Controlled Ovarian Stimulation Induces a Functional Genomic Delay of the Endometrium with Potential Clinical Implications

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Context: Controlled ovarian stimulation induces morphological, biochemical, and functional genomic modifications of the human endometrium during the window of implantation.

Objective: Our objective was to compare the gene expression profile of the human endometrium in natural *vs*. controlled ovarian stimulation cycles throughout the early-mid secretory transition using microarray technology.

Method: Microarray data from 49 endometrial biopsies obtained from LH+1 to LH+9 (n = 25) in natural cycles and from human chorionic gonadotropin (hCG) +1 to hCG+9 in controlled ovarian stimulation cycles (n = 24) were analyzed using different methods, such as clustering, profiling of biological processes, and selection of differentially expressed genes, as implemented in Gene Expression Pattern Analysis Suite and Babelomics programs.

Results: Endometria from natural cycles followed different genomic patterns compared with controlled ovarian stimulation cycles in the transition from the pre-receptive (days LH/hCG+1 until LH/hCG+5) to the receptive phase (day LH+7/hCG+7). Specifically, we have demonstrated the existence of a 2-d delay in the activation/repression of two clusters composed by 218 and 133 genes, respectively, on day hCG+7 *vs*. LH+7. Many of these delayed genes belong to the class window of implantation genes affecting basic biological processes in the receptive endometrium.

Conclusions: These results demonstrate that gene expression profiling of the endometrium is different between natural and controlled ovarian stimulation cycles in the receptive phase. Identification of these differentially regulated genes can be used to understand the different developmental profiles of receptive endometrium during controlled ovarian stimulation and to search for the best controlled ovarian stimulation treatment in terms of minimal endometrial impact. **(*J Clin Endocrinol Metab* 93: 4500–4510, 2008)**

Since the first *in vitro* fertilization (IVF) birth in 1978 (1), assisted reproductive techniques (ARTs) have evolved to reach current figures, revealing that 1–4% of the population in developed countries has been born from using this technology (2). In the last published report in 2002, the number of ART cycles in this year was 440,000 for the United States (3) and Europe (4) together.

The use of controlled ovarian stimulation is associated with ARTs, and aims to recruit a cohort of mature oocytes that can be fertilized resulting in high-grade embryos to be selected for transfer to the maternal endometrium. Historically, focus has been placed on the effect of ovarian stimulation protocols on the quality of oocytes/embryos obtained, assuming that the impact of

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Abbreviations: ART, Assisted reproductive technique; GEPAS, Gene Expression Pattern Analysis Suite; GO, gene ontology; GPX3, glutathione peroxidase-3; hCG, human chorionic gonadotropin; IGFBP, IGF binding protein; IVF, *in vitro* fertilization; MAP2K1, MAPK kinase 1; PCA, principal component analysis; SLC, solute carrier family; SOTA, Self-Organising Tree Algorithm; VEGF, vascular endothelial growth factor; WOI, window of implantation.

supraphysiological levels of ovarian steroid hormones and paracrine mediators on the endometrium are minimal as a collateral effect. Despite advances in new stimulation protocols, pregnancy rates are still relatively low and have not significantly increased in the last decade (3, 5–7), suggesting that the endometrium in controlled ovarian stimulation cycles is not an unaffected bystander.

Histological and immunohistochemical observations using window of implantation (WOI) markers have demonstrated endometrial modifications during the luteal phase in controlled ovarian stimulation compared with natural cycles (8). In controlled ovarian stimulation, the luteal phase is abnormal compared with the natural cycle, and has characteristic features such as elevated progesterone concentrations during the early luteal phase, followed by a dramatic and premature decrease in the unsupported mid-luteal phase (9). A morphological advancement in the early luteal phase of the endometrium in controlled ovarian stimulation cycles has been consistently reported in histological examination (10–12) and scanning electron microscopy studies (13, 14).

Supraphysiological concentrations of estradiol and subtle progesterone increases in the late follicular phase lead to a modulated steroid receptor profile resembling that of the early luteal phase (15). Immunohistochemical studies have demonstrated a down-regulation of endometrial estradiol receptor and progesterone receptor (16), and biochemical changes in the endometrial fluid (17) in controlled ovarian stimulation compared with natural cycles. Microarray studies comparing controlled ovarian stimulation with natural cycles in the same patient using agonist (18) or antagonist (19) protocols indicate a profound impact on the endometrial gene expression at implantation.

Different uncontrolled studies suggest that uterine receptivity diminishes during controlled ovarian stimulation used in ARTs compared with natural cycles (20, 21). This deleterious effect on uterine receptivity has been reported as a mediated response, which is worse in high-responder patients to gonadotrophins (22–25). Furthermore, it has been demonstrated that although low doses of estradiol maintain the receptive state of the endometrium, high doses cause it to become refractory in mice (26).

In the present study, we have compared the global gene expression profiling across the WOI in natural *vs.* controlled ovarian stimulation cycles to elucidate the genomic impact of controlled ovarian stimulation on endometrial development and to search for novel gene targets to improve endometrial receptivity in IVF.

Patients and Methods

Study design and tissue collection

This study has been approved by the Ethics Committee of the Instituto Valenciano de Infertilidad, Valencia, Spain, the institution in which the endometrial biopsies were obtained and processed. Written informed consent was obtained from all patients.

