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Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology

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In humans, embryonic implantation and reproduction depends on the interaction of the embryo with the receptive endometrium. To gain a global molecular understanding of human endometrial receptivity, we compared gene expression profiles of pre-receptive (day LH+2) versus receptive (LH+7) endometria obtained from the same fertile woman (n = 5) in the same menstrual cycle in five independent experiments. Biopsies were analysed using the Affymetrix HG-U95A array, a DNA chip containing ~12 000 genes. Using the pre-defined criteria of a fold change ⩾3 in at least four out of five women, we identified 211 regulated genes. Of these, 153 were up-regulated at LH+7 versus LH+2, whereas 58 were down-regulated. Amongst these 211 regulated genes, we identified genes that were known to play a role in the development of a receptive endometrium, and genes for which a role in endometrial receptivity, or even endometrial expression, has not been previously described. Validation of array data was accomplished by mRNA quantification by real time quantitative fluorescent PCR (Q-PCR) of three up-regulated [glutathione peroxidase 3 (GPx-3), claudin 4 (claudin-4) and solute carrier family 1 member 1 (SLC1A1)] genes in independent LH+2 versus LH+7 endometrial samples from fertile women (n = 3) and the three up-regulated genes throughout the menstrual cycle (n = 15). Human claudin-4 peaks specifically during the implantation window, whereas GPx-3 and SLC1A1 showed highest expression in the late secretory phase. In-situ hybridization (ISH) experiments showed that GPx-3 and SLC1A1 expression was restricted to glandular and luminal epithelial cells during the mid- and late luteal phase. The present work adds new and important data in this field, and highlights the complexity of studying endometrial receptivity even using global gene-expression analysis.

Key words: endometrium/gene expression profiles/implantation/receptivity

Introduction

The endometrium is a specialized hormonally regulated organ that is non-adhesive to embryos throughout most of the menstrual cycle in humans and other mammals. In this environment, endometrial receptivity refers to a hormone-limited period in which the endometrial tissue acquires a functional and transient ovarian steroid-dependent status allowing blastocyst adhesion (Psychoyos, 1986). The scientific knowledge of the endometrial receptivity process is fundamental for the understanding of the mechanisms that govern embryonic implantation and human reproduction (Yoshinaga, 1994). This important knowledge can potentially be used to improve fertility in infertile patients whereas the opposite can be applied as an interceptive approach to prevent embryo implantation (Simón, 1996).

The luminal endometrial epithelium acquires receptivity mainly due to the presence of progesterone after appropriate 17β-estradiol (E_2) priming. This implantation window starts after 4–5 days and closes after 9–10 days of ovarian progesterone production or progesterone administration (Navot *et al*., 1991). Therefore, the receptive period is limited to days 19–24 of the menstrual cycle in humans. In fact, using this concept of E_2 and progesterone priming, a clinical endometrial receptivity window is induced routinely in ovum donation programmes to synchronize the timing of embryo transfer (Remohí *et al*., 1997).

Steroids, acting through their nuclear receptors in endometrial epithelial cells (EEC) induce the formation of a receptive phenotype. EEC undergo specific structural and functional changes. The morphological changes include modifications in the plasma membrane (Murphy, 2000) and cytoskeleton (Thie *et al*., 1995; Martín *et al*., 2000). The apical plasma membrane develops transitional adhesive properties by undergoing structural changes; long thin, regular microvilli are gradually converted into irregular, flattened projections and this process is known as the plasma membrane transformation (Murphy, 2000). The remodelling of the epithelial organization, from a polarized to a non-polarized phenotype, might prepare the apical pole for cell-to-cell adhesion (Thie *et al*., 1995). These changes occur within the complexity of the decidualization process that takes place in the stromal compartment (Irwin *et al*, 1989) and the endometrial vasculature. A number of biochemical markers for endometrial receptivity have been proposed over the years (Giudice, 1999) although thus far none of them have proven to be clinically useful.

Advances in gene expression profiling, facilitated by the development of DNA microarrays (Schena *et al*., 1995) represent a major progress in global gene expression analysis. The availability of this

technology makes it possible to investigate the endometrial receptivity process from a global genomic perspective (Carson *et al*., 2002; Kao *et al*., 2002). In the present study, we have used human endometrial samples and oligonucleotides microarray technology (Human Genome U95A Array, Affymetrix GeneChip® Array) to determine global changes in gene expression at the moment of acquiring endometrial receptivity. To gain new insights into this complex process we have taken a different approach than previously published related studies. We have investigated endometrial biopsies obtained from the same woman in pre-receptive (LH+2) versus receptive (LH+7) endometrium, where LH+2 and LH+7 are 2 and 7 days respectively after the LH surge. This study design allows us to avoid masking effects occurring with the use of sample clustering, both by pooling endometria from different women and grouping sampling days. Here, we present an analysis of the observed gene expression profiles at LH+2 versus LH+7. Array data were validated using three selected up-regulated genes. In addition, complementary real time quantitative fluorescent PCR (Q-PCR) and in-situ hybridization studies were performed throughout the menstrual cycle for some outstanding genes.

Materials and methods

Experimental subjects

The study population comprised 23 women (Caucasian; ages 23–39) who were having normal menstrual cycles. They were followed up during their natural cycles. Women were recruited after written informed consent. Overall, 31 biopsies were obtained using Pipelle catheters (Genetics, Belgium). A small portion of each specimen was examined histologically and dated according to the method of Noyes *et al*. (1950). Two endometrial biopsies were obtained within the same cycle from eight volunteers at days 2 (LH+2) and 7 (LH+7) after the LH peak [five women (n = 10 biopsies) for microarray studies and three women (n = 6 biopsies) for validation studies]. The LH surge was confirmed by urinary analysis, and contrasted with the histological results thus guaranteeing that the samples were taken in the pre-receptive status [early secretory phase (LH+2)], and within the window of implantation [receptive endometrium (LH+7)]. For gene expression and localization studies throughout the menstrual cycle, additional endometrial samples (n = 15) (one per woman) were performed. Endometrial biopsies were classified into five groups: early proliferative (days 5–8) (n = 3), mid–late proliferative (days 9–14) (n = 3), early secretory (days 15–18) (n = 3), mid-secretory (days 19–23) (n = 3) and late secretory (24–28) (n = 3). This project was approved by the institutional review board on the use of human subjects in research at the Instituto Valenciano de Infertilidad.

Methods

RNA isolation for chip analyses

Endometrial samples were snap-frozen in liquid nitrogen and stored at –70°C until further processing. Total RNA was extracted using the 'TRIzol method' according to the protocol recommended by the manufacturer (Life Technologies, Inc., USA). Briefly, homogenized biopsies (1 ml TRIzol reagent/75 mg tissue) were incubated at room temperature for 5 min. After addition of chloroform (0.15× volume of TRIzol), samples were incubated for 2.5 min at room temperature; thereafter, they were centrifuged for 15 min at 12 000 *g* (4°C). The aqueous phase was precipitated with an equal volume of 2-propanol, stored on ice for 10 min, and centrifuged for 30 min at 12 000 *g* (4°C). The pellet was washed with 75% ethanol and dissolved in DEPC-treated H_2O. The samples were kept on ice for 15 min and subsequently incubated for 10 min at 60°C. Approximately 1–2 µg of total RNA was obtained per mg of endometrial tissue. RNA quality was checked by agarose gel electrophoresis and RT–PCR.

Affymetrix chip hybridization

The analysis of hybridization onto the Affymetrix HG-U95A chip was carried out by Gene Logic (USA). Probe generation was performed as described in (Tackels-Horne *et al*., 2001). In brief, 1–5 µg total RNA was used to create double-stranded cDNA using the SuperScript Choice system (Life Technologies). First strand cDNA synthesis was primed with a T7-(dT24) oligonucleotide, extracted with phenol/chloroform and precipitated with ethanol to a final concentration of 1 µg/µl. From 2 µg of cDNA, cRNA was synthesized using Ambion's (USA) T7 MegaScript In Vitro Transcription Kit. To label the cRNA with biotin, nucleotides Bio-11-CTP and Bio-11-UTP (ENZO Diagnostics Inc., USA) were added to the reaction. After a 37°C incubation step for 6 h, the labelled cRNA was cleaned up according to the RNeasy Mini kit protocol (Qiagen). Then, cRNA was fragmented in fragmentation buffer (40 mmol/l Tris–acetate, pH 8.1, 100 mmol/l potassium acetate, 30 mmol/l magnesium acetate) for 35 min at 94°C. As per Affymetrix protocol, 55 µg of fragmented cRNA was hybridized on the HG_U95A chip for 24 h at 60 rpm in a 45°C hybridization oven. Chips were washed and stained with streptavidin phycoerythrin (SAPE; Molecular Probes, USA) in Affymetrix fluidics stations. To amplify staining, we added SAPE solution twice with an anti-streptavidin biotinylated antibody (Vector Laboratories, USA) staining step inbetween.

Hybridization of the probe arrays was detected by fluorometric scanning (Hewlet Packard Corporation, USA). After hybridization and scanning, the microarray images were analysed for quality control, examined for major chip defects or abnormalities in hybridization signal. After all the chips had passed quality control, the data were analysed using Affymetrix GeneChip software and the GeneExpress® (2001) release 1.3 version.

