooled within their respective groups. Total RNA (5 μg) was subjected to one round of amplification according to the Eberwine procedure (Van Gelder *et al*., 1990) using the RiboAmp RNA kit (Arcturus, Mountain View, CA, USA) according to the manufacturer instructions. After amplification, antisense RNA (aRNA) was used to make fluorescence labeled cDNA targets using the LabelStar Array kit (Qiagen, Valencia, CA, USA). About 5 μg of aRNA was used as starting template and subjected to reverse transcription driven by random hexamers allowing direct labeling of DNA with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Piscataway, NJ, USA).

Hybridization on glass cDNA microarrays

Hybridization of cDNA microarrays was performed as described (DeRisi *et al*., 1997). Briefly, the appropriate Cy3 and Cy5 targets were combined, along with 20 μg of Human COT-1 DNA (Invitrogen), 20 μg of poly-d$(A)_{40-60}$ (Amersham Biosciences), 2.6 μl of 20X Sodium chloride-sodium citrate buffer (SSC), 1 μl of 10% (w/v) sodium dodecyl sulphate (SDS) and Tris–EDTA to a final volume of 40 μl. The hybridization solution was heated for 2 min at 99°C and centrifuged for 10 min at 16 000 g in an Eppendorf centrifuge. Slides were hybridized in a water bath overnight (14–16 h) at 55°C. After hybridization, slides were washed for 1 min in 2× SSC and 0.1% SDS, in 1× SSC, for 1 min and in 0.05× SSC for 10 s, then spun until dry.

Scanning, feature extraction and analysis

Fluorescent images from microarray slides were captured using the GenePix 4000 Scanner (Axon Instruments Inc., Foster City, CA, USA) at 10-μm resolution. Photomultiplier voltage settings were set to obtain maximum signal intensities with >1% probe saturation. Feature extraction was done with GenePix Pro 4.0 (Axon Instruments Inc.) software. Spots with high local background or aberrant spot shape were flagged by the software and checked manually. For each slide, the average foreground signal intensity adjusted for local channel specific background was calculated. Spots with signal intensity <100 in both channels were excluded. If at least one channel had intensity above 100, the intensity under 100 was set at 100.

Analysis of array data

Both image and signal intensity data were stored in the NCI Microarrays Data Base (mAdb) supported by the Centre for Information Technology of NIH (http://nciarray.nci.nih.gov). Normalization of microarray data was done on single individual slides using the global normalization method which assumes that the red and green intensities are related by a constant factor. Cy3:Cy5 intensity ratio was calculated for each spot and subsequently adjusted to ratios of overall signal intensity from the corresponding channel to make the median value of log2 ratio equal to zero.

In all data sets included in the analysis, ratios of overall signal intensity ranged from 0.7 to 1.25. Ratios extracted from microarray images exhibited normal distribution, constant coefficient of variation and high positive signal. Ratios of 2 or larger and 0.5 or smaller were considered indicative of differential transcript level between two samples hybridized to the same array spot.

Control pools from Groups B and C were hybridized with a dye swap in duplicate (four microarray slides in total) and a histogram of ratios was performed. Most ratios were between 0.51 and 1.9 (98.2%), whereas ratios ≤0.5 or ≥2.0 were 0.96 and 1.81%, respectively. None of the differentially expressed genes found in Group A with regard to Groups B and C were among the genes with ratios over 2-fold between Groups B and C.

Real time RT–PCR verification of gene expression determined by microarray analyses

First-strand cDNA was synthesized from total RNA from each endometrial sample in duplicate by reverse transcription using the Omniscript Reverse Transcriptase (Qiagen), according to the manufacturer's protocol.

Real time RT–PCR was performed using an ABI PRISM 7900HT sequence detection system (TaqMan) according to the manufacturer's instructions. Prevalidated primers and probes (Assays-on-demand, PE Applied Biosystems, Foster City, CA, USA) were used for all genes submitted to Real Time RT-PCR confirmation, except CXCR4, to determine their respective transcript levels. The primers and probe for CXCR4 were designed by using PRIMER EXPRESS V.5.0 software (Applied Biosystems): forward CCTGCCCTCCTGCTGACTA, reverse GGGTAGAAGCGGTCACAGAT and probe TCCCGACTTCATCTTTG. Expression values for all transcripts analysed were normalized against those from the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Assays-on-demand, PE Applied Biosystems) to account for differing amounts of starting material.

The thermal cycling conditions included an initial activation step at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation and annealing-amplification (95°C for 15 s, 60°C for 1 min). QPCR Reference Total RNA, Human (Stratagene, La Jolla, CA, USA) was used as a reference expression level.

Results

A total of 17 women were treated as recipients in a mock oocyte donation cycle, and an endometrial biopsy was taken on Day 7 of treatment with progesterone. The anthropometric and functional parameters of participating women did not show relevant differences between groups (Table I).

At the time of performing the microarrays, only three samples from Group A and all the samples from control groups were available. In order to identify differentially expressed genes, RNA samples within each control group were pooled and were compared with the RNA from each individual sample of Group A.