Endometrial samples (n = 50) were collected from healthy fertile cycling donors (aged 23–39 yr), with a body mass index of 19–25 kg/m^2, who underwent either natural cycles (n = 25) or controlled ovarian stimulation treatment (n = 25) consisting of a long protocol with leuprolide acetate and FSH/haptoglobin, human menopausal gonadotropin and human chorionic gonadotropin (hCG), as described previously (18). Endometrial biopsies were obtained on days LH+1 (n = 5), LH+3 (n = 5), LH+5 (n = 5), LH+7 (n = 5), and LH+9 (n = 5) in natural cycles, and at hCG+1 (n = 5), hCG+3 (n = 5), hCG+5 (n = 5), hCG+7(n = 5), and hCG+9 (n = 5) in controlled ovarian stimulation cycles. In both cases no luteal phase supplementation was administered. All of them were obtained from different women.

In natural cycles a daily assessment of the urinary LH levels beginning on cycle d 10 was performed using a commercially available ovulation predictor kit (Donacheck ovulación; Novalab Ibérica, S.A.L, Coslada, Madrid, Spain), and the day of the urinary LH surge was considered to be LH=0. Biopsies were obtained from the uterine fundus using a Pipelle catheter (Genetics, Namont-Achel, Belgium) under sterile conditions. Endometrial dating was performed using the criteria of Noyes *et al.* (27) by two different pathologists who were blinded to the day on which the specimen was obtained. The histological analysis was performed for endometrial biopsies in natural and controlled ovarian stimulation cycles. Only endometrial samples considered "in phase" were included in the present study for microarray interrogation. One of the samples corresponding to hCG+5 was excluded for microarray analysis due to its inconsistency with the histological dating.

Total RNA isolation and microarray hybridization

Endometrial samples were snap frozen in liquid nitrogen and stored at −70 C. Total RNA was extracted using the "TRIzol method" according to the protocol recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD). Approximately 1–2 mg total RNA was ob tained per mg endometrial tissue. RNA quality was assessed by loading 300 ng total RNA onto an RNA Labchip and was an-

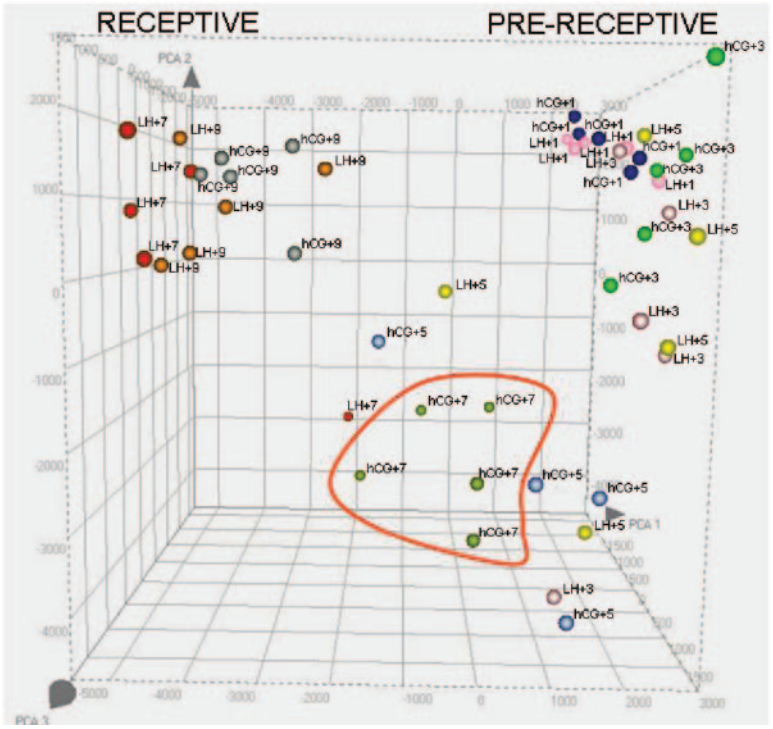

FIG. 1. PCA of human endometrium throughout the development of the luteal phase in natural (LH+n) and controlled ovarian stimulation cycles (hCG+n).

