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Effect of an Intrauterine Device on the Gene Expression Profile of the Endometrium

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Context: The human endometrium acquires the ability to allow embryo attachment just for a specific period of time during each menstrual cycle. Understanding of the opposite functional status, referred to as refractoriness, can potentially be used to improve receptivity in infertile patients or as an interceptive approach to prevent gestation.

Objective: The objective of the study was to analyze the endometrial gene expression profile induced by an inert intrauterine device (IUD) at the time of implantation.

Design: We used a microarray containing more than 16,000 cDNAs to investigate the gene expression profile of receptive *vs.* refractory endometrium in the same women induced by the presence of an IUD. We compared the gene expression profile of endometrium obtained at LH+7 (window of receptivity) from the same women (n = 5) at the following time points: month 1, corresponding to the natural cycle before IUD insertion; month 3, just before IUD removal; and months 5 and 15. Data were validated by quantitative RT-PCR for IGF binding protein-3, peroxisome proliferative activated receptor-γ, glycodelin, and leukemia inhibitory factor and immunohistochemistry for glycodelin.

Results: We identified 147 genes significantly dysregulated in the refractory endometrium (78 up- and 69 down-regulated). Interestingly, 52 of these genes have previously been reported to be regulated during window of implantation. Surprisingly, the majority of genes (96.6%) remained dysregulated 2 months after IUD removal, but 1 yr later most of them (80%) returned to normal.

Conclusions: Our results reveal that a refractory endometrium in a fertile woman produced by an IUD is induced by preventing the normal transition to a receptive gene expression profile through effects on a specific subset or cluster of genes that impact on endometrial receptivity. **(*J Clin Endocrinol Metab* 91: 3199–3207, 2006)**

SUCCESSFUL HUMAN EMBRYONIC implantation requires a functionally normal embryo and a receptive endometrium. The window of implantation (WOI) is a self-limited period, in which the endometrial epithelium acquires a functional ability to support blastocyst adhesion. Uncovering the molecular basis of endometrial receptivity is fundamental for the understanding of the mechanisms that govern embryonic implantation and human reproduction (1–3).

In the last 3 yr, several studies (4–8) investigated the gene expression profile of the human endometrium during the WOI, compared with other phases of the menstrual cycle. These analyses have generated long lists of genes that are up- or down-regulated during this specific period in which the endometrium is receptive (9). However, it is not clear from these studies which of the many genes altered during the WOI are functionally important. Additional strategies have been designed to investigate the genomics of the endometrium in subfertile conditions such as endometriosis (10, 11), RU486 treatment (12, 13), or in patients with controlled ovarian stimulation in *in vitro* fertilization (14–16). These approaches have generated indirect evidence of the functional relevance of WOI genes.

Over the past four decades, intrauterine devices (IUDs) have been established as one of the most effective interceptive methods with a typical Pearl index around 0.5 (number of pregnancies per 100 women per year) (17). IUDs induce changes in the endometrium causing refractoriness that prevents embryonic implantation. We hypothesize that the refractoriness induced by the IUD must be due to changes in the WOI endometrial gene expression profile. Until now, only morphology and few studies of individual genes have been reported in the literature using IUDs with levonorgestrel (18, 19). In this work, we investigated the global gene expression profile of the endometrium during the WOI in the same fertile woman, in the presence or absence of an inert IUD. We also analyzed the short- and long-term effect of the IUD on the gene expression pattern of the endometrium.

Subjects and Methods

Study design and tissue collection

This study was conducted in accordance with the guidelines in The Declaration of Helsinki and was approved by the ethics committee of the institution at which the endometrial biopsies were obtained (Génesis Unidad de Fertilidad y Reproducción, Caracas, Venezuela) and processed (Instituto Valenciano de Infertilidad, Valencia, Spain). Written informed consent was obtained from all patients. Healthy fertile volunteers (aged 23–39 yr, with a body mass index between 19

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Abbreviations: IGFBP, IGF binding protein; IUD, intrauterine device; LIF, leukemia inhibitory factor; PPAR, peroxisome proliferative activated receptor; WOI, window of implantation.

TABLE 1. Endometrial genes up-regulated (A) and down-regulated (B) in the presence of the IUD and their outcome 2 months and 1 yr after IUD removal