Data analysis

All samples were prepared as described and hybridized onto the HG-U95A array (Affymetrix) which contains ~12 000 full length sequences. The chip contains 16–20 oligonucleotide probe pairs per gene or cDNA clone. The probe pairs include perfectly matched sets and mismatch sets, both of which are necessary for the calculation of the average difference, or expression value, a measure of the intensity difference for each probe pair, calculated by subtracting the mismatch from the intensity of the perfect match. This takes into consideration variability in hybridization among probe pairs and other hybridization artefacts that could affect fluorescence intensities. Expression and fold change values for each woman were calculated using the GeneExpress® (2001) release 1.3 version software. All expression values that were <20 (or negative) were set at a default level of 20. Genes that gave absent calls in LH+2 and LH+7 samples were eliminated from the analysis. Analyses were performed in two steps. First, fold change levels (ratio between the LH+7 and LH+2 intensities from the same woman) for all individual women were calculated. Genes that were regulated with a fold change ⩾3 in at least four out of five women were selected. Secondly, for these genes the average expression and fold change levels were calculated based on all five women.

Principal component analyses (PCA; Joliffe *et al*., 1986) was performed on the original data set and consists of a matrix having the 10 different endometrial samples (statistical units) as rows and expression levels of 2000 random genes (statistical variables) as columns. The PCA Tool in Spotfire DecisionSite 6.3® projects this multidimensional space into a two-dimensional plot spanned by new variables called principal components ordered in decreasing amount of variability. The preserved variability for the first two components is 89% (for the first three components 93%).

Quantitative gene expression analysis by Q-PCR

Q-PCR assays were performed to validate the microarray data as well as for complementary studies throughout the menstrual cycle. Total RNA extraction and cDNA synthesis was performed as described (Martín *et al*., 2000). The ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems, USA) was used to determine relative gene expression quantification of glutathione peroxidase 3 (GPx-3), solute carrier family 1 member 1 (SLC1A1) and claudin-4 genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as the control housekeeping gene. The SYBR® Green I double-stranded DNA binding dye was the chemistry of choice for these assays. The Detector System, even running SYBR® Green chemistry, provides a broad linear dynamic range (at least five orders of magnitude) for detecting specific PCR products provided there are no associated by-products. Oligonucleotides (see sequences in Table I, in bold type) were designed using Primer Express® software. All Q-PCR assays were run using SYBR® Green PCR Master Mix and the universal thermal cycling parameters as indicated by the manufacturer. The relative quantification was performed by the standard curve method using the SYBR® Green I dye.

Table I. Oligonucleotides used in Q-PCR (bold type) and in-situ hybridization experiments (plain type)

Gene	Direction	Sequence (5′–3′)
GAPDH	**Forward**	**GAAGGTGAAGGTCGGAGTC**
GAPDH	**Reverse**	**GAAGATGGTGATGGGATTTC**
GPx-3	**Forward**	**GGTGGAGGCTTTGTCCCTAA**
GPx-3	**Reverse**	**AGCGCATGATGGGTATACCA**
CEP-R	**Forward**	**GCGCCCTCGTCATCATCA**
CEP-R	**Reverse**	**GGCCACCAGCGGATTGTA**
SLC1A1	**Forward**	**GTCCTGACTGGGCTTGCAA**
SLC1A1	**Reverse**	**CAACGGGTAACACGAATCGA**
GPx-3	Forward	CGATTTAGGTGACACTATAGCATGGGTGTACAGCCACGTG
GPx-3	Reverse	CGTAATACGACTCACTATAGGGGGGCCTTAGCCTGAATGCAC
SLC1A1	Forward	CGATTTAGGTGACACTATAGTGGCCCTATTCATTACATCCTC
SLC1A1	Reverse	CGTAATACGACTCACTATAGGGGGGTAGAACCATTTAGCCCAAG

Data are presented as a relative average value ± SEM after normalization with the average value of the housekeeping gene obtained in each designated group of the menstrual cycle. No direct comparison among different genes can be performed as the standard was composed of different cDNA species, each at different concentrations.

In-situ hybridization

Generation of sense and antisense RNA probes

With gene-specific primers containing either a T7 and/or SP6 RNA polymerase site, a unique part of the gene was amplified. The PCR product was precipitated overnight, centrifuged (14 000 *g*), washed in 70% ethanol and subsequently dissolved in H_2O. After purification on GFX columns (Pharmacia) the probe was diluted to a final concentration of 100 ng/µl. RNA probes were generated by in-vitro transcription, with 500 ng of template (according to the manufacturer, Boehringer–Roche) in the presence of digoxigenin (DIG) labelling mix (DIG-UTP, unlabelled nucleotides, blocking agents), transcription buffer, 10 mmol/l dithiothreitol (DTT), 1 IU/µl RNase inhibitor and 2–4 IU/µl the proper RNA polymerase. Incubations were performed at 37°C for 2 h and stopped by adding 25 mmol/l EDTA (pH 8.0), 400 mmol/l LiCl and an excess of 100% ethanol. The labelled product was precipitated overnight, centrifuged, washed in 70% ethanol and subsequently dissolved in H_2O with RNase inhibitor. Probe concentrations were estimated (according to the manufacturer Boehringer–Roche), 200 and 500 ng of probe was used for the in-situ hybridization. Endometrial samples were fixed in 4% formaldehyde for a maximum of 24 h and then in 70% ethanol. Fixed tissues were included in paraffin. Tissue sections were baked at 60°C for 2 h, dewaxed in xylene and rehydrated with decreasing ethanol concentrations. Subsequently the sections were treated for 20 min in 200 mmol/l HCl, washed in DEPC-treated Milli Q water and digested with proteinase K (1 µg/ml) in digest buffer (100 mmol/l Tris–HCl, 50 mmol/l EDTA pH 8.0) for 30 min at 37°C. Digestion was stopped in prechilled 0.2% (w/v) glycine in phosphate-buffered saline (PBS) for 10 min at room temperature. The slides were acetylated for 5 min with 0.25 % (w/v) acetic anhydride in 100 mmol/l triethanolamine buffer, followed by two washes in DEPC-treated Milli Q. Sections were prehybridized at hybridization temperature in a humid chamber with prehybridization mix, containing 52% (v/v) formamide, 21 mmol/l Tris–HCl, 1 mmol/l EDTA, 0.33 mol/l NaCl, 10% (v/v) dextran sulphate, 1× Denhardt's solution, 100 µg/ml salmon sperm DNA, 100 µg/ml tRNA and 250 µg/ml yeast total RNA. The slides were covered with a glass coverslip. After 2 h prehybridization mix was replaced with probe hybridization mix containing prehybridization mix with the following additions: 0.1 mmol/l DTT, 0.1% sodium thiosulphate, 0.1% (w/v) sodium dodecyl sulphate and 200 or 500 ng of DIG-labelled probe. The hybridization was carried out overnight (16 h) in a humid chamber at 50°C.

Slides were washed in 2× standard saline citrate (SSC) for 15 min at room temperature, followed by washes in 2× SSC, 1× SSC and 0.1× SSC for 15 min at hybridization temperature. Sections were digested by Ribonuclease A (20 µg/ml) in RNase buffer (0.6 mol/l NaCl, 20 mmol/l Tris–HCl, 10 mmol/l EDTA, pH 8.0) for 1 h at 37°C. After two washes (5 min at room temperature) in prechilled PBS and one wash in buffer 1 (100 mmol/l maleic acid, 150 mmol/l NaCl), the sections were incubated for 30 min with blocking solution [1 g/ml blocking reagent (Boehringer–Roche) in buffer 1]. Then the sections were incubated with anti-DIG-AP (Boehringer–Roche), diluted 1:500 in blocking solution, for 1 h at room temperature. After two washes in buffer 1 (15 min at room temperature) the slides were carefully wiped dry around the tissue and the sections were encircled with a Dako-pen®. The sections were covered with NBT/BCIP colour development reagent (Boehringer–Roche) and incubated in a humid chamber at room temperature for 2 h followed by an overnight incubation at 4°C. Finally, the slides were rinsed in water and counterstained with 0.1% (w/v) methyl green for 30 s. Slides were mounted in Kaiser's glycerol–gelatin.

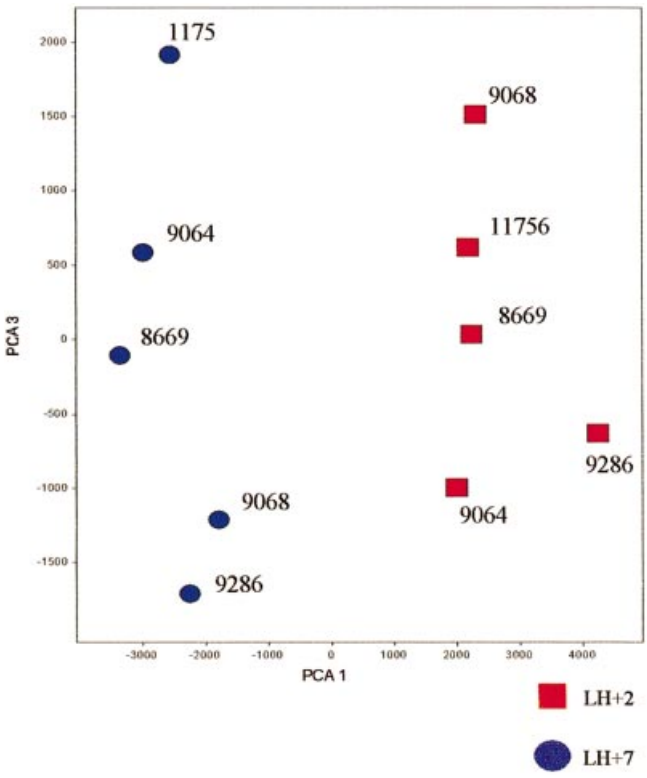

Figure 1. Principal component analyses (PCA) performed to cluster the samples based on the expression profile of 2000 randomly chosen genes. The PCA tool in Spotfire DecisionSite 6.3® software was used and showed a clear distinction between the LH+2 and LH+7 samples. Numbers refer to the individual women used. *x*- and *y*-axis show distinction between the expression values of the individual samples in arbitrary units (see Materials and methods).