Fourteen genes were differentially expressed by at least 2-fold in 3/3 samples from Group A, compared with the pool of samples from Group B (Table II). Sixteen genes were differentially expressed by at least 2-fold in 3/3 samples of Group A when they were compared with the pool of samples from Group C (Table III). Nine of the differentially expressed genes were common to both comparisons (Table III, bold genes), suggesting a very similar expression profile between the two control groups, considering that nearly 10 000 transcripts were compared.

When a filter with less stringency, *i.e*. two-third samples, was applied to the comparison between Groups A and B, 63 transcripts showed a ≥2-fold difference in intensity. This larger list of 63 transcripts was contrasted with a database constructed with previously reported transcript level changes from non-receptive to receptive stage (Carson *et al*., 2002; Kao *et al*., 2002; Borthwick *et al*., 2003; Riesewijk *et al*., 2003;

Table I. Characteristics of women participating in the study and parameters evaluated during the hormonal replacement cycle.

	Group A ($n = 5$)[a]	Group B ($n = 6$)	Group C ($n = 6$)
Age (years)	35.4 (26–43)	41.9 (34–46)	41.3 (36–47)
Body mass index	25.2 (22.6–29.4)	25.6 (22.5–27.4)	25.4 (23.3–28.1)
Reason for oocyte donation	Premature ovarian failure ($n = 3$) Poor ovarian response[b] ($n = 1$) Surgical castration ($n = 1$)	Premature ovarian failure ($n = 2$) Poor ovarian response[b] ($n = 4$)	N/A
Requirement for administration of GnRH agonist prior to hormonal replacement therapy	$n = 1$[c]	$n = 4$	$n = 6$
Plasma progesterone[d] (*n*mol/l), range in parenthesis	75.7 (38–122)	88.1 (36–192)	63.7 (43.3–75)
Endometrial thickness[d] (mm)	10.8 (9–12)	9.1 (8–10)	11.2 (8.5–12.5)

Mean and range in parenthesis is indicated except for infertility diagnosis.
[a]Within this group of five there are three patients whose endometrial samples were used for the microarray analysis and real time RT–PCR and other two only for real time RT–PCR. One of those two corresponded to surgical castration and the other to premature ovarian failure. [b]Poor ovarian response defined as failure to respond to ovulation induction therapy with appropriate follicular development, despite having normal basal FSH levels (<10 IU/l). [c]Sample used for microarray analysis and real time RT–PCR. [d]On the day of biopsy.

Table II. Genes whose transcripts displayed ≥2-fold difference in level in Group A when compared with Group B in the microarray analyses.

Gene name	Description ($n = 14$)	Fold change
IGHG1	Immunoglobulin heavy constant gamma 1 (G1m marker)	0.10
PAEP	Progestagen-associated endometrial protein, glycodelin	0.15
RAP1GAP	RAP1 GTPase activating protein	0.17
RRM1	Ribonucleotide reductase M1 polypeptide	0.18
C4BPA	Complement component 4 binding protein, alpha	0.21
SOD2	Superoxide dismutase 2, mitochondrial	0.26
NNMT	Nicotinamide *N*-methyltransferase	0.34
FLJ39046	cDNA FLJ39046 fis, clone NT2RP7010612	0.34
DHX36	DEAH (Asp-Glu-Ala-His) box polypeptide 36	0.37
EDNRB	Endothelin receptor type B	0.37
CLU	Clusterin	0.38
ASPN	Asporin (LRR class 1)	0.38
MGST1	Microsomal glutathione S-transferase 1	0.40
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	2.88

All, except ankyrin 3, appeared down-regulated in Group A.

Table III. Genes whose transcripts displayed ≥2-fold difference in level between Groups A and C in the microarray analyses.

Gene name	Description ($n = 16$)	Fold change
MGC27165	Hypothetical protein MGC27165	0.08
RAP1GAP	**RAP1 GTPase activating protein**	0.19
PAEP	**Progestagen-associated endometrial protein**	0.19
C4BPA	**Complement component 4 binding protein, alpha**	0.22
RRM1	**Ribonucleotide reductase M1 polypeptide**	0.23
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	0.28
MGST1	**Microsomal glutathione S-transferase 1**	0.29
CCNB1	Cyclin B1	0.31
SLC7A5	Solute carrier family 7, member 5	0.32
FLJ39046	**cDNA FLJ39046 fis, clone NT2RP7010612**	0.35
RNASE4	Ribonuclease, RNase A family, 4	0.36
SOD2	**Superoxide dismutase 2, mitochondrial**	0.38
TOP3A	Topoisomerase (DNA) III alpha	0.38
EDNRB	**Endothelin receptor type B**	0.39
C9orf3	Chromosome 9 open reading frame 3	0.44
ANK3	**Ankyrin 3, node of Ranvier (ankyrin G)**	2.83

All, except ankyrin 3, appeared down-regulated in Group A. Gene names in bold coincide with those shown in Table II.

Mirkin *et al*., 2005), and 14 coincident genes were identified (Table IV).