Gene name	Gene symbol	GenBank accession no.	IUD	Gene recovery	
				2 months	1 yr
A					
Von Ebners gland protein 1	Vegp1	X52016	13.53		√
Calpain 6	**CAPN6**	**BC000078**	**11.20**		√
Serine proteinase inhibitor, clade A, member 5	SERPINA5	BC005353	10.28		√
Cytochrome P450, family 26, subfamily A, polypeptide 1	CYP26A1	H11516	8.44		√
Glycoprotein hormones, α-polypeptide	CGA	AA476314	8.03		√
Tissue factor pathway inhibitor 2	**TFPI2**	**L27624**	**7.32**		
α-2-Glycoprotein 1, zinc	AZGP1	AI041156	7.04		√
Matrix metalloproteinase 12	MMP12	H48216	7.04	√	√
MAPK kinase 6	**MAP2K6**	**U39657**	**6.57**		√
Transcribed locus	PITPN	AI028602	6.56		
Transmembrane 4 liter six family member 4	TM4SF4	R22914	6.49		√
Catenin, α2	**CTNNA2**	**R13007**	**6.40**		√
Glyoxylate reductase/hydroxypyruvate reductase	GRHPR	AI028668	6.08		
Epithelial V-like antigen 1	EVA1	H69578	6.02		√
Sorbitol dehydrogenase	**SORD**	**AI025167**	**5.87**		
Serum amyloid A2	SAA1	BC007038	5.62		√
ATPase, Ca^{2+} transporting, plasma membrane 2	ATP2B2	R19470	5.11		√
Hypothetical protein MGC11242	MGC11242	BC007685	5.06		√
S100 calcium binding protein P	**S100P**	**BC006819**	**5.05**		√
Major histocompatibility complex, class II, DOβ[ρ]	**HLA-DOB**	**BC008921**	**4.93**		√
Carbonic anhydrase II	CA2	BC011949	4.61		√
PPARγ	PPARG	BC006811	4.56		
Chemokine ligand 1	**CXCL1**	**X12510**	**4.51**		√
Keratin 8	**KRT8**	**BC008200**	**4.36**		√
Nuclear factor I/B	**NFIB**	**H43668**	**4.24**		√
Annexin A3	ANXA3	BC000871	4.08		√
Discs, large homolog 5	DLG5	AA490581	4.08		√
Branched chain keto acid dehydrogenase E1, β-polypeptide	**BCKDHB**	**T80553**	**4.06**		√
3′-phosphoadenosine 5′-phosphosulfate synthase 1	PAPSS1	AA001143	3.92		√
Phosphodiesterase 9[a]	PDE9A	BC009047	3.89		√
Solute carrier family 43, member 1	SLC43A1	R91733	3.79		√
Spermidine/spermine N1-acetyltransferase	SAT	BC001386	3.79		√
Annexin III	ANXA3	R33139	3.78		√
Matrix metalloproteinase 10	**MMP10**	**X07820**	**3.75**	√	√
Major histocompatibility complex, class II, DMβ	HLA-DMB	W72230	3.67		√
Tumor protein D52-like 1	TPD52L1	BC001653	3.55		√
Aldehyde dehydrogenase 3 family, member B2	**ALDH3B2**	**BC005807**	**3.52**		√
Cystatin B	CSTB	BC010532	3.41		√
Phosphoserine aminotransferase 1	PSAT1	BC002865	3.41		√
Carboxypeptidase B2	CPB2	N53447	3.37		√
G protein-coupled receptor 109B	GPR109B	R05515	3.35	√	√
NDRG family member 2	**NDRG2**	**BC010458**	**3.35**		
Secretoglobin, family 2A, member 1	SCGB2A1	AA441787	3.34		√
Interferon-γ receptor 1	IFNGR1	J03143	3.31		√
V-crk sarcoma virus CT10 oncogene homolog	CRK	BC008506	3.12		√
Metallothionein 1X	**MT1X**	**BC018190**	**3.04**		√
Atearoyl-CoA desaturase	SCD	BC008807	2.95		√
Hemoglobin, α2	HBA2	BC008572	2.91	√	√
Cancer susceptibility candidate 4	ANK3	AI024298	2.90		
Putative anti-CNGα1 cation channel translation product	CNGA1	W73475	2.89		√
Serine proteinase inhibitor, clade B, member 3	SERPINB3	AA393164	2.79	√	√
PHD finger protein 8	PHF8	AI024940	2.76		
Pirin	PIR	BC000644	2.75		√
Adenosylmethionine decarboxylase 1	AMD1	BC000171	2.73		√
CNDP dipeptidase 2	CNDP2	BC004909	2.73		√
IAP homolog C	BIRC3	U37546	2.72		√
Chromosome 6 open reading frame 142	C6orf142	BC009010	2.71		√
Sulfotransferase family, cytosolic, 1C, member 1	SULT1C1	BC005348	2.68		√
Serine hydrolase-like	SERHL	AI018678	2.67		√
Mitochondrial ribosomal protein L3	**MRPL3**	**R19209**	**2.61**		√
ATP-binding cassette, subfamily G, member 1	ABCG1	R13431	2.60		√
Glycerophosphodiester phosphodiesterase domain containing 3	MGC4171	BC002730	2.59		√
Family with sequence similarity 13, member A1	FAM13A1	H69334	2.57		√
Carnitine palmitoyltransferase 1A	CPT1A	BC001755	2.54		√