Table II. Genes up-regulated in endometrium from LH+7 versus endometrium from LH+2 with a fold increase ⩾3 in at least four out of five women (plain text) and in all women (i.e. five out of five) (bold type)

Description	Accession ID	LH+2 average	LH+7 average	Fold change average	Functional Category
progestagen-associated endometrial protein (placental protein 14)	**J04129**	**35**	**3776**	**107**	**Secreted glycoprotein**
glutathione peroxidase 3 (plasma)	**D00632**	**30**	**1964**	**66**	**Enzyme**
nicotinamide N-methyltransferase	**U08021**	**24**	**916**	**39**	**Enzyme**
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	**AI928365**	**23**	**724**	**31**	**Transporter**
complement component 4-binding protein, alpha	**M31452**	**20**	**576**	**29**	**Immune response**
decay accelerating factor for complement (CD55, Cromer blood group system)	**M31516**	**36**	**809**	**22**	**Immune response**
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	**AF085692**	**20**	**442**	**22**	**Transporter**
transmembrane 4 superfamily member 3	**M35252**	**23**	**455**	**20**	**Transmembrane protein**
putative lymphocyte G0/G1 switch gene	**M69199**	**23**	**448**	**20**	**Regulatory protein**
aldehyde oxidase 1	**AF017060**	**20**	**378**	**19**	**Enzyme**
claudin 4	**AB000712**	**66**	**1139**	**17**	**Cell adhesion**
defensin, beta 1	**AI309115**	**75**	**1276**	**17**	**Antimicrobial peptide**
transcobalamin I (vitamin B12 binding protein, R binder family)	**J05068**	**20**	**339**	**17**	**Transporter**
tubulin, alpha 1 (testis specific)	**X06956**	**20**	**322**	**16**	**Cytoskeletal protein**
ephrin-A1	**M57730**	**26**	**402**	**16**	**Signal transduction**
dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	**X60708**	**20**	**306**	**15**	**Immune response**
laminin, beta 3 [nicein (125 kDa), kalinin (140 kDa), BM600 (125 kDa)]	**U17760**	**20**	**303**	**15**	**Cell adhesion**
monoamine oxidase A	**AA420624**	**39**	**583**	**15**	**Enzyme**
cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	**L32137**	**27**	**402**	**15**	**Structural protein**
amiloride binding protein 1 [amine oxidase (copper-containing)]	**U11863**	**23**	**338**	**15**	**Enzyme**
stanniocalcin 1	**U25997**	**21**	**311**	**14**	**Hormone**
S100 calcium-binding protein P	**AA131149**	**20**	**291**	**14**	**Calcium-related**
insulin-like growth factor binding protein 1	M74587	29	410	14	Regulatory protein
secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	**AF052124**	**147**	**1685**	**11**	**Structural protein**
Gastrin	**V00511**	**42**	**474**	**11**	**Regulatory protein**
immediate early response 3	**S81914**	**48**	**503**	**11**	**Membrane protein**
lipocalin 2 (oncogene 24p3)	AI762213	22	225	10	Protection factor
ceruloplasmin (ferroxidase)	**M13699**	**72**	**718**	**10**	**Enzyme**
Nuclear Factor 1, Variant Hepatic	**HG2339-HT2435**	**20**	**197**	**10**	**Nuclear factor**
superoxide dismutase 2, mitochondrial	X07834	31	297	10	**Enzyme**
Thrombomodulin	**J02973**	**22**	**212**	**10**	**Receptor**
thrombospondin 2	**L12350**	**34**	**318**	**9**	**Cell adhesion**
phospholipase A2, group IIA (platelets, synovial fluid)	**M22430**	**20**	**189**	**9**	**Enzyme**
short-chain dehydrogenase/reductase 1	**AF061741**	**111**	**1041**	**9**	**Enzyme**
Human complement component C4A	**U24578**	**163**	**1528**	**9**	**Enzyme**
leiomodin 1 (smooth muscle)	**X54162**	**24**	**224**	**9**	**Cytoskeletal protein**
carbonic anhydrase XII	**AF037335**	**27**	**246**	**9**	**Enzyme**
growth arrest and DNA-damage-inducible, alpha	**M60974**	**81**	**714**	**9**	**Regulatory protein**
granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	M18737	57	485	8	Enzyme
endothelial cell growth factor 1 (platelet-derived)	**M63193**	**47**	**398**	**8**	**Enzyme**
interferon, gamma-inducible protein 30	J03909	74	591	8	Enzyme
ribonuclease, RNase A family, 4	**D37931**	**47**	**372**	**8**	**Enzyme**
similar to rat HREV107	**X92814**	**49**	**373**	**8**	**Cell proliferation**
EST	**AW016815**	**59**	**451**	**8**	**Unknown**
EST	**AF070632**	**31**	**234**	**8**	**Unknown**
Granulysin	**M85276**	**161**	**1202**	**7**	**Antimicrobial protein**
myosin, heavy polypeptide 11, smooth muscle	AF001548	24	177	7	Muscle protein
protocadherin 17	AF029343	20	149	7	Cell adhesion
p8 protein (candidate of metastasis 1)	**AI557295**	**109**	**782**	**7**	**Unknown**
gamma-glutamyltransferase 1,gamma-glutamyltransferase 2	**M30474**	**42**	**299**	**7**	**Enzyme**
transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	M55153	73	522	7	Enzyme
dickkopf (Xenopus laevis) homolog 1	**AB020315**	**82**	**579**	**7**	**Development**
D component of complement (adipsin)	**M84526**	**85**	**590**	**7**	**Enzyme**
S100 calcium-binding protein A1	**X58079**	**22**	**150**	**7**	**Calcium-related**
decidual protein induced by progesterone	**AB022718**	**63**	**424**	**7**	**Unknown**
S100 calcium-binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	**W72186**	**93**	**618**	**7**	**Calcium-related**
inhibin, beta B (activin AB beta polypeptide)	**M31682**	**24**	**160**	**7**	**Hormone**
cathepsin W (lymphopain)	AF013611	31	201	7	Enzyme
EST	**W28438**	**20**	**131**	**7**	**Unknown**
EST	**W26466**	**26**	**165**	**6**	**Unknown**
endothelin receptor type B	**D13168**	**20**	**128**	**6**	**Receptor**
fibulin 5	AF093118	50	317	6	Matrix protein
clusterin (complement lysis inhibitor, apolipoprotein J)	**M25915**	**227**	**1427**	**6**	**Apoptosis**
EST	**AA203487**	**57**	**355**	**6**	**Unknown**
retinoic acid receptor responder (tazarotene induced) 3	**AF060228**	**132**	**818**	**6**	**Receptor**
inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	**AL022726**	**73**	**450**	**6**	**Unknown**