The 63 transcripts were also contrasted with genes differentially expressed in endometrial biopsies taken during the receptive period: from women with or without endometriosis (Kao *et al*., 2003) and from women with or without an inert IUD (Horcajadas *et al*., 2006). Seven genes coincided (Table V), and five of them were represented also in Tables II and III.

In order to confirm differences in transcript levels found in the microarrays, a selected set of transcripts (Table VI) was submitted to real time RT–PCR. Selection was done according to the following criteria: (i) transcripts that consistently displayed up- or down-regulation in the cDNA microarrays analysis in 3/3 samples from Group A compared with pooled samples from Groups B and C; (ii) transcripts that were differentially expressed in two-third samples from Group A compared with Group B and that also were coincident with genes whose expression profile has been reported to change with acquisition of receptivity, or are differentially expressed in women with endometriosis or with an inert IUD. Transcripts selected with these criteria are listed in Table VI. Endometrial samples used for this purpose were from Groups A ($n = 5$), B ($n = 6$) and C ($n = 6$). The results are shown in Fig. 1 (panels a–p). mRNA levels for genes AVIL, C4BPA, MMP-7, MAO-A, MGST1, NNMT, CXCR4, CLU, SERPINB9, PAEP and RRM1 were reduced in Group A compared with both control groups in agreement with the microarray data. The transcript level of CXCR4 was also different between Groups B and C (Wilcoxon Rank-Sum test, $P < 0.05$). The mRNA levels for ANK3 appeared to be greater in Group A compared with the control groups, but the differences were not statistically significant. RAP1GAP, EDNRB, SOD2 and FLJ39 046, which displayed differences in the microarray analysis, did not show statistically significant differences either in this independent assay.

Discussion

The present study assessed the expression level of ~10 000 genes and expressed sequence tags (Ests) in endometrial

Table IV. Genes whose transcript level differed ≥2-fold in two of three samples of Group A in comparison with Group B, which in addition coincided with those previously found to change during acquisition of endometrial receptivity.

A versus B	Kao *et al.* (2002)	Carson *et al.* (2002)	Borthwick *et al.* (2003)	Riesewijk *et al.* (2003)	Mirkin *et al.* (2005)	UniGene ID	Gene name	Description ($n = 14$)
↓		↑				Hs.190783	HAL	Histidine ammonia-lyase
↓	↑		↑	↑		Hs.1012	C4BPA	Complement component 4 binding protein, alpha
↓				↑		Hs.387871	TNFSF10	TRAIL = Apo-2 ligand
↓		↑				Hs.584854	AVIL	Advillin
↓		↑				Hs.104879	SERPINB9	Serpin peptidase inhibitor, clade B, member 9
↓	↑		↑	↑		Hs.532325	PAEP	Progestagen-associated endometrial protein
↓	↑		↑		↑	Hs.183109	MAOA	Monoamine oxidase A
↓				↑		Hs.436657	CLU	Clusterin
↓	↓	↓	↓	↑		Hs.2256	MMP7	Matrix metalloproteinase-7
↓		↓				Hs.67928	ELF3	E74-like factor 3
↓		↓				Hs.421986	CXCR4	Chemokine receptor 4
↓		↓		↑		Hs.503911	NNMT	Nicotinamide *N*-methyltransferase
↓	↓					Hs.558393	RRM1	Ribonucleotide reductase M1 polypeptide
↑				↓		Hs.499725	ANK3	Ankyrin 3, node of Ranvier

Upward and downward arrows mean up- and down-regulated genes.

tissue, during the receptive period in mock oocyte donation cycles of three women who apparently had an endometrial defect impeding embryo implantation (Group A). Their profiles were compared with the one obtained from endometria which in the same oocyte donation program had been receptive to embryo implantation (Group B), or which exhibited receptivity in natural spontaneous cycles (Group C). The data show unequivocally a strong association between defective gene expression in the endometrium and implantation failure. Since all women were subjected to the same steroid hormone stimulation protocol prior to taking the biopsies, differentially expressed genes in Group A would likely reflect a permanent dysregulation of gene expression in their endometrium i.e. not compatible with implantation.

Transcript level differences found between women from Group A with regard to Groups B and C can be attributed to dysregulation in transcriptional control or in messenger stability although transcript level regulation occurs mainly at the rate of transcription.

Real time RT–PCR reactions confirmed that the level of several transcripts in Group A was significantly lower than in Groups B and C, and showed a tendency for some transcript levels of Group C to be lower than in Group B, the difference being statistically significant in the case of chemokine receptor 4. The reason for such behavior is not clear, but may be an epiphenomenon related to biological differences between Groups B and C, that made women of the former group candidates for oocyte donation.

Differential expression of a set of 11 genes in the group refractory to implantation was confirmed by real time RT–PCR. Some of them have been reported before to be involved in endometrial receptivity, whereas others are circumstantially associated with such process for the first time in this report.