TABLE 1. *Continued*

Gene name	Gene symbol	GenBank accession no.	IUD	Gene recovery	
				2 months	1 yr
Folate receptor 1	**FOLR1**	**BC004555**	**2.53**		√
Septin 4	SEPT4	AI018682	2.51		√
Creatine kinase, brain	**CKB**	**AI005571**	**2.50**		√
Aldehyde dehydrogenase 6 family, member A1	**ALDH6A1**	**BC002714**	**2.50**		√
Cordon-bleu homolog	COBL	AA449163	2.45		√
Cytochrome b5 reductase 2	CYB5R2	BC001346	2.44		√
Dicarbonyl/liter-xylulose reductase	DCXR	BC002517	2.38		√
Vestigial like 1	VGLL1	BC003362	2.36		√
Fucosyltransferase 2	FUT2	BC003176	2.36		√
G protein-coupled receptor 132	GPR132	BC007783	2.35		√
Keratin 6C	KRT6A	BC002947	2.31		√
Similar to common salivary protein 1	LOC124220	BC009722	2.27		√
Hypothetical protein LOC285458	LOC285458	BC008915	2.25		√
CD9 antigen	CD9	BC011988	2.15		√
B					
Glutathione peroxidase 3	**GPX3**	**AI040990**	**−13.40**		
Glycodelin (placental protein 14 precursor)	**PP14**	**M34046**	**−10.18**		√
FXYD domain containing ion transport regulator 2	**FXYD2**	**R07511**	**−9.43**		
Left-right determination factor 2	LEFTY2	U81523	−9.35		√
Dipeptidylpeptidase 4	**DPP4**	**N30001**	**−8.47**		√
Similar to G-protein coupled receptor 111 [Pan troglodytes]	LEFTB	R63646	−7.94		
Truncated B-cell CLL/lymphoma 11B/T-cell receptor-δ constant region fusion protein	IGHM	W86653	−6.76		
Dehydrogenase/reductase member 3	DHRS3	BC003507	−5.03		√
Apolipoprotein C-II	APOC2	BC007022	−4.81		√
Transglutaminase 2	**TGM2**	**H17615**	**−4.61**		√
KIAA0664 protein	KIAA0664	H11775	−4.59		√
Melanophilin	MLPH	BC002503	−4.59		√
LIF	**LIF**	**NM_002309**	**−4.57**		√
Chemokine ligand 15	CCL14	R89491	−4.55		
Tetraspanin 8	TM4SF3	AI027644	−4.41		
Integral membrane protein 2[a]	**ITM2A**	**N30237**	**−.17**		
IL-2 receptor, β[ρ]	**IL2RB**	**AA292860**	**−3.92**		
3′-phosphoadenosine 5′-phosphosulfate synthase 2	**PAPSS2**	**AI066594**	**−3.86**		√
Complement component 4B, telomeric	C4A	BC012372	−3.86		√
CD52 antigen	CDW52	BC002434	−3.86		√
Collectin subfamily member 11	COLEC11	BC001899	−3.85		√
P8 protein	**P8**	**BC000654**	**−3.83**		√
IGFBP-3	**IGFBP3**	**AI032308**	**−3.76**		√
γ-Aminobutyric acid A receptor, α2	GABRA2	H07053	−3.66		
Putative lymphocyte G0/G1 switch gene	**G0S2**	**BC009694**	**−3.58**		
EPH receptor A1	EPHA1	M18391	−3.53		√
Growth arrest and DNA-damage-inducible, α[ρ]	**GADD45A**	**M60974**	**−3.52**		√
Src-like-adaptor	**SLA**	**R52482**	**−3.45**		√
Hyaluronan binding protein 2	**HABP2**	**N54494**	**−3.42**		
Endothelin receptor type B	**EDNRB**	**L06623**	**−3.41**		√
Vitronectin	VTN	BC006097	−3.30		√
ST3 β-galactoside α-2,3-sialyltransferase 5	SIAT9	R16334	−3.29		√
Homeodomain-only protein	**HOP**	**H48606**	**−3.27**		√
Guanine nucleotide binding protein, γ-transducing activity polypeptide 2	GNGT2	BC008663	−3.13		√
Leiomodin 1	**LMOD1**	**BC000730**	**−3.10**		√
5-hydroxytryptamine receptor 2B	**HTR2B**	**W61324**	**−3.08**		
Aspartoacylase 3	ACY3	BC008689	−3.07		
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NFATC3	AI016619	−3.07		√
Apolipoprotein C-I	**APOC1**	**W84514**	**−3.06**		√
Neural cell adhesion molecule 1	NCAM1	U63041	−3.02		√
Calponin 1, basic, smooth muscle	**CNN1**	**R21282**	**−2.86**		√
Serine proteinase inhibitor, clade G, member 1,	**SERPING1**	**BC011171**	**−2.83**		√
Cystatin F	CST7	BC015507	−2.83		√
Prolactin receptor	PRLR	R76436	−2.82		
IGFBP-6	**IGFBP6**	**BC004927**	**−2.80**		√
Clusterin	**CLU**	**AL048744**	**−2.79**		√
Fibulin 5	**FBLN5**	**H29224**	**−2.78**		√
Lumican	LUM	BC005302	−2.77		√
TNF superfamily, member 12	TNFSF13	NM_003809	−2.77		√
Transgelin	**TAGLN**	**BC004863**	**−2.73**		√
Complement component 3	C3	K02765	-2.65		√

TABLE 1. *Continued*

Gene name	Gene symbol	GenBank accession no.	IUD	Gene recovery	
				2 months	1 yr
Lymphocyte cytosolic protein 1	**LCP1**	**AI015062**	**−2.63**		√
Fibroblast growth factor 7	FGF7	M60828	−2.60		√
Proteoglycan 2, bone marrow	PRG2	R25649	−2.58		
Chromosome 6 open reading frame 48	C6orf48	AI015058	−2.56		√
Proline-serine-threonine phosphatase interacting protein 1	PSTPIP1	BC008602	−2.52		√
Laminin, α4	**LAMA4**	**R00706**	**−2.51**		
Cytochrome P450, family 2, subfamily C, polypeptide 9	**CYP2C9**	**R92994**	**−2.49**		
Thromboxane A synthase 1	TBXAS1	M80646	−2.43		
Cadherin 3, type 1, P-cadherin	CDH3	X63629	−2.38		√
Tryptophanyl-tRNA synthetase	WARS	N36174	−2.35		√
TAF1 RNA polymerase II, TATA box binding protein -associated factor, 250 kDa	**TAF1**	**R26894**	**−2.34**		√
Frizzled homolog 4	FZD4	AB032417	−2.33		√
Inositol −1 -monophosphatase 2	**IMPA2**	**T86604**	**−2.23**		√
Transgelin 3	TAGLN3	BC015329	−2.21		
UDP glucuronosyltransferase 1 family, polypeptide A9	UGT1A6	BC020971	−2.19		√
Proline dehydrogenase 1	**PRODH**	**W79586**	**−2.07**		√
Actin, α2, smooth muscle, aorta	**ACTA2**	**AI028256**	**−2.00**		
DEAD box polypeptide 46	DDX46	BC012304	−2.00		

WOI genes are in *bold*. √, Normal gene expression recovery.