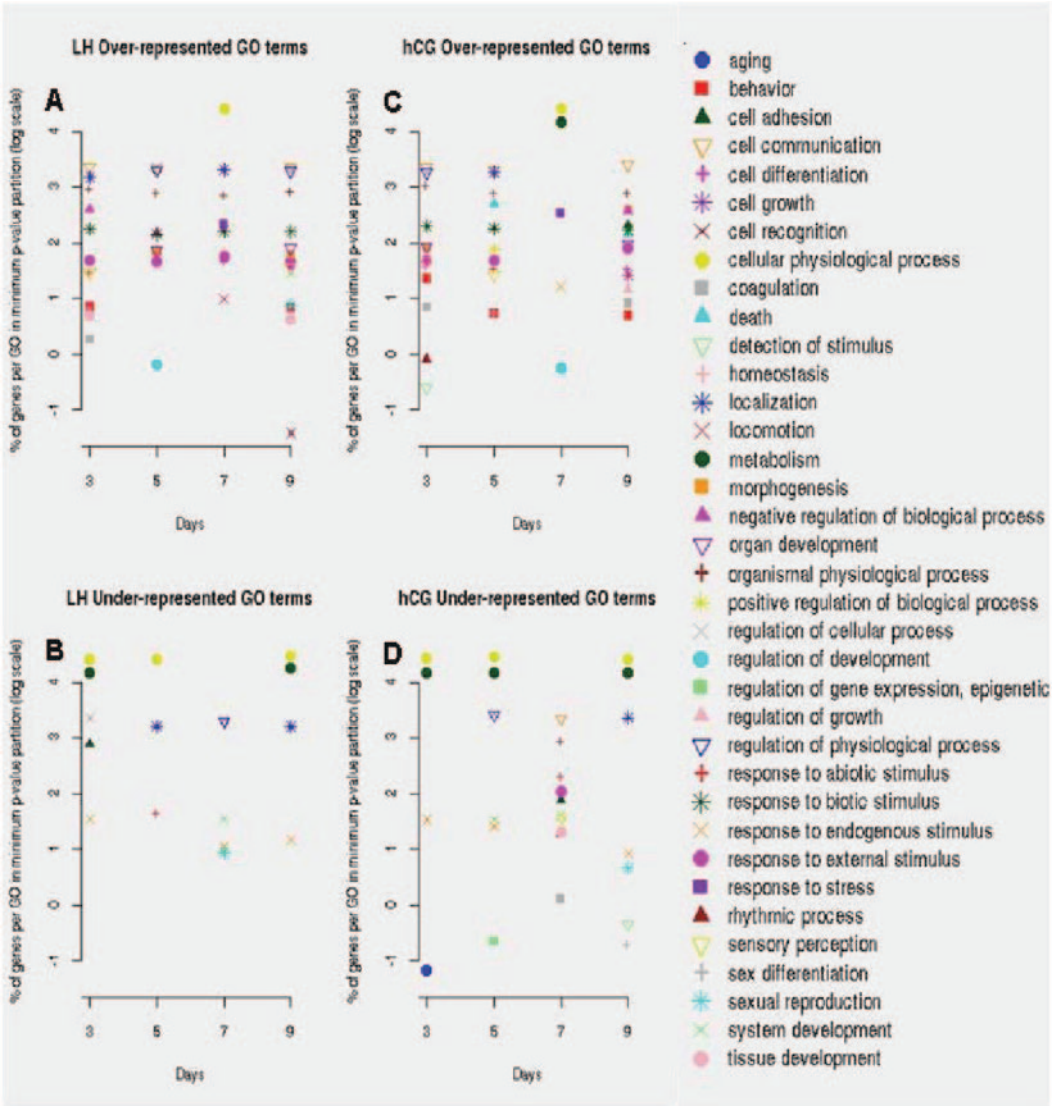

FIG. 2. Dynamical profile of up-regulated and down-regulated functionalities across the time series d 1–9 in the LH and hCG set of samples. The gene expression of each day was compared with the day before (day of reference) by a *t* test using TRex. It shows the overrepresented and underrepresented terms in the natural cycle (A and C, respectively) and in controlled ovarian stimulation cycles (B and D, respectively). *Symbols* are explained to the *right* of the figure.

alyzed in an A2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Hybridization onto Affymetrix HG-U133A chip (Affymetrix, Inc., Santa Clara, CA) was performed by Gene Logic an Ocimum Biosolutions Co. (Gaithersburg, MD) as described (28).

Data analysis

Bioinformatics analysis

The microarray analysis was performed using the Gene Expression Pattern Analysis Suite (GEPAS) version 3.1, available at http://www.gepas.org (29). The functional annotation of the analysis results was performed using the Babelomics suite, available at http://www.babelomics.org (30).

Preprocessing

Output data from the microarray normalization process were preprocessed before performing the microarray analysis. Multiple probes mapping to the same gene were merged using the average as the summary of the hybridization values.

Sample clustering

Samples were grouped into clusters using a self-organizing neural network with tree topology, the Self-Organising Tree Algorithm (SOTA) algorithm (31). We applied a euclidean distance and an unrestricted growth condition to obtain only one sample per cluster.

Differential gene expression

We applied a *t* test for the difference in the mean expression between two groups of arrays (classes). We compared expression patterns for samples at each time point (days) between the LH and hCG classes, obtaining *P* values for each gene in the experiment. We also applied a *t* test between the LH+1 and LH+7, and between the hCG+1 and hCG+7 sample sets. To account for multiple testing effects, we corrected the *P* values using the false discovery rate (32).

Functional analysis of the results

To detect activations or deactivations in biological functions or pathways, we have used the FatiScan (33), a gene set based algorithm that detects significantly up-regulated or down-regulated blocks of functionally related genes in lists of genes ordered by differential

expression. FatiScan is part of the Babelomics suite (30). FatiScan can search blocks of genes functionally related by different criteria, such as gene ontology (GO) terms Kyoto Encyclopedia of Genes and Genomes pathways, and others.

Clustering of gene expression patterns

The SOTA method (31) was used to cluster expression profiles of both genes or samples. The SOTA is included in the GEPAS (29). For the visualization and evaluation of the clusters' quality, we used Environment of Tree Exploration, a module of the GEPAS. Functional enrichment of the clusters was studied with the FatiGO+ tool (34, 35).

Microarray validation: quantitative-PCR and immunohistochemistry

To verify the results obtained from the cDNA microarray, real-time PCR was performed for four selected genes: IGF binding protein (IGFBP)-3; glutathione peroxidase-3 (GPX3); solute carrier family (SLC) 1 (neuronal/epithelial high-affinity glutamate transporter system Xag) member 1 (SLC1A1); and glycodelin (also known as placental protein 14). The relative expression levels of each gene in the total RNA from the endometrium was determined by real-time RT-PCR using specific primers for each gene: primer sequence (5′-3′) for IGFBP-3, forward 5′-GCACAGATACCCAGAACTTCTCC and reverse 5′-CAGGTGATTCAGTGTGTCTTCCA; GPX3, forward 5′-CGATTTAGGTGACACTATAGCATGGGTGTACAGCCACGTG and reverse 5′-CGTAATACGACTCACTATAGGGGGGCCTTAGCCTGAATGCAC; SLC1A1, forward 5′-GTCCTGACTGGGCTTGCAA and reverse 5′-CAACGGGTAACACGAATCGA; and glycodelin, forward 5′-TGGTCTGTGGTGTCCCGG and reverse 5′-AGGGAGATGTTGTTGGTCGC.