C4b-binding protein (C4BP), also known as proline-rich protein, is a regulatory protein in the complement system and works mainly in the classical pathway. It binds to the activated complement component C4b and also to C3b, though very weakly, through the α chain. It works as cofactor in the degradation of C3b and C4b by factor I and/or in preventing the formation of C3/C5 convertase (Liszewski *et al.*, 1996; Ogata *et al.*, 1993; Blom *et al.*, 2001). The complement system activity has been suggested to be present in the endometrium throughout the menstrual cycle (Nogawa Fonzar-Marana *et al.*, 2006), and it is postulated that the complement system might be conferring immunity to the uterine cavity, defending it against bacterial infection. Complement-regulatory molecules are up-regulated in human endometrium during the

Table V. Genes whose transcript level differed ≥2-fold in two of three endometrial samples of Group A in comparison with Group B, and coincided also with those whose expression profile differs in women with endometriosis or with an IUD.

A versus B	Kao *et al.* (2003) (endometriosis)	Horcajadas *et al.* (2006) (IUD)	UniGene ID	Gene name	Description ($n = 7$)
↓	↓		Hs.1012	**C4BPA**	Complement component 4 binding protein, alpha
↓	↓	↓	Hs.82269	**PAEP**	Placental protein 14
↓	↓		Hs.148178	**RAP1GAP**	RAP1, GTPase activating protein 1
↓		↓	Hs.450230	IGFBP3	Insulin-like growth factor binding protein 3
↓		↓	Hs.82002	**EDNRB**	Endothelin receptor type B
↑		↑	Hs.73769	FOLR1	Folate receptor 1
↑	↑	↑	Hs.75893	**ANK3**	Ankyrin 3, node of Ranvier [ankyrin G]

Upward and downward arrows indicate up- and down-regulation. Gene names in bold listed here coincide with some of the genes in bold in Table III.

Table VI. Transcripts exhibiting a ≥2-fold difference in level in microarrays of samples of Group A versus B, which were subsequently submitted to confirmation by real time RT–PCR.

Gene name	UniGene ID	Description ($n = 16$)
C4BPA	Hs.1012	Complement component 4 binding protein, alpha
NNMT	Hs.503911	Nicotinamide *N*-methyltransferase
RRM1	Hs.558393	Ribonucleotide reductase M1 polypeptide
MGST1	Hs.389700	Microsomal glutathione S-transferase 1
ANK3	Hs.499725	Ankyrin 3, node of Ranvier
PAEP	Hs.532325	Progestagen-associated endometrial protein
AVIL	Hs.584854	Advillin
SERPINB9	Hs.104879	Serpin peptidase inhibitor, clade B, member 9
MAOA	Hs.183109	Monoamine oxidase A
MMP7	Hs.2256	Matrix metalloproteinase-7
CXCR4	Hs.89414	Chemokine (C-X-C motif) receptor 4
CLU	Hs.436657	Clusterin
RAP1GAP	Hs.148178	RAP1 GTPase activating protein
FLJ39046	Hs.595560	cDNA FLJ39046 fis, clone NT2RP7010612
EDNRB	Hs.82002	Endothelin receptor type B
SOD2	Hs.487046	Superoxide dismutase 2, mitochondrial

secretory phase, suggesting a protective role in maintaining the epithelial integrity of human endometrium (Young *et al*., 2002; Nogawa Fonzar-Marana *et al*., 2006). However, these complement regulatory molecules might be protecting the embryo since decreased expression of an inhibitor of the complement system activation could increase the chance of a misdirected complement attack on the embryo if perceived as a semiallograft. C4BP has been reported previously to be abnormally diminished in endometrial tissue during the receptive phase in women with endometriosis (Isaacson *et al*., 1989; Kao *et al*., 2003).

Glycodelin, also known as progestagen-associated endometrial protein (PAEP), placental protein 14 or placental α_2-macroglobulin (Seppala *et al*., 1998, 2002) is the main progesterone-regulated glycoprotein secreted into uterine luminal cavity. Glycodelin has immunosuppressive activity including inhibition of NK cell activity (Okamoto e*t al*., 1991) and its high concentration at the feto-maternal interface may contribute to protect the embryo against immune system attack.

Advillin is a member of the gelsolin/villin family of actin regulatory proteins. Due to the structural similarity of advillin with gelsolin family members, it is thought to play an important role in dynamic changes in the actin cytoskeleton during a variety of forms of cell motility (Kwiatkowski, 1999). Gelsolin severs assembled actin filaments in two, and caps the fast-growing plus end of a free or newly severed filament. Northern blot analysis has shown high levels of advillin mRNA expression in murine uterus and *in situ* mRNA analysis of adult murine tissues demonstrates that the message is most highly expressed in the endometrial epithelium (Marks *et al*., 1998). If this protein is expressed in the human endometrial epithelial cells as well, its function may mediate the cytoskeleton modification these cells undergo from a polarized to a nonpolarized phenotype, in preparation for cell-to-cell adhesion (Thie *et al*., 1995; Martin *et al*., 2000).