and 25 kg/m^2) (n = 5) were monitored as follows: month 1 was a natural cycle before IUD insertion; in month 2, the IUD was inserted; in month 3, the IUD was removed; and from month 4 onward, natural cycles without any intervention were monitored. Endometrial biopsies were obtained at months 1, 3, 5, and 15 (biopsies 1, 2, 3, and 4, respectively) from each woman at LH+7 as determined by assaying the serum LH surge. Transvaginal ultrasound was performed in late follicular phase and early luteal phase to localize the dominant follicle or corpus luteum to assure that ovulation occurred. Patients resumed menstrual cycles consistent with their previous gynecological history. Overall, 18 biopsies (n = 5 at cycle 1, 3, and 5 and n = 3 for endometrial biopsy at cycle 15) were obtained using a Pipelle catheter (Genetics, Namont-Achel, Belgium) under sterile conditions from the uterine fundus. Endometrial dating was performed using the Noyes criteria (20). The inert IUD used in this study (Lippes Loop Intrauterine Double-S; Ortho Pharmaceutical Corp., Raritan, NJ) was chosen because of the absence of any hormone associated that could modify the refractoriness gene expression profile.

Gene expression profiling

Total RNA was extracted from human endometrium using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions and treated with RQ1 DNase I (Promega, Southampton, UK) for 30 min at 37 C and then reextracted with Trizol. RNA quality was assessed by loading 300 ng of total RNA onto an RNA Labchip and analyzed on an A2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany).

The microarray was printed on two slides (HMN1 and HMN2). The manufacturing of the slides is described by Rossi *et al.* (13) and Evans *et al.* (21). A full list of the cDNAs is available (http://www.path.cam.ac.uk/resources/microarray/microarrays/).

Array hybridization

The generation of the amplified labeled cDNA targets and the chip hybridization was performed using the method of Petalidis *et al.* (22). The fluorescence signal on the microarrays was acquired by using a Genepix 4100 microarray scanner (Axon Instruments, Foster City, CA). The scanned images were processed by using the GenePix Pro 3.0 software (Axon Instruments).

Statistical analysis

The raw data were normalized per spot and per chip using GeneSpring version 7.0 software with intensity dependent (Lowess) normalization (percent of the data used for smoothing 10%) and per chip normalized to 50th percentile. Low hybridization signals were removed to give an average of 10,000 different RNA transcripts expressed above background. For each cDNA spot on the array, a ratio was derived in which the signal from the test sample (Cy5) was expressed relative to its expression in the Cy3-labeled control sample (same women before IUD) hybridized to the array at the same time. Genes that showed statistically different expression levels were identified by performing pairwise comparisons between the different time points using Welch's *t* test with Benjamini and Hochberg multiple testing correction. Transcripts with a $P < 0.05$ were selected for inclusion in Table 1. In addition, fold change ratios between groups (*i.e.* LH+7 pre-IUD *vs.* LH+7 in the presence of the IUD) were subsequently derived, and up- and down-regulated genes were selected on the basis that they showed a change of at least 3-fold in three of five women and also had an overall median fold change over 2 and a $P < 0.05$ (23, 24).

Array validation

To verify the results obtained from the cDNA microarray, real-time PCR (Taqman) was performed for four selected genes: IGF binding protein (IGFBP)-3, peroxisome proliferative activated receptor (PPAR)-γ glycodelin (also known as placental protein 14) and leukemia inhibitory factor (LIF). Immunohistochemistry was also performed for glycodelin. It was carried out on endometrial sections using an LSAB peroxidase kit (Dako Corp., Barcelona, Spain) with a protocol previously described (25). It used glycodelin polyclonal antiserum diluted 1:1000 (this antibody was kindly provided by Dr. Koistinen, Helsinki, Finland).

Relative expression levels of each gene in total RNA from endometrium was determined by real-time RT-PCR using specific primers and FAM-labeled probes for each gene: primer sequence (5′-3′) for IGFBP-3 forward, GCACAGATACCCAGAACTTCTCC, reverse, CAGGTGATTCAGTGTGTCTTCCA, and probe, AGACAGAATATGGTCCCTGCCGCA; PPARγ forward, CAGAGCAAAGAGGTGGCCAT, reverse, GCTTTTGGCATACTCTGTGATCTC, and probe, CATCTTTCAGGGCTGCCAGTTTCGC; glycodelin forward, TGGTCTGTGGTGTCCCGG, reverse, AGGGAGATGTTGTTGGTCGC, and probe, ACATCCCCCAGACCAAGCAGGACCT. LIF transcript levels were measured using primers provided as an assay on demand (Applied Biosystems, Warrington, UK). Primers were labeled with 5′FAM and 3′TAMRA. Real-time PCR was performed using an ABI PRISM 7700 sequence detection system (TaqMan) according to the manufacturer's instructions (Applied Biosystems). cDNA was produced from each RNA sample by reverse transcription with random hexamers using 5 μg of total RNA with 200 IU Superscript RT (Invitrogen Life Technologies). The expression values obtained were normalized against those from the control ribosomal 18S to account for differing amounts of starting material.

Expression levels in different endometrial biopsies from each patient were compared using the paired *t* test; statistical significance was accepted when $P < 0.05$.

Results

Identification of genes involved in endometrial refractoriness

In the presence of the IUD (biopsy 2 *vs.* biopsy 1), 78 transcripts were up-regulated and 69 genes down-regulated. Table 1 shows the fold increase (A) and decrease (B) of the 147 genes with known identity that were dysregulated at the time of implantation. WOI genes (7, 15) characterize the normal transition from nonreceptive to a receptive state. In our study, 52 of them (35%) corresponded to WOI genes and are presented in Table 1 (*bold*).