Immunohistochemistry was performed, as previously described (36), for three selected genes at 3 different days of natural cycles: IGFBP-7, growth arrest and DNA damage inducible gene-45, and glycodelin (PP14). Microarray data validation (data not shown) has been presented to the reviewers.

Results

Cluster analysis

Principal component analysis (PCA) is a statistical method that allows projecting higher-dimensional data onto a lower-dimensional space. When PCA is applied to data, samples with similar trends in their gene expression profiles tend to cluster close together in the plot. Figure 1 shows clusters corresponding to the development of the endometrium in natural *vs*. stimulated cycles. Endometrial samples corresponding to LH+1 in natural cycles and to hCG+1 in stimulated cycles showed a very similar gene expression profile (Fig. 1, *upper-right corner*). Similar results were obtained for LH+3 and hCG+3 (Fig. 1, *right side*), LH+5 and hCG+5, and LH+9 and hCG+9 (Fig. 1, *upper-left corner*). The only group that showed statistical differences in its gene profile corresponded to LH+7 in natural cycles *vs*. hCG+7 in stimulated cycles (Fig. 1). The exception was one LH+7 sample that segregated close to the hCG+7 cluster and was histologically classified as "in phase." Samples for LH+7 and LH+9 in natural cycles and hCG+9 in stimulated cycles showed very similar gene expression profiles at the receptive part.

Hierarchical clustering was also applied to the microarray gene expression profiles of the 49 well-characterized endometrial samples. The SOTA (31) was applied to the complete gene expression matrix to obtain a tree of the relationships among all the samples of the experiment. Similar results to the PCA were obtained (data not shown).

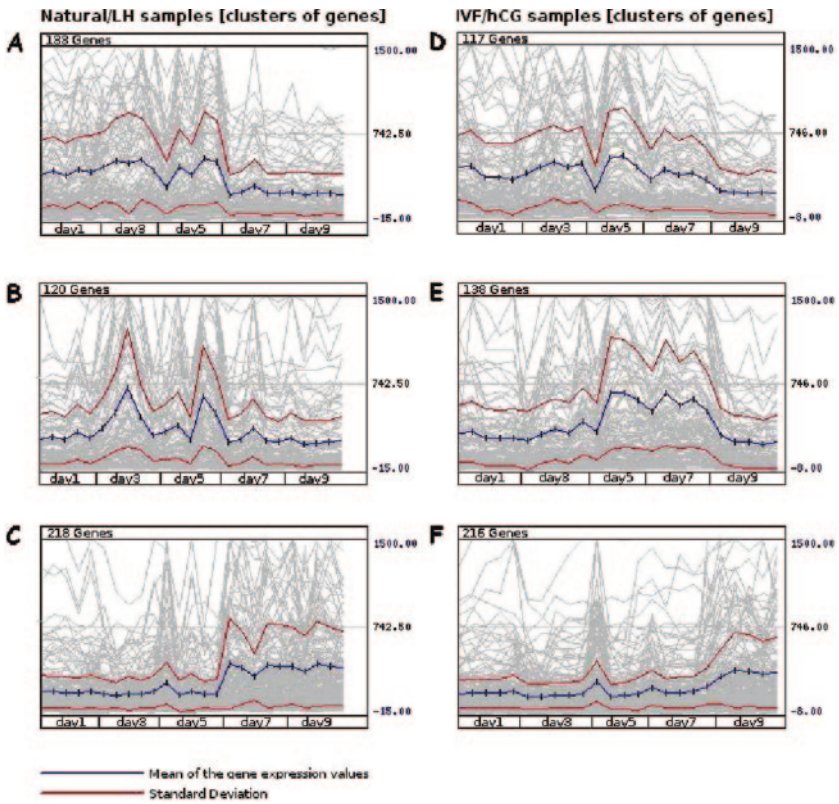

FIG. 3. Schematic graphs of six gene clusters derived from genes that are differentially expressed throughout the luteal phase

Temporal functional profiling in natural *vs*. controlled ovarian stimulation cycles along the WOI

To understand how cellular functionalities are activated and deactivated along the WOI in natural *vs*. controlled ovarian stimulation cycles, we analyzed their corresponding temporal functional profiles. For that end, we used the first day as reference, and we compared each subsequent day with this reference time by a gene set enrichment analysis, as implemented in the FatiScan tool of Babelomics. This method allows us to trace the functional blocks (GO, Kyoto Encyclopedia of Genes and Genomes pathways, *etc.*) that were significantly up-regulated and down-regulated on each day of the WOI.

In Fig. 2, we described overrepresented (A and C) and underrepresented (B and D) GO functions in natural *vs*. controlled ovarian stimulation cycles. Many overrepresented biological terms were shared in both natural and controlled ovarian stimulation categories, particularly on d +3 and +5, suggesting a similar development on the first days of the WOI.

