Gene expression profile of the human endometrial receptivity: comparison between natural and stimulated cycles for the same patients

D. Haouzi[a,b,c,d], S. Assou[a,c,d], K. Mahmoud[e], S. Tondeur[a,c,d], T. Rème[a,c,d], B. Hedon[b], J. De Vos[a,c,d], S. Hamamah[a,b,c,d,f].

[a] CHU Montpellier, Institut de Recherche en Biothérapie, Hôpital Saint-Eloi, Montpellier, F-34000 France ; [b] CHU Montpellier, Département de Médecine et Biologie de la Reproduction, Hôpital Arnaud de Villeneuve, Montpellier, F-34000 France ; [c] INSERM, U847, Montpellier, F-34000 France; [d] Université Montpellier1, UFR de Médecine, Laboratoire 'Développement embryonnaire précoce et cellules souches embryonnaires humaines', Montpellier, F-34000 France; [e] Centre de FIV, Clinique les Jardins, Tunis, Tunisia.

Running title: Human endometrial receptivity in stimulated cycles

[f] corresponding author
Pr. Samir Hamamah
ART/PGD Division, Département de Médecine et Biologie de la Reproduction
Hôpital Arnaud de Villeneuve
INSERM U 847
34295 Montpellier, France
Tél : 04 67 33 64 04
Fax : 04 67 33 62 90
Mail: S-hamamah@chu-montpellier.fr

Abstract

Background: The adjunction of exogenous hormones for controlled ovarian stimulation (COS) may alter endometrial receptiveness. In order to identify the genes mis-regulated under COS, we compared the endometrium gene expression profiles, from the same patients, in a natural cycle and in a subsequent COS cycle. **Methods**: For the same normal-responder patients (n=21), endometrial biopsies (n=84) were collected during the pre-receptive (LH+2) and receptive stages (LH+7) of a natural cycle and, subsequently, on oocyte retrieval day (hCG+2) and on transfer day (hCG+5) of a stimulated cycle. Samples were analyzed using DNA microarrays. Gene expression profiles and biological pathways involved in the endometrial receptivity were analyzed. **Results**: Although endometrium transition profiles from pre-receptive to receptive phases are similar between patients, COS regimens alter endometrial receptivity in comparison with natural cycle. Under COS conditions, two endometrial profiles were identified and were associated either with a moderately altered receptivity profile for the majority of the patients or a strongly altered profile for a sub-category of patients. The receptive endometrium transcription profile under COS was defective for biological functions such as TGFβ signalling, leukocyte transendothelial migration and the cell cycle. In addition, several biological functions specifically activated under COS regimens were identified. **Conclusion**: Gonadotropin treatments in COS cycles led to disruptions of the transcriptional activation of genes involved in normal endometrial receptivity. We propose that when the receptiveness of the endometrium is seriously compromised by the COS protocol, fresh embryo replacement should be cancelled, the embryo frozen, and thawed embryo replacement should be performed under natural cycles.

Key Words: human endometrium, microarray, endometrial receptivity, natural cycle, COS.

Introduction

In assisted reproductive technology (ART), pregnancy and birth rates following IVF attempts remain low. Indeed, 2 out of 3 IVF cycles fail to result in pregnancy and more than 8 out of 10 transferred embryos fail to implant (Kovalevsky and Patrizio, 2005). Indeed, successful implantation requires a competent embryo, a receptive endometrium and a synchronized dialogue between maternal and embryonic tissues. Although the selection of competent oocyte, embryo and endometrial status assessment has experienced important technological progress, implantation rates are still low under COS for IVF (Donaghay and Lessey, 2007). It is assumed that two-thirds of implantation failures are associated with inadequate endometrial receptivity or with defects in the embryo-endometrium dialogue (Paulson *et al*., 1990; Simon *et al*., 1998).