We then sought to determine the effect of removal of the IUD on the endometrial gene expression. Two months after IUD, biopsy 3 was taken at LH+7 in the same patient. When we compared biopsy 3 (LH+7, 2 months after IUD insertion) *vs.* biopsy 1 (LH+7 before IUD insertion), 142 of the 147 dysregulated transcripts (96% of the total) remained dysregulated. Only five transcripts recovered their normal expression 2 months after IUD insertion: matrix metalloproteinase 12 (macrophage elastase), matrix metalloproteinase 10 (stromelysin 2), G protein-coupled receptor 109B, hemoglobin-α2 and serine (or cysteine) proteinase inhibitor, and clade B (ovalbumin) member 3. Interestingly, only one gene, matrix metalloproteinase 10, was a known WOI gene.

To examine the long-term effect imposed by the IUD on endometrial receptivity, we analyzed the gene expression profile in biopsy 4 obtained at LH+7, 1 yr after IUD removal. Only three endometrial biopsies could be collected: one patient refused to continue the study and the other one was under oral contraceptive treatment. Of the three biopsies, one was excluded because the histology revealed that the tissue showed incorrect dating. The gene expression profiles of these samples were compared with biopsy 1 (pre-IUD) from the same women. We found that, 1 yr later, 118 genes (80% of the total) recovered their normal expression at the time of implantation. Figure 1 shows the expression profile time line after the IUD removal of those 147 genes that were up- and down-regulated in the presence of IUD. It is remarkable that most of the genes (96%) showed very similar expression levels in the presence of IUD and 2 months after IUD. These results show an unexpectedly long-term effect of the IUD on the endometrial gene expression profile. However, most of the genes recovered their normal expression 1 yr after IUD removal (80%) (Fig. 1).

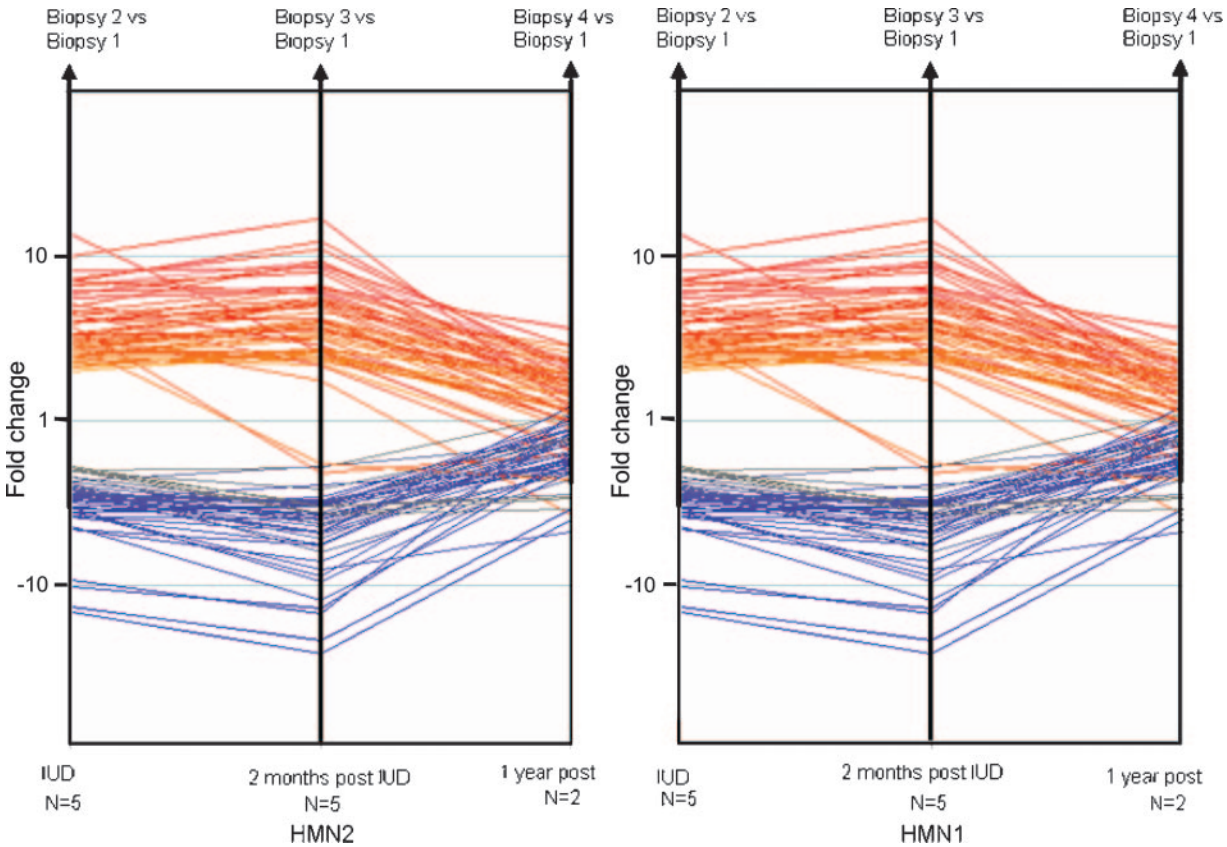

FIG. 1. Graphs indicating median ratio across each experimental group for final gene list. Only genes identified as altered by the presence of an IUD are shown (listed in Table 1). The fold change for each gene was calculated relative to expression of the same gene in biopsy 1 from the corresponding patient, and then the median for that gene in all the patients was plotted at each time point in the vertical axis: line 1 (biopsy 2 *vs.* biopsy 1), line 2 (biopsy 3 *vs.* biopsy 1), and line 3 (biopsy 4 *vs.* biopsy 1). Each *panel* represents one slide, HMN1 and HMN2. All genes are by definition identified as altered in expression in biopsy 2 (IUD present) *vs.* biopsy 1 (pre-IUD). The majority remain altered in biopsy 3 (2 months after IUD removal) *vs.* biopsy 1. However, 1 yr after biopsy removal, the expression of most genes has returned to similar levels as seen in biopsy 1.