TABLE 1. Down-regulated and up-regulated WOI genes delayed in COS cycles on d +7 compared with natural cycles

Abbreviation	Gene name
Down-regulated genes (clusters A and D)	
ALPL	Alkaline phosphatase, liver/bone/kidney
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)
ARF4 liter	ADP-ribosylation factor 4-like
C11orf8	Chromosome 11 open reading frame 8
C7orf24	Chromosome 7 open reading frame 24
CKB	Creatine kinase, brain
CREG1	Cellular repressor of E1A-stimulated genes 1
CSRP2	Cysteine and glycine-rich protein 2
CYB5	Cytochrome b-5
DNCI1	Dynein, cytoplasmic, intermediate polypeptide 1
F3	Coagulation factor III (thromboplastin, tissue factor)
GREB1	GREB1 protein
HLA-DOB	Major histocompatibility complex, class II, DO β
KCNG1	Potassium voltage-gated channel, subfamily G, member 1
KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3
KIAA0274	KIAA0274
NDRG2	NDRG family member 2
NUDT9	Nudix (nucleoside diphosphate linked moiety X)-type motif 9
OFD1	Oral-facial-digital syndrome 1
PART1	Prostate androgen-regulated transcript 1
PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)
PIP5K1B	Phosphatidylinositol-4-phosphate 5-kinase, type I, β
PKP2	Plakophilin 2
QPRT	Quinolinate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating)
RAB4A	RAB4A, member RAS oncogene family
SERPINA4	Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 4
SLC15A2	Solute carrier family 15 (H+/peptide transporter), member 2
Up-regulated genes (clusters C and F)	
CXCL13	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
SLC15A1	Solute carrier family 15 (oligopeptide transporter), member 1
DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5
HAL	Histidine ammonia-lyase
PROS1	Protein S (α)
C4.4A	GPI-anchored metastasis-associated protein homolog
DAF	Decay accelerating factor for complement (CD55, Cromer blood group system)
KIAA1199	KIAA1199
ANXA2P1	Annexin A2 pseudogene 1
MET	Met proto-oncogene (hepatocyte growth factor receptor)
MAOA	Monoamine oxidase A
S100A1	S100 calcium binding protein A1
SOD2	Superoxide dismutase 2, mitochondrial
MAPK6	Mitogen-activated protein kinase 6
RNASE4	Ribonuclease, RNase A family, 4
ITGA3	Integrin, α 3 (antigen CD49C, α 3 subunit of VLA-3 receptor)
ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
VEGF	Vascular endothelial growth factor
ENPEP	Glutamyl aminopeptidase (aminopeptidase A)
FOSL2	FOS-like antigen 2
TPM1	Tropomyosin 1 (α)
FLII	Flightless I homolog (Drosophila)
ALS2CR3	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3
EPAS1	Endothelial PAS domain protein 1
PISD	Phosphatidylserine decarboxylase
C4BPA	Complement component 4 binding protein, α
CP	Ceruloplasmin (ferroxidase)

(*Continued*)

TABLE 1. (*Continued*)

	Gene name
Up-regulated genes (clusters C and F)	
Abbreviation	
SFN	Stratifin
PAX8	Paired box gene 8
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
TNFAIP2	Tumor necrosis factor, α-induced protein 2
DF	D component of complement (adipsin)
C1orf34	Chromosome 1 open reading frame 34
LAMB3	Laminin, β 3
SCYE1	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
ACADSB	Acyl-Coenzyme A dehydrogenase, short/branched chain
GUCY1B3	Guanylate cyclase 1, soluble, β 3
BF	B-factor, properdin /// B-factor, properdin
GPX3	Glutathione peroxidase 3 (plasma)
MITF	Microphthalmia-associated transcription factor
SLC22A4	Solute carrier family 22 (organic cation transporter), member 4
TRPC6	Transient receptor potential cation channel, subfamily C, member 6
DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6
PPP2R5A	Protein phosphatase 2, regulatory subunit B (B56), α isoform
GADD45A	Growth arrest and DNA-damage-inducible, α
PAEP	Progestagen-associated endometrial protein (placental protein 14, pregnancy-associated endometrial α-2-globulin, α uterine protein)
IGFBP3	Insulin-like growth factor binding protein 3
SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
HNMT	Histamine N-methyltransferase /// histamine N-methyltransferase
RARRES3	Retinoic acid receptor responder (tazarotene induced) 3
RAB21	RAB21, member RAS oncogene family
C1orf38	Chromosome 1 open reading frame 38
RFP	Ret finger protein
PLXNC1	Plexin C1
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)
BENE	BENE protein
DUSP5	Dual specificity phosphatase 5
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
LGMN	Legumain
IMPA2	Inositol(myo)-1(or 4)-monophosphatase 2
AGR2	Anterior gradient 2 homolog (Xenopus laevis)
LPHN2	Latrophilin 2
PPP1R14B	Protein phosphatase 1, regulatory (inhibitor) subunit 14B
RBP1	Retinol binding protein 1, cellular
DUSP6	Dual specificity phosphatase 6
IGFBP7	Insulin-like growth factor binding protein 7
FHL2	Four and a half LIM domains 2
LAMC2	Laminin, γ 2
CA12	Carbonic anhydrase XII

This information will be deposited in the Endometrial Data Base (http://www.endometrialdatabase.com) after publication.

However, on d +7 the natural cycle showed a higher number of overrepresented biological terms, such as "localization," "response to external stimulus," "locomotion," "response to biotic stimulus," and others (Fig. 2A). Interestingly, most of these GO terms are not present in the transition from day hCG+5 to hCG+7 in controlled ovarian stimulation cycles. Only two GO terms are conserved in the transition from the pre-receptive to receptive state in natural and controlled ovarian stimulation cycles; these terms are the "response to the stress" and "cellular physiological process."

We also found similarities in the biological terms underrepresented in the pre-receptive endometrium, except on d +7 when more differences were observed. On d +7 no common biological term was identified in natural and controlled ovarian stimulation cycles. Furthermore, some terms appeared to be underrepresented in hCG+7 of controlled ovarian stimulation cycles, such as response to external stimulus or organismal physiological process, which are overrepresented in LH+7 of natural cycles (Fig. 2A).