Several parameters have been suggested for assessing endometrial receptivity, including endometrial thickness which is a traditional criterion, endometrial morphological aspect, and endometrial and sub-endometrial blood flow. However, their positive predictive value is still limited (Friedler *et al*., 1996; Pierson, 2003; Alcázar, 2006). More recently, transcriptomic approaches have been driven to identify bio-markers of the endometrial receptivity. Using microarray technology in human endometrial biopsy samples, several studies have reported modifications related to the gene expression profile associated with the transition of the human endometrium from a pre-receptive (early-secretory phase) to a receptive (mid-secretory phase) state during a natural cycle (Carson *et* al., 2002; Riesewijk *et al*., 2003; Mirkin *et al*., 2005; Talbi *et al*., 2006; Haouzi *et al*., 2009). However, recent data suggested that COS regimens may have a negative impact on endometrial receptivity compared with natural cycles (Paulson *et al*., 1990; Horcajadas *et al*., 2005; Simon *et al*., 2005; Horcajadas *et al*., 2008; Liu *et al*., 2008). It has been suggested that the impairment of

endometrial receptivity is due to high concentration of steroids resulting from COS (Krikun *et al*., 2005; Liu *et al*., 2008).

The divergence between the reports analyzing the effects of stimulation protocols on endometrial receptivity may have several explanations: differences in the day of the endometrial biopsies, different patient profiles, different COS protocols, and inadequate numbers of endometrial samples studied. In addition, no study has compared the early and the mid-secretory phases during a natural and stimulated cycle for the same patients, which seems an essential condition to minimize the impact of inter-patient variability.

The aim of this study was to evaluate the global gene expression profile of human endometrial biopsies of normal responder patients during the early-secretory phase and the mid-secretory phase of natural and stimulated cycles in the same patients.

Materials and methods

Patient characteristics and endometrial biopsies

The study population included 21 normal responders patients (age 30.9 years ± 3.3) referred for ICSI for male infertility factor recruited after written informed consent. All patients had normal serum FSH, LH and estradiol on day 3 and were normal-responders during a previous first ICSI attempt. This project has received institutional review board (IRB) approval. During the same natural cycle that preceded a second ICSI attempt, two endometrial biopsies were obtained in all women at day 2 (LH+2) and day 7 (LH+7) after the LH peak and two others were obtained at day 2 (hCG+2) and day 5 (hCG+5) after hCG administration during COS cycles. The LH surge was estimated by the patient herself according to the first day of her menstruation. Histologic analysis was not performed to verify that the LH timing was accurate. Therefore, the possibility for a delay of one day from the first day of the

menstruation cannot be excluded. Each biopsy sample was frozen at -80°C in RLT RNA extraction buffer (RNeasy kit, Qiagen, Valencia, CA, USA).

Complementary RNA (cRNA) preparation and microarray hybridization

Endometrial samples RNA were extracted using the micro RNeasy Kit (Qiagen). The total RNA quantity was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA was prepared with two rounds of amplification according to the manufacturer's protocol "double amplification" (Two-Cycle cDNA Synthesis Kit, Invitrogen) starting from total RNA (100 ng). Labeled fragmented cRNA (12 µg) was hybridized to oligonucleotide probes on Affymetrix HG-U133 Plus 2.0 arrays containing 54 675 sets of oligonucleotide probes ("probe set") which correspond to ≈ 30 000 unique human genes or predicted genes. Each endometrial sample was put individually on a microarray chip.

Data processing

Scanned GeneChip images were processed using Affymetrix GCOS 1.4 software to obtain an intensity value signal and a detection call (present, marginal or absent) for each probe set, using the default analysis settings and global scaling as the first normalization method, with a trimmed mean target intensity value (TGT) of each array arbitrarily set to 100. Probe intensities were derived using the MAS5.0 algorithm. This algorithm also determines whether a gene is expressed with a defined confidence level or not ("detection call"). This "call" can either be "present" (when the perfect match probes are significantly more hybridized than the mismatch probes, FDR < 0.04), "marginal" (for FDR > 0.04 and <0.06) or "absent" (FDR >

0.06). The microarray data were obtained in our laboratory in agreement with the Minimal Information about Microarray Experiment MIAME recommendations.

Microarray data analysis

The Significant Analysis of microarrays (SAM, Stanford University, USA, Tusher *et al.* 2001) was used to identify genes whose expression varied significantly between the LH+2 (n=21) and LH+7 (n=21) samples and between the hCG+2 (n=21) and hCG+5 (n=21) samples. SAM provides mean or median fold change values (FC) and a false discovery rate (FDR) confidence percentage based on data permutation. To perform the comparison of gene expression profiles between endometrial sample groups, a probe set selection using the Absent/Present "detection call" and a coefficient of variation (CV) (≥ 40%) between samples was first performed before the SAM.