We compared the expression changes of the 52 dysregulated WOI genes with the natural cycle during the transition from LH+2 (nonreceptive) to LH+7 (receptive). Thirty-two of 33 WOI genes down-regulated at LH+7 in the presence of an IUD are normally up-regulated during the transition from LH+2 to LH+7. Relevant genes include glycodelin or LIF, two of the genes normally up-regulated at the time of implantation. On the other hand, of the 19 WOI genes increased at LH+7 with the IUD, 15 of them are usually down-regulated between LH+2 and LH+7. These genes include α-catenin and nuclear factor I/B.

Array validation

Real time RT-PCR was used to verify the changes in RNA expression levels indicated by the cDNA analysis. Four genes, IGFBP-3, PPARγ, LIF, and glycodelin, which apparently changed on average more than 2-fold in the presence of the IUD and which were highly expressed, were chosen for verification. Levels of these transcripts were measured by real-time RT-PCR in each cDNA sample relative to a reference RNA, and the values were corrected for differences in loading relative to the 18S ribosomal RNA. RNA transcripts for PPARγ showed consistent up-regulation in all five patients in the endometrium in the presence of the IUD (Fig. 2B). PPARγ expression remained significantly elevated two cycles after IUD removal. In contrast, the array analysis indicated that transcripts for IGFBP-3 were down-regulated in the presence of an IUD. Analysis of IGFBP-3 mRNA by real-time RT-PCR confirmed this decrease with the IUD in place (Fig. 2A). However, one of the five patients (patient 4) did not show a decrease in IGFBP-3 on IUD insertion, so this change was not quite statistically significant. As in the case of PPARγ, IGFBP-3 levels remained significantly altered two cycles after IUD removal. Similarly, glycodelin transcripts also decreased in the presence of an IUD with the IUD in place (Fig. 2C). Decreased expression for LIF was also confirmed by RT-PCR (Fig. 2D). As seen with IGFBP-3, one patient (patient 4) did not show this change, but the expression of glycodelin and LIF remained decreased at the time of the biopsy in the other patients two cycles after IUD removal. Although there was some heterogeneity in response between patients (*e.g.* patient 4), the real-time RT-PCR results confirmed the changes identified by the microarray analysis. This indicates that the microarray analysis has reliably detected changes in gene expression after IUD treatment of secretory phase endometrium.

In the natural cycle, immunoreactive glycodelin appears in endometrial glands 5 d after ovulation, and it is detectable in neither luminal epithelium nor stroma (26). Glycodelin immunoreactivity was clearly lower at LH+7 in the presence of