Differential gene expression between natural and controlled ovarian stimulation cycles

A *t* test was applied to each day's samples, which were grouped into two classes, LH and hCG, to find any significant

difference in the gene expression patterns under these conditions. No significant differences were found between the LH and hCG gene expression profiles except in the LH+7/hCG+7 comparison, which showed a high number of significantly overexpressed and underexpressed genes in LH+7 *vs.* hCG+7.

When genes differentially expressed (false discovery rate-adjusted P value < 0.01) were clustered using the SOTA, six main cluster expression profiles were found, three belonging to natural cycles and three to controlled ovarian stimulation cycles (Fig. 3). In natural cycles, cluster 1 (Fig. 3A) was formed by 133 genes that were down-regulated at LH+7 and LH+9. Cluster 2 comprised 120 genes, and was homogeneous throughout the WOI, peaking at LH+3 and LH+5 (Fig. 3B). Cluster 3 was the largest in the natural cycle with 218 genes and showed an up-regulation at LH+7 that was maintained at LH+9 (Fig. 3C). In controlled ovarian stimulation cycles, cluster 1 was composed of 117 genes that maintained a similar profile from hCG+1 to hCG+7, and decreased at hCG+9 (Fig. 3D). The 138 genes of cluster 2 displayed a clear peak at hCG+5 and hCG+7, and decreased thereafter (Fig. 3E). Finally, cluster 3 was formed by 216 genes with no changes during the WOI until day hCG+9, when their expression suddenly increased (Fig. 3F).

Interestingly, when we analyzed the list of genes of the different clusters, we found that 97 genes corresponding to 73% of the genes from cluster 1 in the natural cycle (Fig. 3A) were present in cluster 1 of controlled ovarian stimulation cycles (Fig. 3D), and 104 genes (87%) of the cluster 2 in the natural cycle (Fig. 3B) were present in cluster 2 of controlled ovarian stimulation cycles (Fig. 3E). Of the 218 genes of cluster 3 in the natural cycle (Fig. 3C), 203 (93%) were included in cluster 3 of the controlled ovarian stimulation cycle (Fig. 3F), and the expression peak of 203 genes underwent a 2-d delay in controlled ovarian stimulation *vs.* natural cycles (LH+7) (Fig. 3F). Interestingly, an important number of the down-regulated and up-regulated genes of clusters A and C of natural cycle (27 and 74 genes, respectively) with a delayed expression are categorized as WOI genes (18) and listed in Table 1. The overrepresented biological processes for these 97 down-regulated (cluster A) and 203 up-regulated (cluster C) delayed genes are listed in Table 2. Therefore, all three clusters described present a 2-d delay in controlled ovarian stimulation *vs.* natural cycles only in the transition from the prereceptive to the receptive phase, demonstrating for the first time a molecular substrate for the impact of controlled ovarian stimulation in the human endometrium.

Finally, we analyzed the biological connections among the 97 down-regulated and 203 up-regulated delayed genes of clusters A and C in controlled ovarian stimulation cycles by String (http://string.embl.de/), and we found that vascular endothelial growth factor (VEGF) and MAPK kinase 1 (MAP2K1) were in connection with a significant number of genes (Fig. 4).

TABLE 2. Biological processes of the 97 down and 203 up-regulated delayed genes

	Count (%)	*P* value
Down-regulated genes: clusters A and D		
Biological term		
Coagulation	3 (3.57)	0.0681
Wound healing	3 (3.57)	0.0785
Blood coagulation	3 (3.57)	0.0656
Hemostasis	3 (3.57)	0.0732
Regulation of body fluids	3 (3.57)	0.0879
Up-regulated genes: clusters C and F		
Biological term		
Localization	48 (25.67)	0.0037
Establishment of localization	47 (25.13)	0.0057
Cell proliferation	13 (6.95)	0.0124
Transport	41 (21.93)	0.0263
Positive regulation of apoptosis	6 (3.21)	0.0299
Positive regulation of programmed cell death	6 (3.21)	0.0307
Negative regulation of biological process	15 (8.02)	0.0331
Angiogenesis	4 (2.14)	0.0359
Blood vessel development	4 (2.14)	0.0400
Vasculature development	4 (2.14)	0.0400
Blood vessel morphogenesis	4 (2.14)	0.0400
Cell motility	7 (3.74)	0.0410
Locomotion	7 (3.74)	0.0410
Localization of cell	7 (3.74)	0.0410
Fructose 6-phosphate metabolism	2 (1.07)	0.0413
Taxis	5 (2.67)	0.0437
Chemotaxis	5 (2.67)	0.0437
Response to stress	19 (10.16)	0.0442
Locomotory behavior	5 (2.67)	0.0492
Cellular physiological process	126 (67.38)	0.0550
Response to chemical stimulus	9 (4.81)	0.0574
Behavior	6 (3.21)	0.0588
Negative regulation of cellular process	13 (6.95)	0.0758
Cellular lipid metabolism	10 (5.35)	0.0785
Development	27 (14.44)	0.0788
Phosphate metabolism	16 (8.56)	0.0793
Phosphorus metabolism	16 (8.56)	0.0793
Negative regulation of cell proliferation	5 (2.67)	0.0805
Negative regulation of cellular physiological process	12 (6.42)	0.0806
Carboxylic acid metabolism	10 (5.35)	0.0824
Organic acid metabolism	10 (5.35)	0.0839
Cell death	11 (5.88)	0.0900

The number of genes in each biological term and the percentage and *P* valued for each one are represented.