To compare profile expressions of endometrial samples (n=84) from the LH+2, LH+7, hCG+2 and hCG+5 sample groups, we performed an unsupervised classification with both principal component analysis (PCA) and hierarchical clustering (Eisen *et al.*, 1998; de Hoon *et al.*, 2004). The PCA involved original scripts based on the R statistics software through the RAGE web interface (http://rage.montp.inserm.fr) (Rème *et al.*, 2008). Hierarchical clustering analysis based on the expression levels of varying probes were performed with the CLUSTER and TREEVIEW software packages. Genetic expression profiles were analyzed with the RAGE supervised analysis module, using a non parametric Mann Whitney U test with multiple testing corrections and were confirmed with the SAM software. Selected gene lists (mean fold change > 2 and FDR < 5%) were submitted to Ingenuity (http://www.ingenuity.com) and FatiGO+ (http://babelomics2.bioinfo.cipf.es) software to identify the biological mechanisms altered by these gene expression variations. FatiGO+ is a part of the Babelomics suite and allows any differential distribution of biological terms (Gene

Ontology, KEGG pathways) to be found between two groups of genes (Al-Shahrour *et al*., 2006).

Ingenuity Pathway Analysis

Selected gene lists were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Those genes with known gene symbols and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. The network identified is then presented as a graph indicating the molecular relationships between genes/gene products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line).

Results

Gene expression profile of endometrial receptivity during natural and stimulated cycles for the same patients

We selected 18,389 probe sets based on a CV ≥40% and a "detection call" present in at least 10 samples per group, and then subjected the 84 samples to an unsupervised clustering (Figure 1A). A first branch allowed the separation of the pre-receptive (LH+2 and hCG+2) from the receptive sample groups (LH+7 and hCG+5), corresponding to 62, 57, 81 and 100% of the samples for LH+2, hCG+2, LH+7 and hCG+5 respectively. A sub-branch allowed to

partition the receptive samples between the natural (LH+7) and stimulated (hCG+5) cycles. A three dimensional PCA was performed (4,237 probe sets), representing 43% of the data information, and confirmed the unsupervised cluster analysis (Figure 1B).

A SAM analysis between the LH+2 and the LH+7 and between the hCG+2 and the hCG+5 sample groups (LH+2 *versus* LH+7; hCG+2 *versus* hCG+5; paired sample analysis) were performed. Among 1071 genes significantly modulated between the pre-receptive and receptive samples during natural cycle, 93 % and 7 % were respectively up- ($2 \leq$ fold change ≤ 465) and down-regulated ($-3.8 \leq$ fold change ≤ -2) in the LH+7 sample group (fold change ≥ 2 and a *FDR* < 5%) as previously described (Haouzi *et al*., 2009) (Supplementary Table I and Figure 4). Analysis of samples during stimulated cycle revealed that 998 genes were significantly modulated between the pre-receptive and receptive stages, including 78 % up-regulated ($2 \leq$ fold change ≤ 59) and 22 % down-regulated ($-5 \leq$ fold change ≤ -2) genes in the hCG+5 sample group (Figure 2A, Figure 4 and Supplementary Table I). A hierarchical clustering with this significant list of genes allowed the separation of the two endometrial sample groups of the stimulated cycle (Figure 2B). Interestingly, more down-regulated genes were observed during endometrial receptivity of the stimulated cycle in comparison with the natural cycle.

A SAM analysis between the LH+2 and hCG+2 and between the LH+7 and hCG+5 sample groups (LH+2 *versus* hCG+2, LH+7 *versus* hCG+5, paired sample analysis) was performed. The number of significantly modulated genes is reported in Table I. In contrast with other studies, the comparison of gene expression profile between natural and stimulated cycles was performed by paired samples (hCG+2 versus their corresponding LH+2), which is an important point to minimize inter-patient variability. For example, the number of genes significantly modulated between LH+2 and hCG+2 samples analysed by paired or unpaired

was dramatically different, highlighting the need to perform paired analyses and explaining differences with other studies.