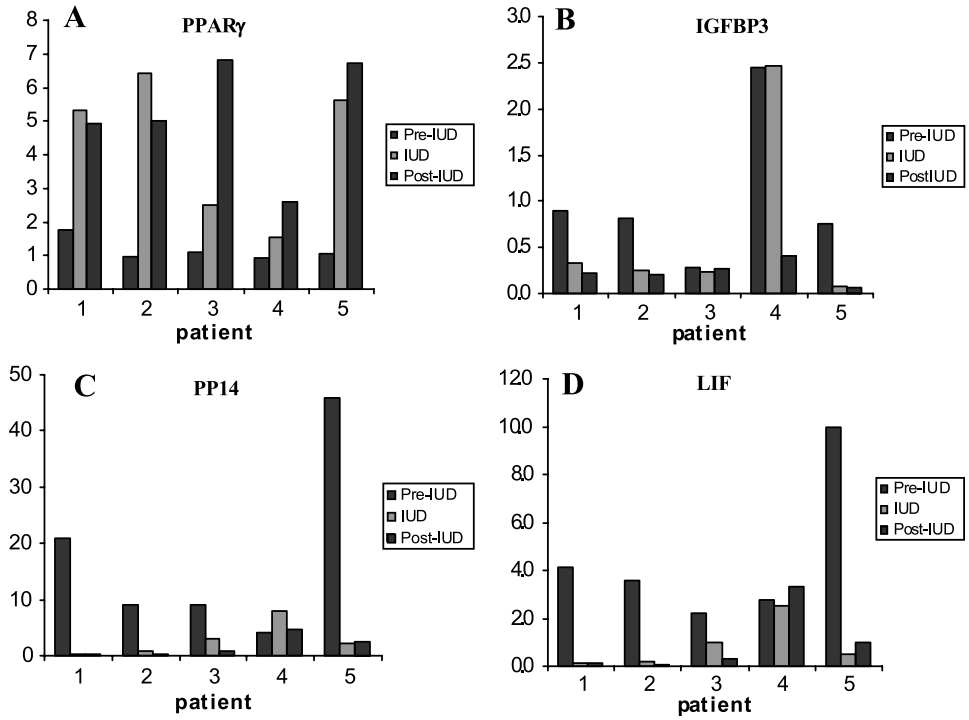

FIG. 2. Real time RT-PCR analysis of transcript levels in endometrium of five patients at LH+7 before IUD insertion (pre-IUD, biopsy 1), two cycles after IUD had been in place (IUD present, biopsy 2), and 2 months after IUD removal (post-IUD, biopsy 3). RNA transcript levels were measured in the five patients relative to a reference endometrial RNA sample, and values are expressed in arbitrary units relative to this reference. A, PPARγ transcript levels increased significantly from a median of 1.1 (range 0.9–1.7) to 5.3 (range 1.5–6.4) in paired biopsies from the same patients with the IUD in place ($P < 0.03$). PPARγ expression remained significantly elevated two cycles after IUD removal, with median expression levels of 5.0 (range 2.6–6.8; $P < 0.01$). B, Transcripts for IGFBP-3 were down-regulated from a median expression level of 0.8 (range 0.3–0.9) to 0.26 (range 0.1–2.5) in the presence of an IUD. IGFBP-3 levels remained significantly altered two cycles after IUD removal with a median expression level of 0.22 (range 0.1–0.4). C, Placental protein 14 (PP14) (glycodelin) transcripts also decreased in the presence of an IUD from a median expression at LH+7 of 9.2 (range 4.1–49) before IUD to a median of 2.10 (range 0.4–8.0) with the IUD in place. This decrease was sustained two cycles after IUD removal. D, LIF expression decreased from a median of 3.6 (range 2.2–10.0) to 0.5 (range 0.1–2.5) with the IUD in place and remained decreased 2 months after IUD removal.

IUD and LH+7, 2 months after the IUD removal in the same fertile woman (Fig. 3). Although the localization in both cases is still present in the glandular epithelium, the endometrium with or after IUD showed a decrease in the number of glands stained.

Discussion

The molecular mechanisms implicated in the uterine refractoriness produced by the IUD are still unknown (27). This is the first time that a genome-wide analysis of the effect of an inert IUD on the endometrium is presented. It is remarkable that the gene expression profile of the endometrium with an IUD showed a consistent pattern in the five women studied. Most of the endometrial biopsies immediately after IUD removal showed inflammatory changes, vascular congestion, and edema consistent with the local effect of the IUD as it has been previously reported (28). We used stringent criteria and reported our results after considering 3.0-fold changes in at least three of five women, a median fold change over 2, and a $P < 0.05$. We identified genes for apoptosis, ion transporters, immunomodulators, secretory proteins, signal transduction, membrane proteins, and transcription factors (Table 1). Interestingly, 52 of the 147 identified genes belong to the class of previously identified WOI genes, which are characteristically up- or down-regulated when the endometrium becomes receptive during the natural cycle (Table 1). These 52 transcripts appeared dysregulated in the presence of an inert IUD, compared with their normal expression during the WOI.

Furthermore, comparing the genomic profile between the receptive (15) and refractory endometrium (present work), we identified four unique groups of genes related to the refractoriness induced by the IUD with potential functional relevance (Table 2). The first group is composed by genes that were up-regulated during the implantation window in women during the natural cycle but significantly decreased in the presence of IUD (22 genes). Group 2 comprises genes that are down-regulated during the WOI but significantly increased in endometrium in fertile women with IUD (10 genes). Group 3 was formed by six genes up-regulated during the WOI and further increased in the presence of IUD. Finally, group 4, composed of one gene, which is down-regulated during the WOI and in the presence of the IUD, is further decreased. All these genes recovered their normal expression 1 yr after the IUD removal. This suggests that one of the primary mechanisms of action of the IUD is to prevent