Clusterin in its predominant form is a secreted sulphated heterodimeric glycoprotein of 75–80 kDa comprised of the disulfide-linked subunits α and β (de Silva *et al*., 1990; Kirszbaum *et al*., 1992). Its mRNA has been shown to be expressed in the endometrial surface and in endometrial glands of mouse and human uterus (Brown *et al*., 1995), and has been suggested as a marker of blastocyst implantation in the mouse (Brown *et al*., 1996). Clusterin inhibits the membrane attack complex of complement proteins activated as a result of inflammation (Murphy *et al*., 1988; Choi *et al*., 1989; Jenne and Tschopp, 1989; McDonald and Nelsestuen, 1997) and interacts with immunoglobulin G, increasing the rate of formation of insoluble immune complexes (Wilson *et al*., 1991). Since gene expression of this molecule has been reported to increase from the pre-receptive to the receptive state of the endometrium, it seems that clusterin could be another modulator of the immune system in the endometrium playing an immunosuppressive role during the receptive period.

Monoamine oxidase (MAO) is an enzyme of the mitochondrial outer membrane (Johnston, 1968) critical in the neuronal metabolism (Castro Costa *et al*., 1980) that preferentially degrades 5-hydroxy tryptamine (serotonin, 5-HT) and norepinephrine (Zhu *et al*., 1992). Progesterone provokes a selective rise of MAO-A activity in the rat uterus (Mazumder *et al*., 1980) and in human endometrium its activity markedly increase during the mid-secretory phase of the menstrual cycle, coincident with plasmatic progesterone peak levels and endometrial receptivity (Ryder *et al*., 1980). Promoter sequence analysis for the gene coding for MAO-A has shown response elements to progesterone, suggesting direct transcriptional regulation by this hormone (Borthwick *et al*., 2003). Enzymes responsible for monoamine synthesis have been demonstrated in normal endometrium as well as in early pregnancy deciduas (Manyonda *et al*., 1998). Conditioned media from human embryos induce the expression of β-adrenergic receptors in endometrial cell cultures (Bruzzone *et al*., 2005), suggesting the occurrence of a signaling pathway in the endometrium, mediated by catecholamines. 5-HT has been shown to inhibit decidualization (Mitchell *et al*., 1983; Maekawa and Yamanouchi, 1996). Expression of MAO-A gene might possibly represent a protective mechanism, which maintains low levels of 5-HT thereby assuring decidualization.

Matrix metalloproteinase-7 (MMP-7, matrilysin or uterine metalloproteinase) degrades casein, fibronectin and gelatin types I, III, IV and V (Muller *et al*., 1988; Imai *et al*., 1995). MMP-7 has been shown to be localized only to endometrial glandular or luminal epithelium during the proliferative and premenstrual/menstrual stage of the cycle. (Rodgers *et al*., 1994; Bruner *et al*., 1995). MMP-7 is down-regulated by progesterone in human endometrium and strongly up-regulated during menses. We found by real time RT-PCR that MMP-7 transcript levels were further decreased in the infertile group. This finding suggests that MMP-7 is expressed during the receptive phase, although to a small extent that cannot be detected by less sensitive techniques, as its transcript has been reported in other study using microarrays in secretory human endometrium (Yanaihara *et al*., 2004). The proteolytic activity of MMPs is regulated by zymogen activation and inhibition by physiologic tissue inhibitors (TIMPs) (Chambers and Matrisian, 1997; Gomez *et al*., 1997; Nagase and Woessner, 1999), so the participation of MMP-7 in endometrial receptivity has yet to be determined.