Table II. *Continued*

Description	Accession ID	LH+2 average	LH+7 average	Fold change average	Functional Category
myosin regulatory light chain 2, smooth muscle isoform	J02854	149	913	6	Muscle protein
keratin 7	**AJ238246**	**71**	**430**	**6**	**Structural protein**
DKFZP564J102 protein	**AL080065**	**29**	**175**	**6**	**Unknown**
epithelial protein up-regulated in carcinoma, membrane associated protein 17	U21049	31	182	6	Membrane protein
solute carrier family 15 (oligopeptide transporter), member 1	**U21936**	**33**	**197**	**6**	**Transporter**
Transgelin	**M95787**	**197**	**1152**	**6**	**Muscle protein**
matrix metalloproteinase 7 (matrilysin, uterine)	L22524	72	420	6	Enzyme
growth arrest-specific 1	L13698	53	306	6	Cell cycle
natural killer cell group 7 sequence	S69115	54	305	6	Membrane protein
retinol-binding protein 4, interstitial	X00129	20	112	6	Transporter
killer cell lectin-like receptor subfamily C, member 1,killer cell lectin-like receptor subfamily C, member 3	AJ001685	51	286	6	Receptor
related RAS viral (r-ras) oncogene homolog	M14949	23	126	6	Ras-related
insulin-like growth factor binding protein 3	**M35878**	**121**	**658**	**5**	**Regulatory protein**
guanylate binding protein 2, interferon-inducible	**M55543**	**38**	**202**	**5**	**GTP-binding protein**
UDP glycosyltransferase 1 family, polypeptide A9,UDP glycosyltransferase 2 family, polypeptide B	M57951	20	105	5	Enzyme
KIAA1077 protein	AB029000	24	127	5	Unknown
acyl-Coenzyme A dehydrogenase, short/branched chain	U12778	58	305	5	Enzyme
EST	AF038198	71	366	5	Unknown
retinoic acid receptor responder (tazarotene induced) 1	AI887421	20	102	5	Receptor
killer cell lectin-like receptor subfamily C, member 2	AJ001684	52	263	5	Receptor
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	AF004230	88	442	5	Membrane protein
prominin (mouse)-like 1	AF027208	70	351	5	Membrane protein
Oncogene Tls/Chop, Fusion Activated	HG2724-HT2820	30	146	5	Oncogene
Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	M33195	24	116	5	Immune response
fibrinogen-like 2	**AI432401**	**20**	**96**	**5**	**Coagulation factor**
small inducible cytokine subfamily C, member 1 (lymphotactin)	D63789	47	226	5	Chemotaxis
guanylate cyclase 1, soluble, beta 3	**X66533**	**23**	**110**	**5**	**Enzyme**
EST	AF070569	29	138	5	Unknown
serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	**X54486**	**687**	**3248**	**5**	**Inhibitor**
downregulated in ovarian cancer 1	U53445	33	155	5	Unknown
T cell receptor delta locus	X73617	30	135	5	Receptor
EST	AL049471	220	991	5	Unknown
death-associated protein kinase 1	**X76104**	**55**	**246**	**5**	**Enzyme**
secretory leukocyte protease inhibitor (antileukoproteinase)	X04470	544	2448	5	Inhibitor
tumor necrosis factor receptor superfamily, member 1B	AI813532	20	91	4	Receptor
crystallin, alpha B	AL038340	62	274	4	Inhibitor
synaptic nuclei expressed gene 2; KIAA1011 protein	AL080133	44	195	4	Unknown
actin, alpha 2, smooth muscle, aorta	X13839	519	2281	4	Muscle protein
CD3Z antigen, zeta polypeptide (TiT3 complex)	J04132	30	131	4	Receptor
interferon stimulated gene (20kD)	U88964	60	259	4	Unknown
apolipoprotein D	J02611	194	838	4	Transporter
integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	M59911	24	105	4	Receptor
anterior gradient 2 (Xenepus laevis) homolog	**AF038451**	**106**	**454**	**4**	**Unknown**
solute carrier family 16 (monocarboxylic acid transporters), member 3	U81800	132	569	4	Transporter
stromal cell-derived factor 1	U19495	36	153	4	Unknown
hyaluronan-binding protein 2	D49742	20	86	4	Enzyme
sialyltransferase 1 (beta-galactoside alpha-2,6-sialytransferase)	**X62822**	**55**	**232**	**4**	**Enzyme**
uridine phosphorylase	X90858	20	84	4	Enzyme
Human mRNA for annexin II, 5″UTR	D28364	91	367	4	Calcium-related
B-cell CLL/lymphoma 6 (zinc finger protein 51)	**U00115**	**94**	**377**	**4**	**Transcription factor**
protein S (alpha)	**M15036**	**34**	**137**	**4**	**Cofactor**
amine oxidase, copper containing 3 (vascular adhesion protein 1)	U39447	27	107	4	Enzyme
predicted osteoblast protein	D87120	55	219	4	Unknown
CD53 antigen	M37033	27	106	4	Membrane protein
adaptor-related protein complex 1, gamma 1 subunit,hypothetical protein FLJ20151	AL050025	66	259	4	Unknown
annexin A4	M82809	260	1007	4	Calcium-related
Duffy blood group	**X85785**	**56**	**218**	**4**	**Receptor**
KIAA0367 protein	**AB002365**	**44**	**168**	**4**	**Unknown**
tumor necrosis factor (ligand) superfamily, member 10	U37518	69	265	4	Apoptosis
trinucleotide repeat containing 15	W28281	146	557	4	Unknown
KIAA0843 protein	AB020650	34	128	4	Unknown
G protein-coupled receptor, family C, group 5, member B	AC004131	56	211	4	Receptor
small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	U10117	91	340	4	Cytokine
metal-regulatory transcription factor 1	**X78710**	**134**	**496**	**4**	**Transcription factor**
TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	L06139	40	147	4	Enzyme
KIAA0963 protein	**AC005390**	**36**	**130**	**4**	**Unknown**
t-complex-associated-testis-expressed 1-like	**U02556**	**266**	**952**	**4**	**Unknown**

Table II. *Continued*

Description	Accession ID	LH+2 average	LH+7 average	Fold change average	Functional Category
Tat-interacting protein (30kD)	AF039103	62	218	4	Coactivator
cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 5	J04813	20	70	3	Energy transduction
galactose-4-epimerase, UDP-	L41668	39	136	3	Enzyme
breast carcinoma amplified sequence 1	AF041260	25	86	3	Unknown
mitogen-activated protein kinase kinase kinase 5	U67156	162	556	3	Cell cycle
tumor necrosis factor, alpha-induced protein 2	M92357	191	646	3	Unknown
runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	D43969	21	69	3	Transcription factor
fibulin 2	X82494	158	515	3	Matrix protein
insulin-like growth factor 2 (somatomedin A)	J03242	97	316	3	Growth factor
chondroitin sulfate proteoglycan 2 (versican)	X15998	412	1334	3	Matrix protein
glutamyl aminopeptidase (aminopeptidase A)	L12468	53	170	3	Enzyme
EST	AL080060	22	71	3	Unknown
phosphodiesterase 4B, cAMP-specific (dunce (Drosophila)-homolog phosphodiesterase E4)	L20971	25	79	3	Enzyme
interleukin 15	U14407	66	202	3	Immune response
CGI-49 protein	AA005018	117	352	3	Unknown
natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	X15357	51	152	3	Receptor
microvascular endothelial differentiation gene 1	AL080081	33	100	3	Chaperone
major histocompatibility complex, class II, DQ beta 1	M81141	50	149	3	Glycoprotein
EST	W28743	41	107	3	Unknown
chromosome 11open reading frame 9	AB023171	28	73	3	Unknown

Results

DNA chip hybridization data analysis

Global gene expression profiles were analysed by microarray technology comparing the expression patterns of pre-receptive (LH+2) versus receptive (LH+7) endometrium in the same individual. Even allowing for individual biological divergence, our approach reveals a consistent pattern of differentially expressed genes. Trends of gene expression across samples were studied using PCA, which determines the key variables (principal components) in a multidimensional data set that explain the differences between samples based on the expression profiles of, in our case, 2000 randomly selected genes on the microarray. A clear distinction between the LH+2 samples and the LH+7 samples was obtained (see Figure 1). This indicates that the major consistent differences in gene expression profiles are caused by endometrial development between days LH+2 and LH+7.

We anticipated that the biological variation in gene expression levels between individual women would be substantial. Therefore, we used two endometrial biopsies, within the same menstrual cycle from individual woman at days LH+2 and LH+7. In this way, false positives and negatives were eliminated that could otherwise be introduced either by pooling samples or by comparing an LH+2 sample from one woman with an LH+7 sample from another woman. After we identified the regulated genes in the individual women, we only selected those genes that were regulated in at least four out of the five women participating in the study, suggesting that their regulation is of importance for endometrial receptivity.

Using the pre-defined criteria of a change in regulation >3-fold in at least four out of five women, we identified 211 regulated genes amongst which are 12 Expressed Sequenced Tag (EST). In total, 153 of these genes were specifically up-regulated in the LH+7 samples. In Table II, the average fold change and expression levels based on all five women are listed. Likewise, 58 down-regulated genes were identified and these are presented in Table III. When we applied the more stringent criteria of regulation in all five women, we identified 75 genes as being up-regulated $\geqslant$3-fold in all five women at LH+7 (see genes in bold type in Table II), whereas 10 genes were down-regulated using the same criteria (see genes in bold type in Table III).

In the lists of regulated genes, we identified genes that were already known to be differentially expressed during the receptive phase compared to the pre-receptive phase such as glycodelin (107-fold increase), osteopontin (11-fold increase), insulin-like growth factor binding protein-3 (IGFBP-3; 5.4-fold increase), crystallin alphaB (4.4-fold increase) and integrin, alpha 3 (4.3-fold increase). We also identified a number of genes for which the differential expression between the pre-receptive (LH+2) and the receptive (LH+7) endometria or even the presence in human endometrium has not been described before. These genes can be classified into different groups such as: immune modulatory genes, adhesion molecules, genes related to oxidative stress, cytoskeletal proteins and others (see functional categories in Tables II and III).

Validation of gene expression

Gene expression quantification by Q-PCR

To validate microarray findings, we quantified the expression pattern of four differentially expressed genes by Q-PCR in LH+2 versus LH+7 endometria in three independent women. The selected genes were: GPx-3, claudin-4 and SLC1A1 (up-regulated), and ACAT (down-regulated). Results corroborated the regulation profiles observed with DNA chip hybridization experiments.

In Q-PCR experiments, GPx-3 (Figure 2A) was up-regulated on average 113-fold in three independent LH+7 versus LH+2 samples in agreement with the 66-mean-fold increase obtained in the five women studied by microarray. Human claudin-4 analysis (Figure 2B) showed that this gene was up-regulated on average 2.9-fold in LH+7 versus LH+2 samples whereas in the microarray analysis a mean of 17-fold increase was registered. Validation Q-PCR studies showed that SLC1A1 gene (Figure 2C) was up-regulated on average 75-fold in LH+7 versus LH+2 samples and in the microarray analysis there was a 31-fold increase.