Atypical gene expression profile in endometrium under stimulated cycles

The hierarchical clustering based on the stimulated cycle endometrium signature (Figure 2B) revealed that 14 % (3/21 patients) of the hCG+5 samples were misclassified. Interestingly, the same samples also displayed an altered expression profile at day hCG+2 (Figure 3 and 2B). Hence, gene expression analysis of the endometrium transcriptome after COS can distinguish a "standard" profile shared by most patients, and an "atypical" profile found in 14% of patients. A SAM analysis performed between the hCG+2 and hCG+5 samples reveals that only one gene, TSPAN3 (x39.3, FDR<0.0001) was significantly modulated between these two sample groups in these three "atypical" patients compared to 1296 genes which were modulated in the 18 "standard" patients. This gene was not modulated during the transition from the pre-receptive to the receptive stages in natural nor in the 18 "standard" patients of the stimulated cycles. We also performed a SAM analysis between the hCG+2 of the 18 "standard" and the 3 "atypical" samples. 197 genes were significantly modulated, including 188 up- and 9 down-regulated genes, suggesting a strong difference in gene expression at hCG+2 between "standard" and "atypical" samples. Among these genes, 24 up- and 2 down-regulated genes were specifically restricted to the hCG+2 "atypical" profile. In addition, the SAM analysis between the "standard" hCG+5 sample group and the "atypical" hCG+5 sample group revealed that 14 genes were significantly modulated, among which, 4 up- and 1 down-regulated genes were restricted to the "atypical" samples.

Differential expression of biological functions between natural and stimulated cycles

The two gene lists identified with SAM analyses between the pre-receptive and receptive samples from the natural and stimulated cycles were then intersected to determine their overlap. While 361 up- and 32 down-regulated genes were in common to the natural and stimulated cycles, each category displayed a specific gene expression profile (Figure 4). These specific profiles included 632 up- and 46 down-regulated genes during the natural cycle, 416 up- and 189 down-regulated genes during the stimulated cycle. Interestingly, during the endometrial receptivity of the stimulated cycles, numerous genes which were down-regulated compared to natural cycles, were articulated around the cell cycle function (Figure 5). Among this function, the most representative genes were those coding for Cyclins, Cell Division Cycle (CDC) members, Cyclin Dependent Kinases (CDK), and members of the E2F family of transcription factors. They included: CCNA2 (x-2.7, FDR<0.0001), CCNB1 (x-2.3, FDR<0.0001), and CCNE2 (x-2.1, FDR<0.0001), CDCA8 (x-2.8, FDR<0.0001), CDC25C (x-3.3, FDR<0.0001), CDCA5 (x-2.7, FDR<0.0001), CDC20 (x-2.8, FDR<0.0001), CDCA7 (x-2, FDR<0.0001), CDKN3 (x-2.9, FDR<0.0001), E2F1 (x-2.1, FDR<0.0001), E2F7 (x-2.9, FDR<0.0001), E2F8 (x-3.2, FDR<0.0001), as well as FOXM1 (x-2.1, FDR<0.0001), MYBL2 (x-2.8, FDR<0.0001), TK1 (x-2.2, FDR<0.0001), PBK (x-3.2, FDR<0.0001), CHAF1B (x-2.2, FDR<0.0001), BUB1 (x-2.7, FDR<0.0001), AURKA (x-2.2, FDR<0.0001), CHEK1 (x-2, FDR<0.0001), HDAC (x-3.5, FDR<0.0001), DHFR (x-2, FDR<0.0001) and CDT1 (x-2.5, FDR<0.0001) (Figure 5).