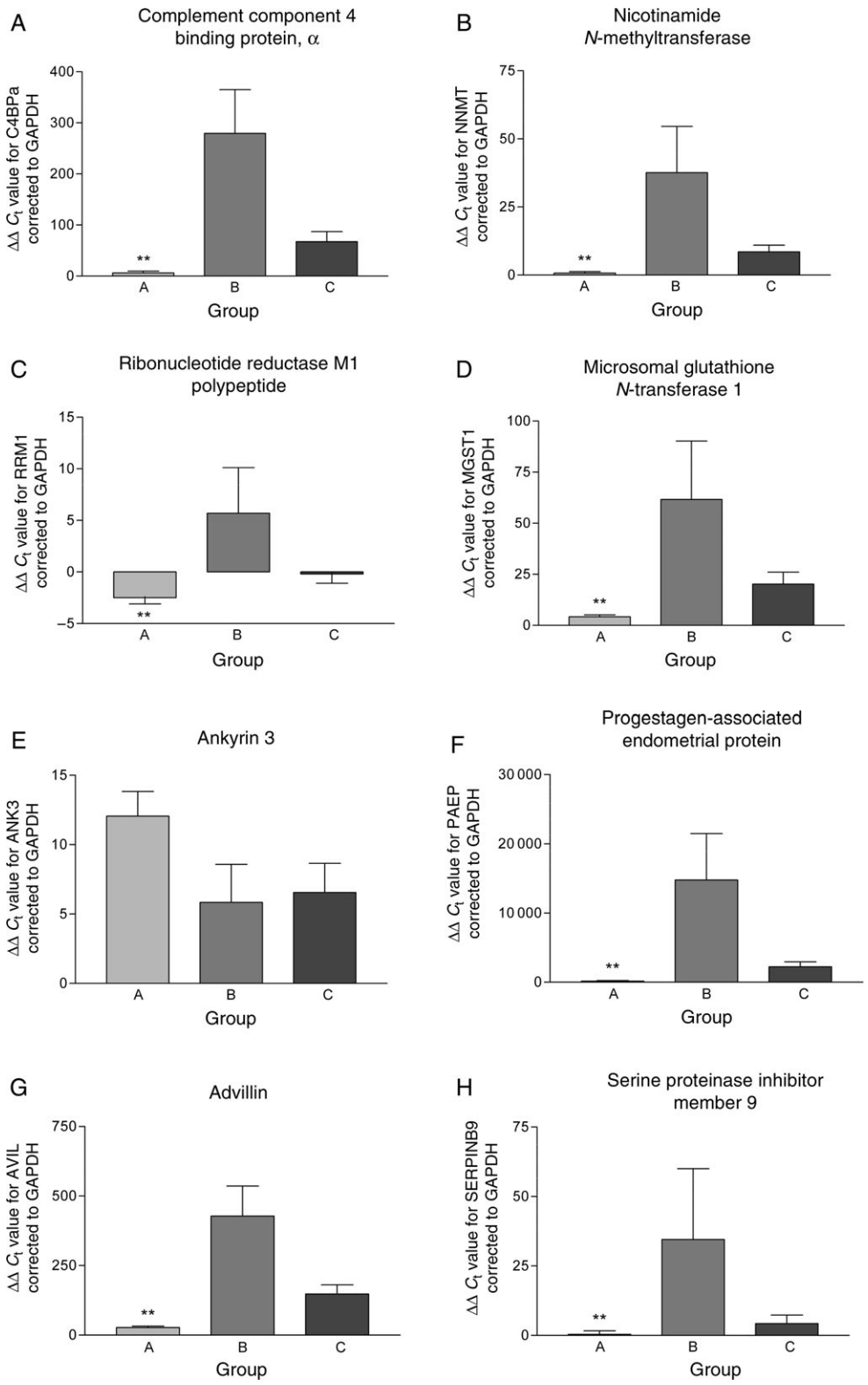

Figure 1: Relative expression of selected transcripts determined by real time RT–PCR in endometrial samples of Group A (refractory endometrium from infertile women), Group B (receptive endometrium from infertile women) and Group C (fertile women) after normalization to GAPDH (panels a–p)
Data are presented as mean ± SEM. ** is significantly different from Groups B and C, $P < 0.05$ and *** is significantly different from Group C, $P < 0.05$; Wilcoxon Rank-Sum test)

CXC chemokine receptor-4 (CXCR4) is the only physiological receptor for stromal cell-derived factor-1 (SDF-1) and has a potent chemotactic activity for lymphocytes (Bleul *et al.*, 1996). CXCR4 mRNA and protein levels are up-regulated during the implantation window in natural and HRT cycles. Chemokine receptors are up-regulated in