Quantitative gene expression analysis by Q-PCR throughout the menstrual cycle

To further corroborate our findings we investigated gene expression of the selected up-regulated genes GPx-3, claudin-4 and SLC1A1

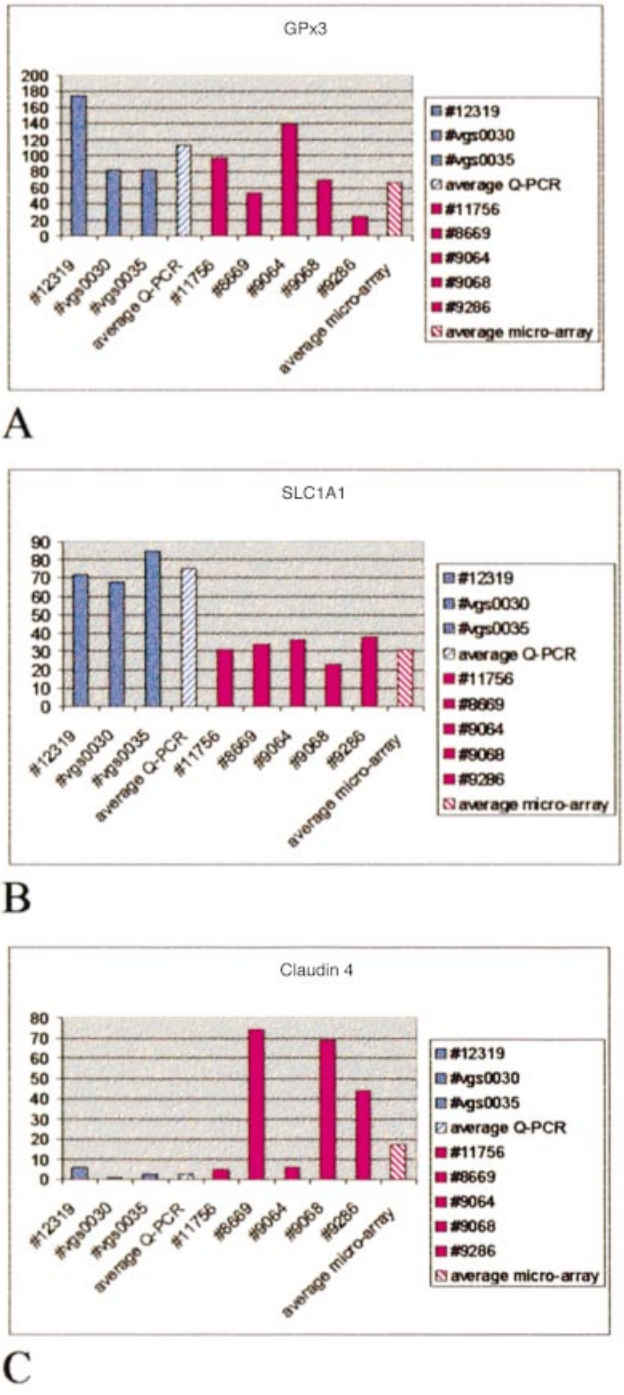

Figure 2. Validation studies. Schematic representation of the fold changes observed in endometrial samples obtained from individual women LH+2 and LH+7 for GPx-3, claudin-4 and SLC1A1 (up-regulated), by Q-PCR and microarray analyses. Bars indicate LH+7/LH+2 ratios using Q-PCR assays (Q1–Q3) and microarrays (A1–A5). The corresponding averaged values for each technique are also shown. Note the individual variation between the samples obtained from different women, indicating the need to compare samples from within the same patient as compared with average values.

throughout the entire menstrual cycle (Figure 3). GPx-3 gene expression increased a mean 43-fold during the receptive phase compared with the pre-receptive phase followed by a sharp increase in the late-luteal phase (Figure 3A). Human claudin-4 gene expression increased 4.5-fold during the receptive phase compared with the pre-receptive phase followed by a gradual decline in the late luteal phase, a profile consistent with a specific marker of endometrial receptivity

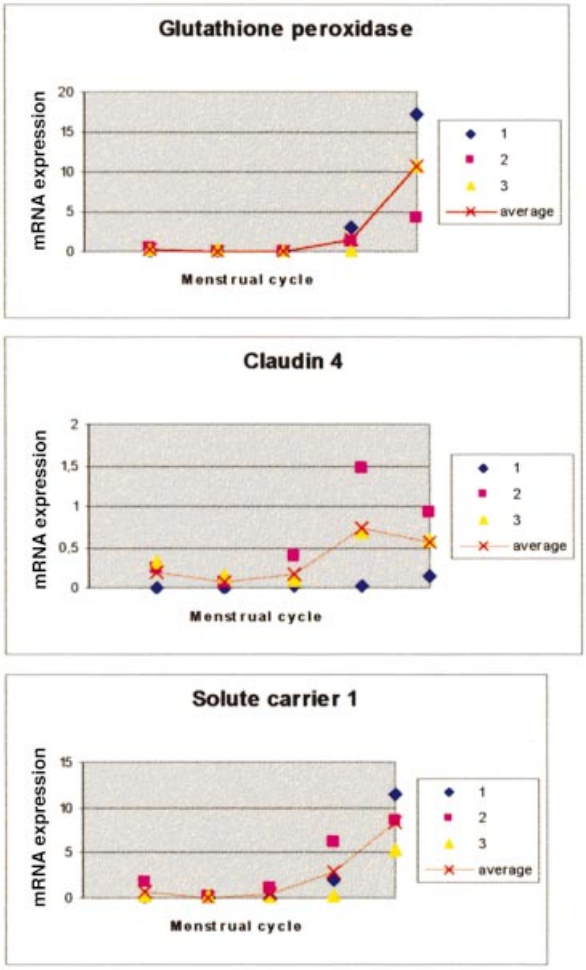

Figure 3. Pattern of mRNA expression of GPx-3, claudin-4 and SLC1A1 determined by Q-PCR analysis throughout the menstrual cycle. *y*-axis corresponds to normalized mRNA values for each experiment to demonstrate inter-individual variability. The relative fold-changes are shown in the Results section. The *x*-axis corresponds to the stage of the menstrual cycle: Group I, early proliferative (days 5–8) (n = 3); Group II, mid–late proliferative (9–14) (n = 3); Group III, early secretory (15–18) (n = 3); Group IV, mid-secretory (19–23) (n = 3); Group V, late secretory (24–28) (n = 3). 1, 2 and 3 indicate the three experiments performed with samples from five different women each. The averaged data are represented as a line.

(Figure 3B). Finally, SLC1A1 increased 7.2-fold during the receptive phase compared to the pre-receptive phase, again followed by a sharp increase in the late luteal phase (Figure 3C).

Gene expression localization in natural cycles by in-situ hybridization

To examine mRNA cellular localization we selected two of the three up-regulated genes (GPx-3 and SLC1A1). In-situ hybridization experiments were performed on three sets of endometrial biopsies as described in Materials and methods. For GPx-3 a clear gene expression pattern was observed, showing low or non-staining in the proliferative phase and increasing amounts in glandular and luminal epithelial expression during the secretory phase, consistent with the pattern observed in the Q-PCR analysis (see Figure 4A–J). In addition, for SLC1A1 we found increased staining in glandular epithelium during the secretory phase compared to mid-luteal glandular expression (see Figure 4K and L respectively), again consistent with the observed expression pattern by Q-PCR analysis.

Table III. Genes down-regulated in endometrium from LH+7 versus endometrium from LH+2 with a fold decrease ⩾3 in at least four out of five women (plain text) and in all women (i.e. five out of five) (bold type)