KEGG pathways between natural and stimulated cycles

We performed a FatiGO+ analysis with the two lists of up-regulated genes during endometrial receptivity of the natural and stimulated cycles. There were numerous differences, between the natural and stimulated cycles, in main systems involved in the implantation process, such as the TGFβ signaling pathway, the complement and coagulation cascades, and leukocyte transendothelial migration (Figure 6). While CHRD (x2.4, FDR<0.0001), BMP6 (x3.5, FDR<0.0001), DCN (x2, FDR=0.02), FST (x8.7, FDR<0.0001), ACVR1B (x2.9, FDR=0.02) and SMAD3 (x2.4, FDR=0.003) were the most representative up-regulated genes around the TGFβ signaling pathway in the natural cycle from the pre-receptive and receptive stages, there were no changes in these genes the stimulated cycle (Figure 6A). Also, MASP1 (x2.2, FDR=0.004), C2 (x3.6, FDR<0.0001), C3 (x4.7, FDR<0.0001), SERPINF2 (x2.2, FDR=0.0006), FGB (x46.4, FDR=0.005), PROS1 (x2.9, FDR=0.0006), and BDKRB2 (x2.1, FDR=0.006) were the major up-regulated genes of the complement and coagulation cascades in the natural cycle, while BDKRB1 (x2.6, FDR=0.008) was the only gene associated with this pathway to be up-regulated during receptivity in the stimulated cycles (Figure 6B). Numerous genes involved in leukocyte transendothelial migration were up-regulated during endometrial receptivity of the natural cycle, such as ITGB2 (x3.1, FDR<0.0001), ITGB3 (x7, FDR=0.002), ITGB4 (x2.6, FDR<0.0001), ITGA3 (x2.2, FDR<0.0001), ITGAL (x2.6, FDR<0.0001), ITGAM (x2, FDR=0.005), CD99 (x3.3, FDR=0.001), VCAM1 (x4.2, FDR=0.003), VIL2 (x2.5, FDR<0.0001) and RAC2 (x2.6, FDR=0.0007). Whereas, only ITGA4 gene (x2.6, FDR=0.003) was up-regulated during endometrial receptivity of the stimulated cycle (Figure 6C).

Biomarkers of the endometrial receptivity during stimulated cycles

We also performed a PCA analysis of the hCG+2 and hCG+5 samples with the predictor list of endometrial receptivity as previously described (Haouzi *et al*., 2009). The majority of the hCG+2 samples were separated from the hCG+5 samples, except for the three "atypical" samples, in this PCA using the first two dimensions representing 39% of the data information (Figure 7). During the pre-receptive to the receptive transition in stimulated cycles, four of our biomarkers of endometrial receptivity were significantly up-regulated. For natural cycles compared to stimulated cycles, NLF2 was increased by a factor 12.6 compared to 2 , PROK1 was increased by a factor of 10.2 compared to 7.8, MFAP5 was increased by a factor of 37 compared to 11.5,and LAMB3 was increased by a factor of 20.4 compared to 11.1. The upregulation observed in the microarray analyses was validated by QRT-PCR (Haouzi *et al*., 2009; Supplementary Figure 1).

ICSI outcome and biomarker genes

Seven ongoing pregnancies were obtained in the patients participating in this study. There were no significantly differences in the modulated genes between the pregnant and non pregnant patients either in the hCG+2 or hCG+5 subgroups.

Discussion

This is the first study analyzing by paired samples the endometrial gene expression profiles from the same patients during the pre-receptive to the receptive transition both in a natural and in a subsequent stimulated cycle. Although endometrium transition profiles are similar between patients, COS regimens affect endometrial receptivity in comparison with natural cycles. Two endometrium genes profiles were observed and associated either with a

294 moderately altered receptivity for the majority of the patients or a strongly altered receptivity
295 under COS conditions.
296
297 ***Differential gene expression profiles of endometrial receptivity between natural and***
298 ***stimulated cycles***
299 In natural as well as stimulated cycles, the majority of the genes modulated during
300 endometrial receptivity were up-regulated (93% in the natural cycle compared to 78 % in the
301 stimulated cycle), suggesting that the receptive endometrium requires transcriptional
302 activation as suggested by Horcajadas *et al*. (2008). Among the up-regulated genes of
303 endometrial receptivity in the stimulated cycles, only 46 % were in common with those of the
304 natural cycles. These data suggest that either the duration or FSH dose in gonadotropins
305 treatment under COS cycles leads to the transcriptional activation of other genes which are
306 not involved in physiological endometrium receptivity. Major differences in biological
307 functions known to be involved in the implantation process, such as the TGFβ signaling
308 pathway (Jones *et al*., 2006), the complement and coagulation cascades and the leukocyte
309 transendothelial migration (Berlin *et al*., 1995; Ivetic *et al*., 2004; Dominguez *et al*., 2005; da
310 Costa Martins *et al*., 2006), were observed between the natural and stimulated cycle. The up-
311 regulation of genes involved in these signaling pathways was found in natural cycles, but was
312 lacking in stimulated cycles. On the other hand, the expression profile of the receptive
313 endometrium in stimulated cycles during COS showed more down-regulated genes than in the
314 natural cycle (7% in natural compared to 22 % in stimulated cycles respectively), suggesting
315 that COS treatment, partly, induced gene repression. The majority of these down-regulated
316 genes was not observed in the natural cycle (2% in common) and were related to the “cell
317 cycle” function, suggesting a negative impact of gonadotropins treatment during COS on this
318 function. Several cyclins (cyclin A2, B1 and E2), cell-division cycles (CDCA5, A8, A7, 20