Discussion

Reproductive endocrinologists involved in IVF have been working worldwide for more than three decades with controlled ovarian stimulation protocols, largely ignoring their possible detrimental effects on endometrial receptivity. Clinical data have suggested a lower implantation rate in controlled ovarian stimulation compared with natural cycles (3, 5–7). However, supporting scientific data in the human endometrium with functional implications have been scarce until now. This study provides the first comparative, genome-wide analysis in natural *vs.* controlled ovarian stimulation cycles across the WOI. As a result we have gained insight into functional genomics that may account for

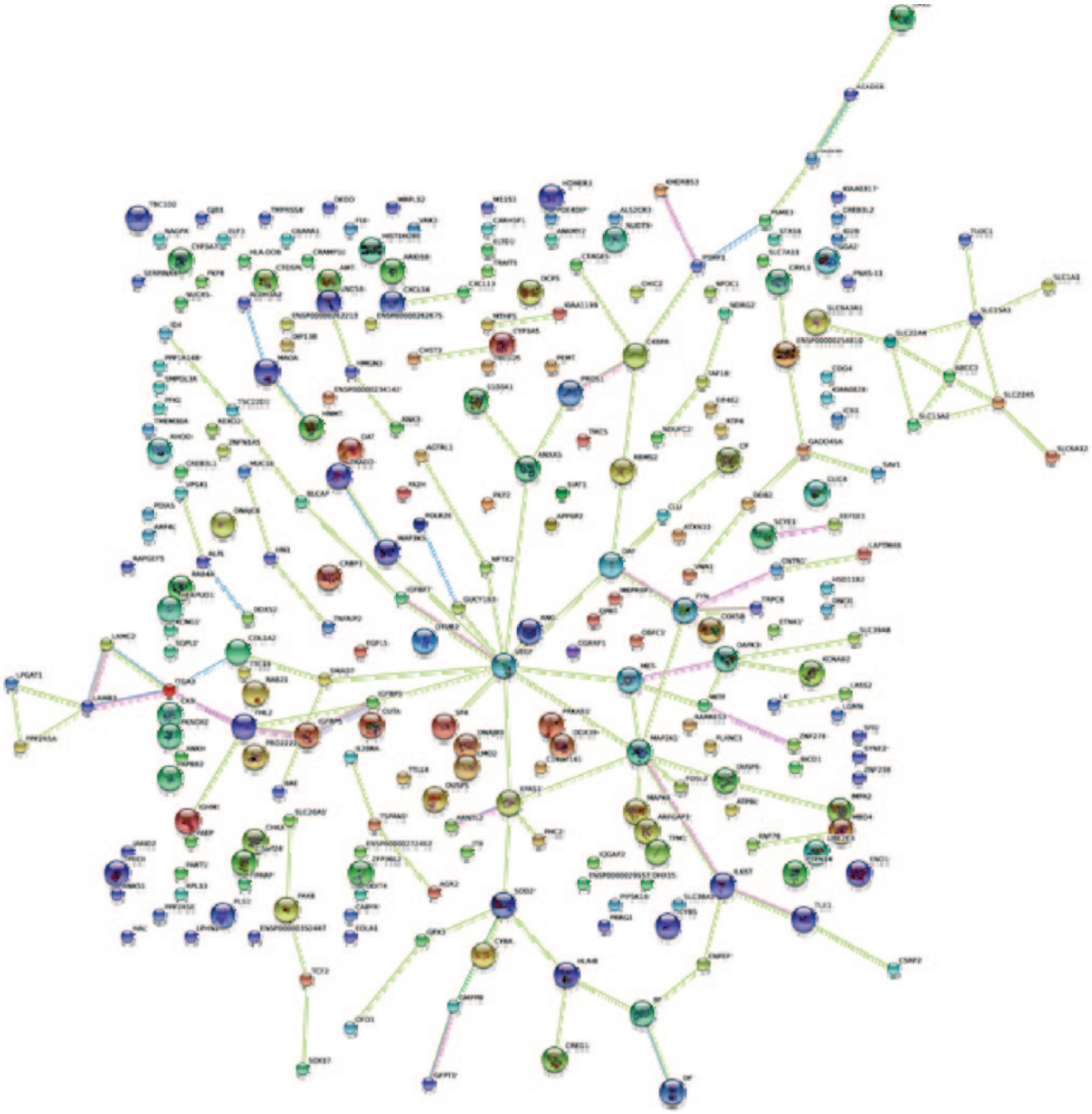

FIG. 4. Biological connections among the 97 up-regulated and 203 down-regulated delayed genes.

different endometrial development in controlled ovarian stimulation *vs.* natural cycles.

The molecular signature of controlled ovarian stimulation cycles during the WOI differs from natural cycles

Different studies have partially addressed the genomics of the human endometrium specifically during the WOI (37–41) or during the complete menstrual cycle (42, 43). Nonetheless, the sequential molecular development of the endometrium during the WOI in natural cycles has not been previously reported. The first conclusion to be drawn from this work is that the development of the human endometrium follows a genetic program with a well-defined molecular transition from the pre-receptive (unable to accept the adhesion of the human blastocysts) to the receptive endometrium, which is comparable among the different subjects investigated.