Description	Accession ID	LH+2 average	LH+7 average	Fold change average	Functional Category
hydroxyprostaglandin dehydrogenase 15-(NAD)	**L76465**	**583**	**42**	**14**	**Enzyme**
alkaline phosphatase, liver/bone/kidney	**AB011406**	**620**	**56**	**11**	**Enzyme**
potassium voltage-gated channel, subfamily G, member 1	AL050404	457	42	11	Channel
solute carrier family 15 (H+/peptide transporter), member 2	S78203	266	30	9	Co-transporter
calbindin 2 (29 kDa, calretinin)	X56667	789	92	9	Calcium-related
thyrotropin-releasing hormona	M63582	195	23	9	Hormona
catenin (cadherin-associated protein), alpha 2	**M94151**	**410**	**50**	**8**	**Cell adhesión**
G protein-coupled receptor 64	X81892	162	20	8	Receptor
opioid receptor, kappa 1	**L37362**	**153**	**20**	**8**	**Receptor**
solute carrier family, member 4	AF030880	155	22	7	Co-transporter
protein kinase C, theta	L01087	230	36	6	Enzyme
cysteine and glycine-rich protein 2	U57646	598	94	6	Development
major histocompatibility complex, class II, DO beta	X03066	654	104	6	Immune response
serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	**M68516**	**928**	**162**	**6**	**Inhibitor**
endothelin 3	J05081	239	42	6	Vasoconstrictor
thymidine kinase 1, soluble	M15205	156	28	6	Enzyme
ubiquitin carrier protein E2-C	U73379	374	67	6	Cell cycle
phosphatidylinositol-4-phosphate 5-kinase, type I, beta	X92493	478	87	5	Receptor
low density lipoprotein receptor-related protein 4	**AB011540**	**344**	**63**	**5**	**Receptor**
CDC20 (cell division cycle 20, S. cerevisiae, homolog)	U05340	178	33	5	Cell cycle
KIAA1069 protein	AB028992	151	28	5	Unknown
cyclin B2	AL080146	114	21	5	Cell cycle
37 kDa leucine-rich repeat (LRR) protein	U32907	142	27	5	Membrane protein
follistatin-like 3 (secreted glycoprotein)	**U76702**	**473**	**90**	**5**	**Regulatory protein**
TED protein	AF087142	126	24	5	Unknown
dynein, cytoplasmic, intermediate polypeptide 1	AI810807	287	58	5	Cytoskeletal protein
hydroxysteroid (11-beta) dehydrogenase 2	U26726	862	180	5	Enzyme
ankyrin 3, node of Ranvier (ankyrin G)	U13616	874	184	5	Membrane protein
chromosome 11 open reading frame 8	U57911	115	25	5	Unknown
olfactomedin related ER localized protein	U79299	333	73	5	Secreted glycoprotein
EST	AL109696	175	39	4	Unknown
N-acylaminoacyl-peptide hydrolase	J03068	91	21	4	Enzyme
neuroblastoma (nerve tissue) protein	D82343	192	45	4	Development
centromere protein A (17 kDa)	U14518	140	33	4	Histone-like
KIAA0888 protein	AB020695	122	29	4	Unknown
BTG family, member 3	D64110	829	200	4	Development
EST	AL050021	1133	277	4	Unknown
mitogen-activated protein kinase kinase 6	**U39064**	**265**	**67**	**4**	**Cell cycle**
forkhead box M1	U74612	166	42	4	Transcription factor
chromosome X open reading frame 5	Y15164	680	178	4	Unknown
EphB3	X75208	88	23	4	Receptor
pituitary tumor-transforming 1	AA203476	234	63	4	Cell cycle
msh (Drosophila) homeo box homolog 2	D89377	241	66	4	Morphogenesis
Arg/Abl-interacting protein ArgBP2	AF049884	153	42	4	Signal transduction
squalene epoxidase	**D78130**	**645**	**179**	**4**	**Enzyme**
KIAA0471 gene product	AB007940	661	185	4	Unknown
msh (Drosophila) homeo box homolog 1 (formerly homeo box 7)	M97676	877	247	4	Morphogenesis
hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	M31642	804	228	4	Enzyme
cyclin B1	M25753	248	71	3	Cell cycle
Ras association (RalGDS/AF-6) domain family 2	D79990	462	133	3	Ras-associated
isocitrate dehydrogenase 1 (NADP+), soluble	**AF020038**	**1904**	**557**	**3**	**Enzyme**
lipophilin B (uteroglobin family member), prostatein-like	AW015055	2568	763	3	Secreted protein
plakophilin 2	X97675	200	61	3	Structural protein
cytochrome b-5	L39945	839	262	3	Energy transduction
prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	U04636	84	26	3	Signal transduction
mitotic spindle coiled-coil related protein	AF063308	80	26	3	Cell cycle
creatine kinase, brain	X15334	2241	727	3	Enzyme
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	AF055001	1185	389	3	Stress-response protein

Table IV. Comparative results by families between genes up- and down-regulated in the receptive phase with a fold change ⩾3.0 in the study by Kao (n = 60 up-regulated and n = 87 down-regulated), Carson (n = 120 up-regulated and n = 152 down-regulated) and the present study with the same criteria. In those genes in which the accession number is different both codes are indicated

Family/accesion number	Gene name	Riesewijk	Kao	Carson
Up-regulated genes comparison				
Secretory proteins				
J04129	Placental protein-14/glycodelin	✓	✓	
M61886				
M57730	Ephrin-A1/B61	✓	✓	
AB020315	**Dickkopf/DKK1 (hdkk-1)**	✓	✓	✓
Inmmune modulators/cytokines				
M31516	Decay accelerating factor for complement (CD55, Cromer blood group system)	✓	✓	
M84526	Adipsin/complement factor D	✓	✓	
D63789	SCM-1 β precursor (lymphotactin)	✓	✓	
U14407	IL-15	✓	✓	
M55543	guanylate binding protein 2, interferon-inducible	✓		✓
X73617	T cell receptor delta locus	✓		✓
Transporter				
AB000712	**Claudin 4/CEP-R**	✓	✓	✓
U81800	Monocarboxilate transporter (MCT3)	✓	✓	
Extracellular matrix/cell adhesion				
U17760	Laminin S B3 chain	✓	✓	
AF052124	**Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)**	✓	✓	
J04765	✓			
J04765				
Proteases/peptidases				
M30474	γ-Glutamyl transpeptidase type II	✓	✓	
L12468	Glutamyl aminopeptidase/aminopeptidase A	✓	✓	
Other cellular functions				
U11863	Amiloide binding protein 1 (amine oxidase (copper-containing))	✓	✓	
X06956	α-Tubulin	✓	✓	
U12778	Acyl-Coenzyme A dehydrogenase	✓	✓	
M69199	Putative lymphocyte G0/G1 switch gene/G0S2 protein	✓	✓	
J02611	**Apolipoprotein D**	✓	✓	✓
AA420624	Monoamine oxidase A (MAOA)	✓	✓	
M68840				
M60974	Growth arrest and DNA-damage-inducible protein (gadd45)	✓	✓	
M22430	Phospholipase A2/RASF-A PLA2	✓	✓	
AF070569	ETS	✓		✓
AB002365	KIAA0367	✓		✓
AB022718	decidual protein induced by progesterone	✓		✓
W72186	S100 calcium-binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	✓		✓ M80563
AL022726	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	✓		✓
AL080065	DKFZP564J102 protein	✓		✓
Total genes analysed		153	60	120
Total matches		29	21	12
Down-regulated genes comparison				
Cell cycle				
U05340	CDC20 (cell division cycle 20, *S. cerevisiae*, homolog)	✓		✓
AL080146	Cyclin B2	✓		✓
M25753	Cyclin B1	✓		✓
Af063308	Mitotic spindle coiled-coil related protein	✓		✓
Transcription factor				
D89377	msh (Drosophila) homeo box homolog 2/MSX-2	✓	✓	
Vasoactive substance				
J05081	**Endothelin 3 (EDN3)**	✓	✓	✓
X52001				
Other cellular functions				
U57646	Cysteine and glycine-rich protein 2 (CSRP2)	✓	✓	
U79299	**Olfactomedin-related ER localized protein**	✓	✓	✓
M15205	Thymidine kinase 1, soluble	✓		✓
D82343	Neuroblastoma (nerve tissue protein)	✓		✓
U14518	Centromere protein A (17 kDa)	✓		✓
Total genes analysed		58	87	152
Total matches		11	4	9

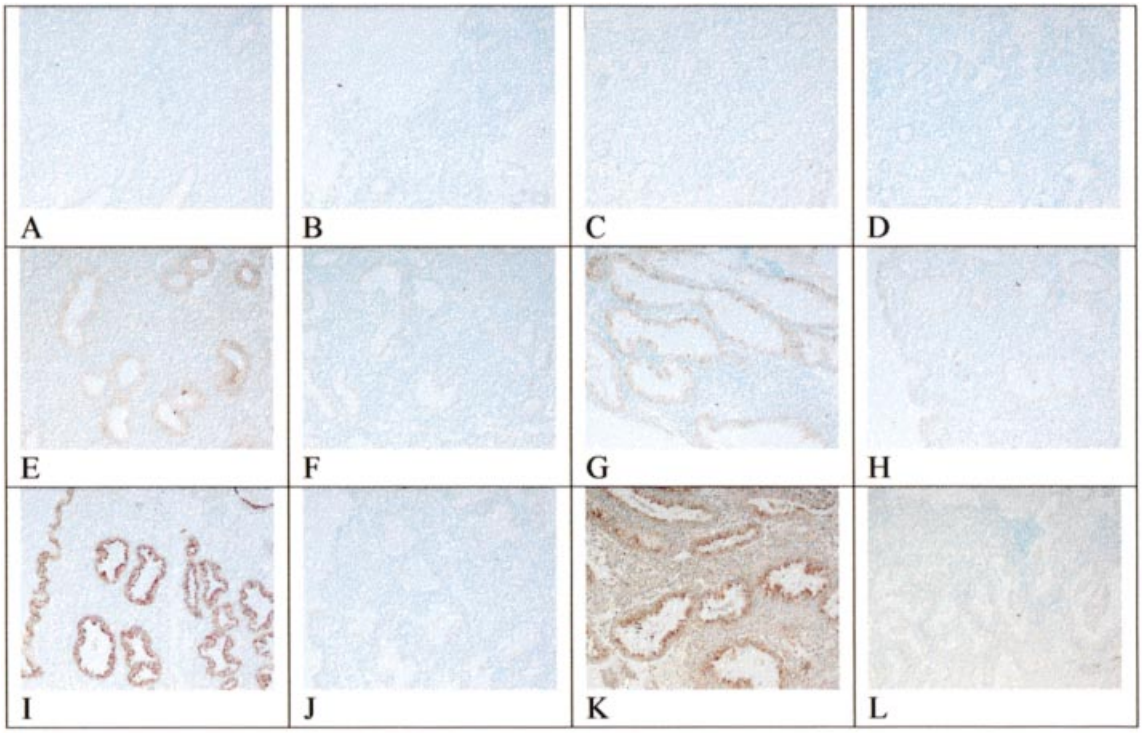

Figure 4. In-situ hybridization experiments for GPx-3 (complete menstrual cycle) and SLC1A (mid-luteal). GPx-3 antisense hybridization is shown in early proliferative (**A**), late proliferative (**C**), early luteal (**E**), mid-luteal (**F**) and late luteal (**I**) phases. GPx-3 sense hybridization was performed in the same sample as control: early proliferative (**B**), late proliferative (**D**), early luteal (**G**), mid-luteal (**H**) and late luteal (**J**) respectively. SLC1A1 antisense (**K**) and sense (**L**) probe staining was shown in mid-luteal endometrium.