and 25C), and members of E2F family (E2F1, 7 and 8), which are key regulators of cell cycle, as well as numerous genes articulated around them, were not upregulated in stimulated cycles. Moreover, genes involved in checkpoint regulation, which are molecular circuits monitoring DNA integrity and cell growth prior to replication and division at the G1/S and G2/M transitions respectively, were also not upregulated in stimulated cycles (Branzei and Foiani, 2008). These data can be considered in conjunction with reports which show that both GnRH antagonist and agonist exert an inhibitory effect on the cell cycle of human endometrial cancer cells in vitro, probably by decreasing the synthesis of growth factors (Emons *et al*., 1993; Hershkovitz *et al*., 1993: Kleinman *et al*., 1993).

In contrast to Horcajadas *et al*. (2005) who showed that genes up-regulated during the formation of the receptive endometrium in the natural cycle tended to be down-regulated during stimulated cycles, we report similar variations but with a lesser amplitude during COS in comparison with natural cycles (Supplementary Tables I and II). One possible explanation is that, this amplitude difference reflects the sampling time of the receptive samples between natural (LH+7) and stimulated cycles (hCG+5). However, as embryos transfer was generally performed on day five after hCG administration (hCG+5) of a stimulated cycle, endometrial biopsies just before embryo transfer fitted with clinical practice. Recently, it has been reported that there is a two-day delay at hCG+7, with hCG+7 samples having a gene expression profile close to day 5 of stimulated cycle samples (Horcajadas *et al*., 2008). However, in the current study, the unsupervised cluster and PCA analyses showed that a proportion of LH+7 (67%) and hCG+5 (30%) samples tend to cluster close together. In addition, the study design (LH+7/9 compared to hCG+7/9 in the Horcajadas's study, LH+2 and LH+7 compared to hCG+2 and hCG+5 in the present study), the size of patient samples (n=5 per group in the Horcajadas's study, n=21 per group in the present study) as well as the samples, which were

obtained from different fertile patients candidates for oocytes donation in the Horcajadas's study, could contribute to these diverging results (Horcajadas *et al*., 2008). By contrast, in the present study the comparison of gene expression profiles between natural and stimulated cycles was performed in the same normal-responder patients, which is an important point to minimize inter-patient variability.

We can wonder about the impact of multiple biopsies performed on the same patient. Kalma *et* al. (2008) reported that the first endometrial biopsy modulated a wide variety of genes in the same cycle as well as in the following cycle, which can consequently mask true gene expression profile differences. However, all patients were subjected similarly to four biopsies. If biopsies affect all samples equally, so this should not prevent the identification of different gene expression profiles. Therefore, gene expression profile differences observed during the pre-receptive to the receptive endometrial transition under COS regimens (hCG+2 compared to hCG+5) between the two described endometrial profiles (endometrial receptivity moderately and strongly altered) suggested, contrariwise, that true differences are not masked. In addition, although Barash *et al*. (2003) showed that multiple endometrium biopsies during the spontaneous menstrual cycle increased implantation and pregnancy rates at the following cycle of treatment, and that Zhou *et al*. (2008) demonstrated that local injury of the endometrium executed in IVF patients during their cycle of treatment, before ovum retrieval, gains the same effect of increasing implantation and clinical pregnancy rates, we don't known the consequences to perform a biopsy on the embryo transfer day. However, in this cohort, pregnancy rate (7 / 21 patients) was similar to those habitually acquired, suggesting a no detrimental effect on implantation process.