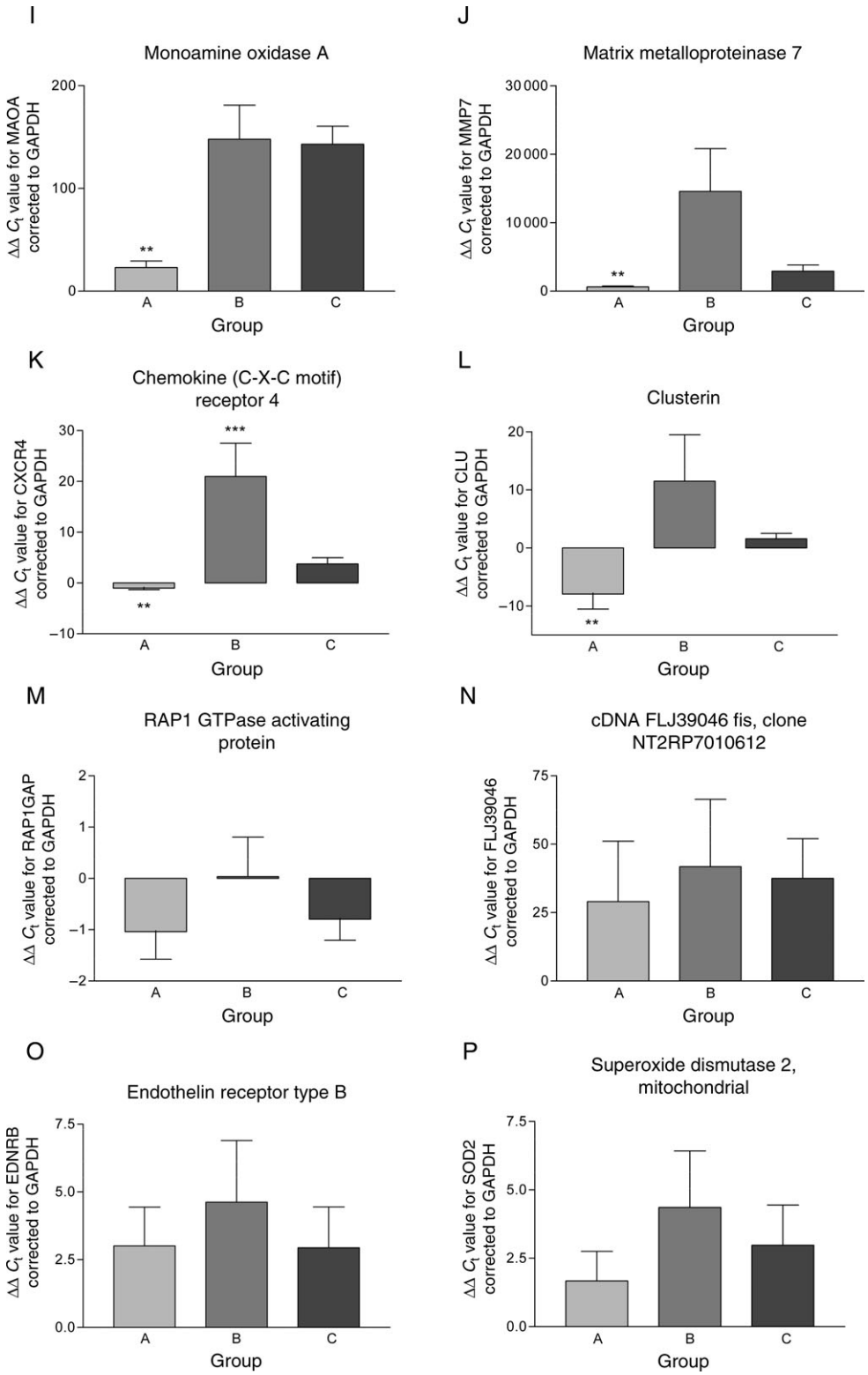

Figure 1: Continued

cultured endometrial epithelial cells and polarization of CXCR4 receptors occurs in the presence of a human blastocyst (Dominguez *et al*., 2003), suggesting that this receptor is implicated in the adhesion phase of human implantation. Moreover, since neutralization of CXCR4 effectively inhibit metastasis in mice (Geminder *et al*., 2001; Muller *et al*., 2001; Taichman *et al*., 2002), it is speculated that trophoblast invasion through the stromal compartment of the endometrium, might be dependent on the SDF-1/CXCR4 pathway. SDF-1 is expressed by invasive trophoblasts and induces the specific migration of $CD56^{+}$ $CD16^{-}$ human natural killer (NK) cells. Such NK phenotype is predominant in the maternal decidua and is found to be in direct contact with the fetal extravillous trophoblast.

The membrane-bound microsomal glutathione transferase 1 (MGST1) is found in abundance in the endoplasmic reticulum and outer mitochondrial membranes. MGST1 is involved in the protection of cells against oxidative damage by membrane lipids (Bannenberg *et al*., 1999; Kelner *et al*., 2000). It has been reported that MGST1 is up-regulated in human endometrial stromal cells *in vitro* by progesterone (Okada *et al*., 2003). Besides the possible protective role this enzyme may play in endometrial cells, its participation in endometrial receptivity remains obscure.

Nicotinamide *N*-methyltransferase catalyzes the N-methylation of nicotinamide, pyridines and structural analogs (Alston and Abeles, 1988). It is involved in the biotransformation of many drugs and xenobiotic compounds. The action of the enzyme in some cases detoxifies its substrates, whereas in other cases it leads to the production of toxic products (Alston and Abeles, 1988). In human endometrium, transcipt levels for NNMT have been reported to be down-regulated from the proliferative to the receptive phase (Carson *et al*., 2002) and up-regulated in the transition from the early secretory phase to the receptive phase (Riesewijk *et al*., 2003). In addition, the function of NNMT in endometrial cells is unclear, so the effects of its down-regulation in infertile women remain to be determined.

Ribonucleotide reductase catalyzes the reduction of all four ribonucleotide diphosphates to their corresponding deoxyribonucleotides, an essential step for DNA synthesis and repair (Cory and Sato, 1983; Reichard, 1993; Tanaka *et al*., 2000). In spite of its essential function in cell proliferation, the role of this enzyme in endometrial receptivity is unclear.

Proteinase inhibitor 9 (PI-9, also designated SERPINB9, cytoplasmic antiproteinase 3) is a 42 kDa member of the ovalbumin family of serpins (Dahlen *et al*., 1997; Sun *et al*., 1997, 1998). PI-9 efficiently inhibits granzyme B (graB) *in vitro* and *in vivo* (Heusel *et al*., 1994; Sun *et al*., 1996; Bird *et al*., 1998), which is found in granules produced by citotoxic T lymphocytes (CTLs), and thus graB-mediated apoptosis. PI-9 is also an inhibitor of caspase-1 and, to a smaller extent, caspase-4 and caspase-8 (Annand *et al*., 1999; Dahlen *et al*., 1999; Kanamori *et al*., 2000). PI-9 is an estrogen-regulated gene (Krieg *et al*., 2001) which is also up-regulated in response to inflammatory stimuli. Since PI-9 inhibits both caspase 1, which is involved in the maturation of inflammatory cytokines, and granzyme B, which is used by CTLs to induce the death of target cells, an anti-inflammatory role for this protein is suggested. PI-9 is expressed at high levels in immune-privileged sites such as placenta and endometrium (Bladergroen *et al*., 2001), increasing in the latter during the window of implantation (Carson *et al*., 2002). Since uterine proinflammatory responses have been suggested to be the result of maternal immunological reactions to the embryo and include localized increased stromal vascular permeability (Psychoyos, 1973), edema (Potts, 1968) and increased levels of prostaglandins (Kennedy, 1977) in the regions of blastocyst implantation, it is reasonable to assume that PI-9 endometrial expression may be controlling those inflammatory processes.