Discussion

DNA microarray technology is a relatively new technology that allows, in a single assay, the simultaneous monitoring of the quantitative expression of thousands of genes. This technological breakthrough has the potential to add a global view to previously scientifically intractable physiological functions, cancer biology or cellular responses to pharmacological treatments (Debouck *et al*., 1999).

Endometrial receptivity is an essential, transient ovarian steroid-dependent status by which the human endometrium develops adhesiveness to the blastocyst allowing implantation and pregnancy to occur. As a crucial process it requires the regulated expression of a large set of genes that provide redundancy to the system as has been shown in the mouse model (Reese *et al*., 2001). In the human, considering specifically the in-vitro decidualization process of stromal cells, 71 differentially regulated genes have been reported (Popovici *et al*., 2000). Until the publication of two recent papers (Carson *et al*., 2002; Kao *et al*., 2002) previous studies on human endometrial receptivity relied on the investigation of individual genes or gene families.

To gain new insights into the endometrial receptivity process, we have taken a different approach that provides a hierarchical overview of the quantitative contribution of different genes obtained after a large simultaneous examination of 12 000 human genes, represented on the Affymetrix HG-U95A microarray. In this work, masking effects that may occur with the use of sample clustering, both by pooling endometrial biopsies and/or grouping sampling days have been avoided by using endometrial samples obtained from the same woman at LH+2 and LH+7 in a given menstrual cycle and repeated in five independent experiments from five fertile women.

From a biological standpoint, the main problem when assaying the expression of thousands of transcripts in complex organs is the biological variability. Partly, variability is due to genotypic differences but also on variation of gene expression independently of genetics. Considering an inbred population of mice genetically alike, it has been demonstrated that 0.8, 1.9 and 3.3% of all transcripts assayed were normally variable in the liver, testis and kidney respectively (Pritchard *et al*., 2001). This represents the level of natural variation of gene expression independently of genetics, but under identical environmental conditions. In contrast, humans are a heterogeneous population, with a variability component resulting from genotypic as well as environmental variation. These considerations emphasize the requirement of a solid experimental design when using genome-wide technology.

As previously stated, we compared endometrial biopsies taken at day LH+2 and LH+7 from one woman in a given menstrual cycle. By analysing five women and selecting only those genes that are regulated in at least four out of five of the women, we rigorously eliminate false positives due to differences in gene expression levels between individuals. Therefore the observed regulations in four or five out of five women suggest true biological relevance.

The present study has identified genes with recognized roles in human endometrial receptivity such as PP-14 (glycodelin) (Julkunen *et al*., 1986), osteopontin (Apparao *et al*., 2001) and IGFBP-3 (Zhou *et al*., 1994; Popovivi *et al*., 2000) (these genes were all up-regulated in five out of five women) and crystallin alphaB (Gruidl *et al*., 1997) (up-regulated in four out of five women). In addition, we have identified highly expressed genes, which are regulated in all five women investigated, which were not previously known to be involved in endometrial receptivity. These genes should now be considered to

have potential roles in endometrial receptivity and require experimental follow-up. We have selected three up-regulated GPx-3, claudin-4, and SLC1A1 genes in which we have validated the chip data by Q-PCR analysis in independent samples with the same design (LH+2 versus LH+7). Also, the expression of the three up-regulated genes has been quantified throughout the menstrual cycle using histologically dated endometrial samples from 15 different women. Moreover, cellular localization of mRNA of two regulated genes has been investigated by in-situ hybridization.

GPx-3, first described in 1991 (Esworthy *et al*., 1991), is a selenoprotein enzyme that protects cells from oxidative damage by catalysing the reduction of hydrogen peroxidase, lipid peroxides and organic hydroxyperoxide, by glutathione. The functional enzyme is a homotetramer secreted into plasma as an extracellular protein. In reproductive tissues of female mice, it is regulated by 17β-estradiol (Waters *et al*., 2001) and selenium. Its expression has been demonstrated to be increased in ovarian (Hough *et al*., 2001), uterine and breast cancer (Gorodzanskaya *et al*., 2001). In this report, we present for the first time the presence and regulation of this gene in human endometrial receptivity development.

GPx-3, as demonstrated in the DNA chip analyses, and further quantified by Q-PCR and localized by in-situ hybridization, showed highest expression levels in the late luteal phase, specifically in the glandular and luminal epithelial cells.

Human claudin-4 was first described by Katahira *et al*. (1997). It is an integral membrane protein and a member of a large family of transmembrane tissue-specific proteins, referred to as claudins, that are essential components of intercellular tight junction structures regulating paracellular ion flux. It is present in multiple tissues and expressed at high levels in prostate cancer (Long *et al*., 2001), pancreatic cancer and other gastrointestinal tumours (Michl *et al*., 2001). It is also up-regulated in ovarian cancer together with other secreted proteins (Hough *et al*., 2000). It has been reported that the expression of this protein is down-regulated by transforming growth factor-β (Michl *et al*., 2001). Tight junctions regulate paracellular conductance and ionic selectivity. Decreases in conductance values correlated directly with the kinetics of claudin-4 induction. Therefore, claudins have an important role in creating selective channels through the tight junction barrier (Van Itallie *et al*., 2001). In humans, the claudin superfamily consists of ⩾18 homologous proteins. They are located both in epithelial and endothelial cells in all tight junction-bearing tissues. Defects in claudins are associated with a variety of human diseases, demonstrating that claudins play important roles in human physiology (Heiskala *et al*., 2001). The present work demonstrates an important quantitative contribution of this gene during the window of receptivity in human endometrium. Human claudin-4 expression peaks specifically during the receptive phase followed by a gradual decline in the late luteal phase; this pattern is consistent with a specific marker of endometrial receptivity (Kao *et al*., 2002; Carson *et al*., 2002). No in-situ experiments were performed for this gene, but according to the existing information the expected localization is in the endometrial epithelium.

SLC1A1 also shows a decidualization-like expression pattern with a good expression in the glandular epithelium and a low expression level in stromal cells. This protein is a neuronal and epithelial glutamate transporter carrying L-glutamate and D-aspartate. It is essential for terminating the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft. It is a sodium-dependent membrane protein. It is expressed in several tissues (Arriza *et al*., 1994; Kanai *et al*., 1994) and now it appears to be modulated in human endometrium.

The mouse has become an indispensable model for the study of endometrial receptivity and implantation. Nevertheless, the comparison of the present study with elegant microarray-based studies in the mouse (Yoshioka *et al*., 2000; Reese *et al*., 2001; Tackels-Horne *et al*., 2001) indicates the existence of important differences in the genomics of endometrial receptivity and implantation between humans and mice. Firstly, there were few genes that were mutually identified in these two models and more importantly, genes functionally crucial for implantation in mice such as leukaemia inhibitory factor (Stewart *et al*., 1992) or cyclooxygenase-2 (Lim *et al*., 1997), as demonstrated by the different knockout models, were not detected as regulated genes in our human study. Even more intriguing is the fact that these genes were not detected in the mouse model during implantation using a similar genome-wide approach (Reese *et al*., 2001). As the authors pointed out, this may be due to highly spatially restricted expression around the implanting blastocyst. It should be mentioned that in the human the timing is not as restricted as it is in the mouse with a window of receptivity of ~3 days (Navot *et al*., 1991).

During the preparation of this manuscript two papers were published describing the use of DNA microarray technology in human endometrial receptivity research (Carson *et al*., 2002; Kao *et al*., 2002). Although we have used the same technology, there are differences in the study design which relate both to the menstrual date of the samples, to the pooling (or not) of the samples and to the analyses of the hybridization data.

Carson *et al*. compared pooled samples of three women in the early luteal phase (LH+2–4) with a pooled sample of three other women in the receptive phase (LH+7–9). Kao *et al*. compared average values of individual samples in the late proliferative stage ($n = 4$) with samples obtained from other individuals at the receptive phase (LH+8–10, $n = 7$). Our experimental design included the analyses of gene expression changes in five individual women during the development of the window of implantation (LH+2 and LH+7). This allowed us to make this comparison for all five fertile women in five independent experiments and to select only those genes that are consistently regulated, i.e. in at least four out of five women. In our view, not pooling the samples, or hybridization data, before the selection of differentially expressed genes will minimize the risk for both false positives and false negatives.