In stimulated cycles the endometrial gene expression pattern is very similar to natural cycles during the WOI in the pre-receptive phase from hCG+1 to hCG+5 (Fig. 1). This observation was confirmed using hierarchical clustering. However, the gene expression profile of the receptive endometrium in the controlled ovarian stimulation cycle at hCG+7 showed significant statistical differences compared with the natural cycle at LH+7

(Fig. 1). These differences have been partially suggested previously by our laboratory (18, 19). Nonetheless, no rigorous statistical determination had been performed until now. We have also analyzed the functional dynamics of the WOI in both natural *vs.* controlled ovarian stimulation cycles using the bioinformatics tools implemented in the GEPAS (29) and Babelomics (30). In general, it is remarkable how most of the biological processes are overrepresented and not underrepresented in the natural transition from the pre-receptive to receptive endometrium in natural and controlled ovarian stimulation cycles (Fig. 2). The development of the WOI is a biological event that is primarily induced by gene activation rather than gene inactivation or repression. We propose the term "transcriptional awakening process" in contrast to a term that produces down-regulation or gene repression, which we propose as the "transcriptional sleeping process." However, some differences can be found between natural and controlled ovarian stimulation cycles, specifically on d +7, in which many overrepresented GO terms in natural cycles do not parallel in controlled ovarian stimulation cycles. Furthermore, some GO terms involving a response to external stimuli have an opposite regulation (Fig. 2, A and D). These differences match the results obtained in sample clustering where the greatest differences were observed precisely on d +7. Clearly, the comparison of the dynamics of biological roles up-regulated and down-regulated in LH *vs.* hCG conditions provide us good insights into their different features as systems.

Analysis of gene clustering reveals a delay in the development of the endometrium in controlled ovarian stimulation cycles

At the gene expression level, natural cycles can be differentiated into two phases in terms of gene expression patterns: pre-receptive (LH+1 to LH+5) and receptive (LH+7 to LH+9). This feature does not occur in controlled ovarian stimulation cycles where the transition from LH+5 to LH+7 is not that evident, suggesting that the biological processes that take place in endometrial development follow different molecular ways, especially in reaching the receptive status in natural (LH+7) and stimulated cycles (hCG+7).

Because we know that this day is crucial for embryonic implantation, we directly compared the differences between LH+7 in the natural cycle and hCG+7 in controlled ovarian stimulation cycles. When we compared the data from LH+7 *vs.* hCG+7, we found that 241 genes were up-regulated, and 291 were down-regulated with a P value less than 0.1 (69 and 73 up-regulated and down-regulated genes, respectively; $P < 0.05$). This is in agreement with two previously cited (18, 19), in which a high number of genes was found to be differently expressed between natural and controlled ovarian stimulation cycles (GnRH agonists or antagonists) and in disagreement with a third one (44). These differences have been discussed in a recent review (45). When we compared gene clusters, a delay in controlled ovarian stimulation *vs.* natural cycles was demonstrated. This is genuinely surprising because it indicates that a large number of WOI genes present a different behavior in controlled ovarian stimulation cycles at this time point. When we performed a GO analysis, we found that many proteins belonged to basic physiological pathways that are necessary for the normal development of cellular functions, at that phase of the endometrial cycle, such as cellular lipid, fructose 6-phosphate, carboxylic acid, organic acid, phosphorus, carboxylic acid, and phosphate metabolisms. Other biological processes in which those proteins were involved were related to the normal development of angiogenesis, blood vessel development, and morphogenesis, also important at the mid-luteal phase. And, finally, other genes were involved in the control of cell number such as negative regulation of cell proliferation, cell death, positive regulation of apoptosis, and positive regulation of programmed cell death. Cellular localization revealed a large amount of membrane proteins such as solute carriers (SLC1A1, SLC15A1, SLC15A2, SLC22A4, and SLC7A11) implicated in ion/molecules transport, and receptors such as RARRES3, MET, ITGA3, ITL6ST, and TRPC6, essential for signal transduction.

Receptiveness is a very active biological process involving a large number of genes in a coordinated manner. These results show that the endometrium in controlled ovarian stimulation cycles does not reach the receptive status in the same manner or, at least, at the same biological time. It also means that the cellular structure of the endometrium in controlled ovarian stimulation cycles could not be built at implantation. Obviously, this could be detrimental for the development of an optimal receptivity.

To understand the reasons for this dys-regulation, we analyzed the net of connections of delayed genes, demonstrating that VEGF and other genes such as MAP2K1 are in the middle of the regulation of a relevant number of genes (Fig. 4). VEGF is an important signaling protein involved in both vasculogenesis and angiogenesis, crucial processes for normal endometrium development. Abnormal angiogenesis may contribute to several different endometrial-related pathologies, including endometrial cancer, endometriosis, menorrhagia, and breakthrough bleeding (reviewed in Ref. 46). It has been also hypothesized that progesterone may modulate vascular permeability changes necessary for implantation by its actions on decidualized cells and VEGF (47). On the other hand, MAP2K1 was also found in the central part of the gene network. This protein acts as an integration point for multiple biochemical signals, and it has been demonstrated to be involved specifically in the signaling pathways of the decidualization process in mice (48).

The discovery of the overrepresented and underrepresented biological processes during the development of the WOI in natural and controlled ovarian stimulation cycles provides invaluable information about the events that take place in the endometrial tissue in reaching receptivity. In this sense, this study contains a very large amount of data and information involving events that occur at the gene, molecular, and structural levels in the human endometrium in two different situations. It allows us to know the biological differences with the WOIs that are clinically induced by hormones. On the one hand, this helps us to go deep into the knowledge of the complex process of endometrial receptivity, whereas, on the other hand, it represents a useful tool to correct the molecular impact of controlled ovarian stimulation treatments on the human endometrium. Furthermore, controlled ovarian stimulation may lead to differences in the timing of endometrial maturation compared with natural cycles (15). This

genomic delay may be of interest to define gene targets for the understanding of endometrial development under controlled ovarian stimulation and search for the optimal stimulation treatments that better mimic the gene expression profile of the natural cycle.

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