The fact that an important proportion of genes displaying diminished expression in Group A with regard to Groups B and C are related to the modulation of the immune system is physiologically relevant because it stresses the importance of up-regulation of immunomodulators to create a milieu permissive for successful implantation. This suggests that implantation failure in this group of women could result from an exaggerated response of the elements that react against foreign proteins leading to rejection of the embryo, even before implantation takes place.

The complement system and NK cells are part of innate immunity and have an important role in protecting exposed epithelial surfaces such as the endometrium. Since the activated complement system can kill self or foreign cells, endometrial cells and an eventual embryo are protected by the local expression of molecules that inhibit complement activation such as complement component 4 binding protein (C4BP) (Ogata *et al*., 1993; Liszewski *et al*., 1996; Blom *et al*., 2001) and clusterin (Murphy *et al*., 1988; Choi *et al*., 1989; Jenne and Tschopp, 1989; McDonald and Nelsestuen, 1997). In addition, glycodelin (PAEP) modulates the activity of NK cells (Okamoto *et al*., 1991) and PI-9 (serpin B) inhibits cytotoxic activity of T lymphocytes and NK cells (Dahlen *et* al., 1997; Sun *et al*., 1997, 1998). All these molecules which modulate immune responses increase their expression level in the secretory phase endometrium, but their transcript level was reduced in Group A.

There are several potential disadvantages and limitations in the model used for the present study. First is the inability to detect differences in gene expression level that may occur in response to embryonic signals. Therefore, the findings are limited to the gene expression profiling resulting from endocrine-driven unfolding of receptivity.

Secondly, contrary to Groups B and C, the uterus of women from Group A had never been exposed to pregnancy, and this could be responsible for the differences found with the other two groups. Since we compared endometrial samples of women who had implantation of transferred embryos with women who never had implantation, we cannot exclude the possibility that pregnancy itself induced an imprinting in the endometrium that could lead to differential expression of certain genes in subsequent menstrual cycles. Experimental data from animals suggest that pregnancy produces a permanent epigenetic change in mammary gland cells, altering their subsequent response to hormones (Ginger *et al*., 2001). To our knowledge, there is no evidence for epigenetic modifications or permanent imprinting in the endometrium attributable to pregnancy. However, we cannot discard *a priori* the possibility that differences found between Group A and the controls, are consequence of previous pregnancy in control groups rather than the cause of implantation failure in the infertile group. Nevertheless, the endometrium of those women who get pregnant for the first time has never been exposed to pregnancy; therefore, the essential gene repertoire for endometrial receptivity must be expressed in such condition.

Thirdly, differential gene expression was determined in a mock oocyte donation cycle, and the possibility that expression

profiles differ between natural and artificially induced endometrial cycles can not be ruled out even if such difference does not affect the rate of implantation. Nevertheless, all three groups had this caveat and we assume that the transcriptional profile displayed by endometria refractory to implantation in previous oocyte donation cycles and in the mock cycle were similar.

Finally, only one-third of the genome was examined; therefore, the alterations found are most likely a partial view of the whole picture.

It is of interest that the genes C4BPA and PAEP whose transcript levels appeared decreased in the endometria of women from Group A have been also reported to be decreased in women with endometriosis (Isaacson *et al*., 1989; Kao *et al*., 2003) and/or in women with an inert IUD (Horcajadas *et al*., 2006). Diminished fertility in endometriosis is likely to be associated with an endometrial defect. Inert IUDs may reduce fertility interfering with several reproductive processes, but their primary effect is to cause an inflammatory reaction at the endometrial level (Croxatto *et al*., 1994). The common defect displayed by these groups of women suggests an important role of the genes in question in embryo implantation.

It is interesting also that several transcripts found to be decreased in Group A have been reported to be up-regulated by chorionic gonadotropin in the baboon endometrium during the window of implantation. They are MMP-7, CXCR4 and PAEP plus three others: serpin A3, complement component 4A and complement component 4B, which belong to the same family as serpin b and C4BPA, reported in the present investigation. Such finding also suggests these genes may have an important role in embryo implantation.

We conclude that repeated implantation failure in some recipients of oocyte donation is associated with an intrinsic defect in the expression of multiple genes in the endometrium at the onset of the implantation window. To our knowledge, reduced levels of several transcripts during the receptive period in endometria that display no other manifest abnormality have been demonstrated for the first time in association with implantation failure.

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Microarrays and PCR analysis—M.V.

Data interpretation—M.V., D.J.M., H.B.C.

Manuscript correction—D.J.M., H.B.C., L.V.

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