The differences in study designs are reflected in the lists of differentially expressed genes identified. Although the data sets of the different studies display a substantial degree of overlap, they are certainly not identical. For example GPx-3, which is highly regulated in our study, was not identified in the other studies. However, a direct comparison between the three studies is quite difficult, not only due to differences in study design, but also due to differences in the software and statistics used for analyses of the hybridization data. As a complex organ, the endometrium is composed of epithelium (luminal and glandular epithelium), stroma, endothelial cells and immune resident cells. Future studies focusing on each separate compartment must be designed in order to dissect out their relative contribution.

Taken together, these data suggest that microarray technology gives us new insights into the quantitative contribution of a large number of genes at given time points during endometrial development. However, the data do provoke a problem of interpretation of the functional relevance of these genes that certainly must be solved by incorporating functional studies. Unlike the mouse model, in which a similar number of down- and up-regulated genes have been found (Yoshioka *et al*., 2000; Reese *et al*., 2001), our data show a broader diversity of genes that are up-regulated (153 with fold increases up to >100) compared with those being down-regulated (58 with maximal fold decease of 14) in the creation of the endometrial implantation window. Finally, in the mouse model, genes typically showed 1.5–3-fold induction, whereas in the human all 211 genes met the pre-defined criterion of a ⩾3-fold

change in at least four out of the five women in order to obtain biologically reliable and relevant data.

In summary, this genome-wide analysis of human endometrial receptivity with DNA microarrays provided results that agree with previous findings as well as identified a significant number of novel genes involved in human endometrial receptivity development. Some of these newly recognized genes are immune modulatory genes, adhesion molecules, genes related to oxidative stress, cytoskeletal proteins and others. The findings presented herein clearly illustrate the differences in gene expression between human and rodent endometrial receptivity. In addition, they underline the problem of interpretation of the data based on different experimental designs and yet the functional relevance of these genes in endometrial receptivity must be solved by incorporating functional studies.

Note added in proof

After the acceptance of this work, one related paper was published: Borthwick, J.M., Charnock-Jones, D.S., Tom, B.D., Hull, M.L., Teirney, R., Phillips, S.C., and Smith, S.K. (2003) Determination of the transcript profile of human endometrium. *Mol. Hum. Reprod.*, **9**, 19–33. These workers performed a comparative genome-wide analysis comprising 60 000 gene targets in pooled samples of five women in the proliferative phase (LH+2–4) versus a further five pooled samples in the secretory phase.

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References

Apparao, K.B., Murray, M.J., Fritz, M.A. *et al*. (2001) Osteopontin and its receptor alpha (v) beta (3) integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *J. Clin. Endocrinol. Metab*., **86**, 4991–5000.

Arriza, J.L., Fairman, W.A., Wadiche, J.I. *et al*. (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.*, **14**, 5556–5569.

Carson, D.D., Lagow, E., Thathiah, A., Al-Shami, R., Farach-Carson, M.C., Vernon, M., Yuan, L., Fritz, M.A. and Lessey, B. (2002) Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol. Hum. Reprod.*, **8**, 971–879.

Debouck, C. and Goodfellow, P.N. (1999) DNA microarrays in drug discovery and development. *Nature Genet.*, **21** (Suppl. 1), 48–50.

Esworthy, R.S., Chu, F.-F., Paxton, R.J. *et al*. (1991) Characterization and partial amino acid sequence of human plasma glutathione peroxidase. *Arch. Biochem. Biophys*., **286**, 330.

Giudice, L.C. (1999) Potential biochemical markers of uterine receptivity *Hum. Reprod.*, **14** (Suppl. 2), 3–16.

Gorodzanskaya, E.G., Larionova, V.B., Zubrikhina, G.N. *et al*. (2001) Role of glutathione-dependent peroxidase in regulation of lipoperoxide utilization in malignant tumors. *Biochemistry*, **66**, 221–224.

Gruidl, M., Buyuksal, A., Babaknia, A. *et al*. (1997) The progressive rise in the expression of alpha crystallin B chain in human endometrium is initiated during the implantation window: modulation of gene expression by steroid hormones. *Mol. Hum. Reprod.*, **3**, 333–342.

Heiskala, M., Peterson, P.A. and Yang, Y. (2001) The roles of claudin superfamily proteins in paracellular transport. *Traffic*, **2**, 93–98.

Hough, C.D., Sherman-Baust, C.A., Pizer, E.S. *et al*. (2000) Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. *Cancer Res.*, **60**, 6281–6287.

Hough, C.D., Cho, K.R., Zonderman, A.B. *et al*. (2001) Coordinately up-regulated genes in ovarian cancer. *Biochem. Biophys. Res. Commun.*, **284**, 3869–3876.

Irwin, J., Kirk, D., King, R. *et al*. (1989) Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. *Fertil. Steril.*, **52**, 761–768.

Joliffe, I. (1986) *Principal Component Analysis*. Springer-Verlag, New York.

Julkunen, M., Koistenen, R., Sjöberg, J. *et al*. (1986) Secretory endometrium synthesizes placental protein 14. *Endocrinology*, **118**, 1782–1786.

Kanai, Y., Stelzner, M., Nussberger, S. *et al*. (1994) The neuronal and epithelial human high affinity glutamate transporter. Insights into structure and mechanism of transport. *J. Biol. Chem.*, **269**, 20599–20606.

Kao, L.C., Tulac, S., Lobo, S. *et al*. (2002) Global gene profiling in human endometrium during the window inplantation. *Endocrinology*, **143**, 2119–2138.

Katahira, J., Sugiyama, H., Inoue, N. *et al*. (1997) Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. *J. Biol. Chem.*, **272**, 26652–26658.

Lim, H., Paria, B.C, Das, S.K. *et al*. (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell*, **91**, 197–208.

Long, H., Crean, C.D., Lee, W.H. *et al*. (2001) Expression of Clostridium perfringens enterotoxin receptors claudin-3 and claudin-4 in prostate cancer epithelium. *Cancer Res.*, **61**, 7878–7881.

Martín, J.C., Jasper, M., Valbuena, D. *et al*. (2000) Increased adhesiveness in cultured endometrial-derived cells is related to the absence of moesin expression. *Biol. Reprod.*, **63**, 1370–1376.

Michl, P., Buchholz, M., Rolke, M. *et al*. (2001) Claudin-4: a new target for pancreatic cancer treatment using Clostridium perfringens enterotoxin. *Gastroenterology*, **121**, 678–684.

Murphy, C.R. (2000) The plasma membrane transformation of uterine epithelial cells during pregnancy. *J. Reprod. Fertil.*, **55** (Suppl.), 23–28.

Navot, D., Bergh, P., Williams, M. *et al*. (1991) An insight into early reproductive processes through the in vivo model of ovum donation. *J. Clin. Endocrinol. Metab*., **72**, 408–414.

Noyes, R.N., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1**, 3–25.

Popovici, R.M., Kao, L.C., Giudice, L.C. (2000) Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology. *Endocrinology*, **141**, 3510–3513.

Pritchard, C., Hsu, L., Delrow, J. *et al*. (2001) Project normal: defining normal variance in mouse gene expression. *Proc. Natl Acad. Sci. USA*, **98**, 13266–13271.

Psychoyos, A. (1986) Uterine receptivity for nidation. *Ann. NY Acad. Sci.*, **476**, 36–42.

Reese, J.K., Das, S., Paria, B. *et al*. (2001) Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. *J. Biol. Chem.*, **276**, 44137–44145.

Remohí, J., Gartner, B., Gallardo, E. *et al*. (1997) Pregnancy and birth rates after oocyte donation. *Fertil. Steril.*, **67**, 717–723.

Schena, M., Shalon, D., Davis, R.W. *et al*. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.

Simón, C. (1996) Potential molecular mechanisms for the contraceptive control of implantation. *Mol. Hum. Reprod.*, **2**, 475–480.

Stewart, C., Kaspar, P., Brunet, L. *et al*. (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature*, **359**, 76–79.

Tackels-Horne, D., Goodman, M.D., Williams, A.J. *et al*. (2001) Identification of differentially expressed genes in hepatocellular carcinoma and metastatic liver tumors by oligonucleotide expression profiling. *Cancer*, **92**, 395–405.

Thie, M., Harrach-Ruprecht, B., Sauer, H. *et al*. (1995) Cell adhesion to the apical pole of epithelium: a function of cell polarity. *Eur. J. Cell Biol.*, **66**, 180–191.

Van Itallie, C., Rahner, C. and Anderson, J.M. (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J. Clin. Invest.*, **107**, 1319–1327.

Waters, K.M., Safe, S. and Gaido, K.W. (2001) Differential gene expression in response to methoxychlor and estradiol through ERalpha, ERbeta and AR in reproductive tissues of female mice. *Toxicol. Sci.*, **63**, 47–56.

Yoshinaga, K. (1994) Endocrinology of implantation. In Tulchinsky, D. and Little, A.B. (eds), *Maternal and Fetal Endocrinology*. Saunders, Philadelphia, pp. 336–349.

Yoshioka, K., Matsuda, F., Takakura, K. *et al*. (2000) Determination of genes involved in the process of implantation: application of GeneChip to scan 6500 genes. *Biochem. Biophys. Res. Commun.*, **272**, 531–538.

Zhou, J., Dsupin, B.A. Giudice, L. *et al*. (1994) Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. *J. Clin. Endocrinol. Metab*., **79**, 1723–1734.

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