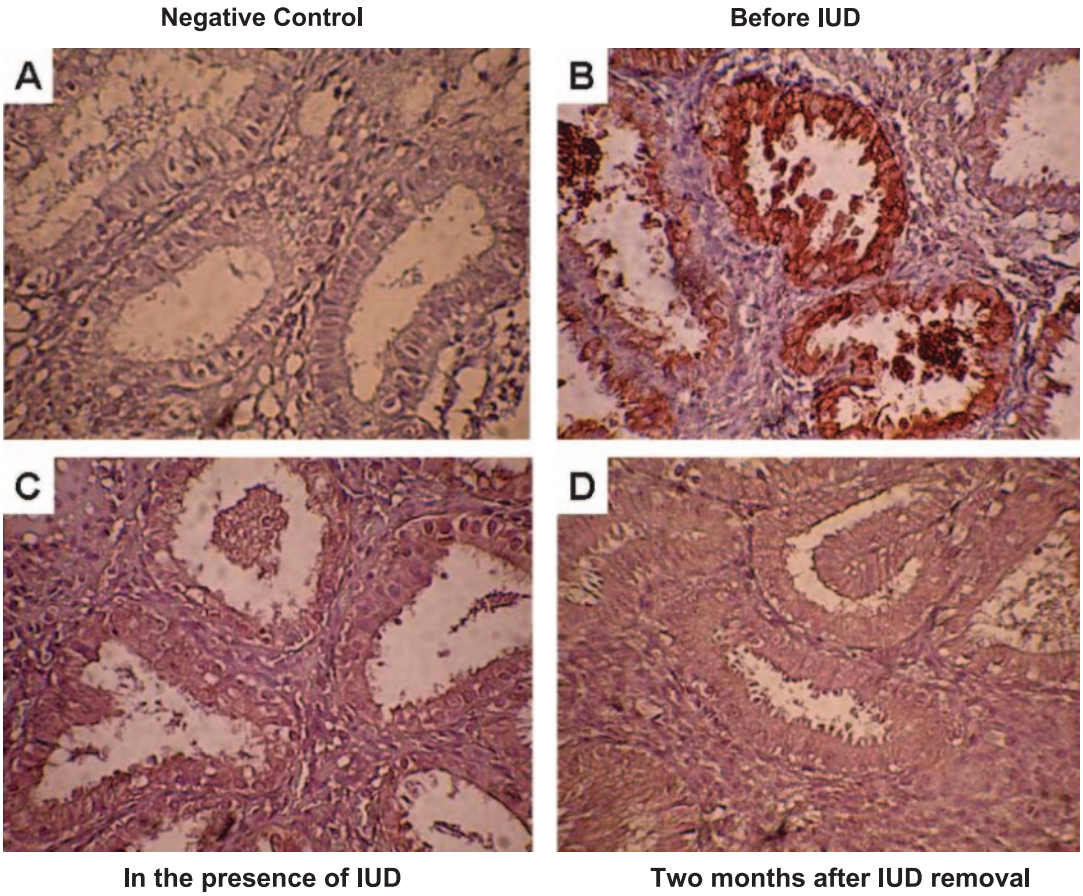

FIG. 3. Immunohistochemical localization of glycodelin in endometrial tissue before, during, and 2 months after IUD use. A, Negative control. B, Endometrial tissue before IUD insertion (biopsy 1). Strong glycodelin immunoreactivity is apparent in glandular epithelium only. We detected some glands strongly stained, whereas other glands were not stained at all. There was no significant staining in the luminal epithelium or stroma. C, Endometrial tissue with IUD in place (biopsy 2). We observed negligible staining in glands. D, Endometrial tissue 2 months after IUD removal (biopsy 3). We detected from weak to strong staining in some glands recovering typical glycodelin expression in human endometrium. Magnification of all microphotographs, ×40.

TABLE 2. List of target genes

Group	Gene name	Function
1	Glycodelin (placental protein 14 precursor)	Secreted glycoprotein
	Dipeptidylpeptidase 4	Immune response
	Transglutaminase 2	Ion binding
	LIF	Differentiation
	3′-phosphoadenosine 5′-phosphosulfate synthase 2	Enzyme
	P8 protein	Cell cycle
	IGFBP-3	Regulatory protein
	Growth arrest and DNA-damage-inducible, α	Cell cycle
	Src-like-adaptor	Intracellular signaling
	Endothelin receptor type B	Receptor
	Homeodomain-only protein	Several functions
	Leiomodin 1	Cytoskeletal protein
	Apolipoprotein C-I	Protein-protein interaction
	Calponin 1, basic, smooth muscle	Muscle protein
	Serine proteinase inhibitor, clade G, member 1,	Immune response
	IGFBP-6	Regulatory protein
	Clusterin	Apoptosis
	Fibulin 5	Matrix protein
	Transgelin	Muscle protein
	Lymphocyte cytosolic protein 1	Ion binding
	Inositol −1-monophosphatase 2	Enzyme
	Proline dehydrogenase 1	Enzyme
2	Calpain 6	Unknown
	Catenin, α2	Cell adhesión
	Keratin 8	Enzyme
	Nuclear factor I/B	Cell cycle
	Branched chain keto acid dehydrogenase E1, β-polypeptide	Regulatory protein
	Aldehyde dehydrogenase 3 family, member B2	Oxydoreductase activity
	Mitochondrial ribosomal protein L3	RNA binding
	Folate receptor 1	Receptor
	Creatine kinase, brain	Enzyme
	Aldehyde dehydrogenase 6 family, member A1	Enzyme
3	MAPK kinase 6	Cell cycle
	S100 calcium binding protein P	Calcium related
	Major histocompatibility complex, class II, DOβ	Immune response
	Chemokine ligand 1	Chemotactic activity
	Matrix metalloproteinase 10	Proteinase
	Metallothionein 1X	Transporter
4	TAF1 RNA polymerase II, TATA box binding protein-associated factor, 250 kDa	Transcription factor

on a large scale the normal transition of gene expression during the WOI. In addition, we found changes in many genes not previously shown to be regulated in the endometrium at the time of implantation.

In this study, we also analyzed the endometrial expression pattern 2 months after IUD removal and found that most of the dysregulated genes in the presence of IUD remained dysregulated. In total, five of 147 genes recovered their normal expression at the time of implantation; specifically only one of 52 WOI genes (matrix metalloproteinase 10) recovered normal expression. This demonstrates that 2 months after IUD removal, the endometrium does not recover its normal gene expression pattern and would predict that it should not have recovered its normal receptivity. Several papers examined the recovery of fertility after the use of various types of IUDs. All these works agree that the pregnancy rates after IUD removal remained lower than in control women for up to 3 months, and 1 yr after IUD removal, pregnancy rates are over 90% (29–32). Cohen (29) estimated that 30% of women become pregnant within 1 month, and another group established a rate of 23.43% after long-term use of IUDs (33). Median time to become pregnant was 3–4 months in one study, depending on the IUD used (30), and 4.4 months in another (31). Our findings indicate that the apparent reduction in pregnancy rate after IUD removal may be due to the transient endometrial genomic modification.

The normalization of the endometrial gene expression profile was confirmed 1 yr after IUD. These results demonstrated that, 1 yr after, the majority (80%) of the genes recovered their normal expression profile at LH+7 corroborating the existing epidemiological data (29–32).

In summary, in an attempt to understand endometrial refractoriness, we examined the gene expression changes induced by the presence of an inert IUD. Significant dysregulation of many genes during the WOI was found, and several mechanisms by which the IUD may induce a nonreceptive state have been put forward. There is a failure to up-regulate many of the genes that are normally increased during the WOI. Second, there is an up-regulation of a group of genes that is not induced at LH+7 including immune response mediators. Finally, we identified abnormal expression of several genes such as LIF and PPARγ that are known to play an essential role in murine implantation. Disruption of these genes may also contribute to the nonreceptive state

in the presence of IUD. We have also shown that this effect persists for some time after IUD removal. The gene expression profile of the endometrium 2 months after the IUD removal at LH+7 does not correspond to a normal endometrium at the time of implantation. This effect is reversible, and our results suggest that within 1 yr the endometrial tissue largely recovers its normal gene expression profile. This correlates with epidemiological studies showing normal implantation rates 1 yr after IUD removal. These results should be taken into consideration in the development of new interceptive strategies.

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