Atypical gene expression profile of endometrial cells during COS

The comparisons of gene expression from the same patients between natural and stimulated cycles revealed endometrial profiles associated either with a moderately altered receptivity in most cases (86%) or a strongly altered receptivity during the COS protocol in a few cases (14%). In these few cases, the transition from the oocyte collection day (hCG+2) to the embryo transfer day (hCG+5) phase introduced only minor differences in gene expression profiles. Only one gene, TSPAN3 was significantly modulated between their pre-receptive to receptive samples. The protein encoded by this gene is a member of the tetraspanin family and plays a role in the regulation of the cell development, activation, growth and motility (Hemler, 2005). Impairment of gene expression of endometrial cells was already apparent at hCG+2 in these samples as judged by the gene number significantly modulated (197 genes) between these samples and those associated with a moderately altered receptivity. However, on hCG+5, there were fewer differences between the two endometrial profiles.

We previously described a predictor list (comprising 60 probe sets) of endometrial receptivity during natural cycle (Haouzi *et al*., 2009). These predictors confirmed the presence of the two endometrial profiles: either receptive or not receptive. However, among these predictors, only four were significantly up-regulated during the pre-receptive to the receptive transition in the stimulated cycle. In addition, the fold changes between the pre-receptive to the receptive phases in endometrial profiles associated with a positive receptive status were less pronounced in stimulated than in natural cycles, suggesting incomplete receptivity during COS. Therefore, our predictors further suggest the moderately altered receptivity status for the majority of the samples, as well as the strongly altered receptivity status for a few patients. When the receptiveness of the endometrium is seriously compromised during COS, fresh embryo replacement could be reconsidered. In these cases, embryos freezing enable the IVF attempt to be saved and the embryo transfer can be done later during a natural cycle. For these

patients, the endometrial receptivity gene expression signature is of high value (Haouzi *et al.*, 2009).

In conclusion, the transcriptomic pattern of endometrial cells in natural and stimulated cycles in the same patients reveals either moderate or strong alterations of endometrial receptivity under COS protocols. This information could open new perspectives, particularly in patients with multiple implantation failures. In this case, analysis of the endometrial profile could reveal a strongly altered profile during COS protocols, prompting the clinician to either adapt the IVF stimulation protocol or to perform embryo transfer later during a natural cycle.

Acknowledgements

We wish to thank the Ferring, Merck-Serono Pharmaceutical, Schering-plough companies for their partial financial support of this study. We thank the direction of the University-Hospital of Montpellier for support and the ART team for their assistance during this study.

References

Alcázar JL. Three-dimensional ultrasound assessment of endometrial receptivity: a review. *Reprod Biol Endocrinol*. 2006;**9**;4:56.

Al-Shahrour F, Minguez P, Tárraga J, Montaner D, Alloza E, Vaquerizas JMM, Conde L, Blaschke C, Vera J, Dopazo J. BABELOMICS: a systems biology perspective in the functional annotation of genome-scale experiments. *Nucleic Acids Research* (Web Server issue) 2006;**34**: W472-W476.

Barash A, Dekel N, Fieldust S, Segal I, Schechtman E, Granot I. Local injury of the endometrium doubles the incidence of successful pregnancies in patients undergoing in-vitro fertilization. *Fertil Steril* 2003;**79**:1317–22.

Berlin C, Bargatze RF, Campbell JJ, vonAndrian U H, Szabo MC, Hasslen SR, Nelson RD, Berg EL, Erlandsen SL, and Butcher EC. Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 1995;**80**,413–422.

Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol*. 2008;**9**(4):297-308.

Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, 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(9):871-9.

da Costa Martins PA, van Gils JM, Mol A, Hordijk PL, Zwaginga JJ. Platelet binding to monocytes increases the adhesive properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins. *J Leukoc Biol.* 2006;**79**(3):499-507

de Hoon, M.J., Imoto, S., Nolan, J. and Miyano, S. Open source clustering software. *Bioinformatics* 2004;**20**:1453-4.

Dominguez F, Yáñez-Mó M, Sanchez-Madrid F, Simón C. Embryonic implantation and leukocyte transendothelial migration: different processes with similar players? *FASEB J.* 2005;**19**(9):1056-60.

Donaghay M, Lessey BA. Uterine receptivity: alterations associated with benign gynecological disease. *Semin Reprod Med*. 2007;**25(**6):461-75.

Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genomewide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America* 1998;**95**:14863-8.

Emons G, Schröder B, Ortmann O, Westphalen S, Schulz KD, Schally AV. High affinity binding and direct antiproliferative effects of luteinizing hormone-releasing hormone analogs in human endometrial cancer cell lines. *J Clin Endocrinol Metab.* 1993;**77**(6):1458-64.

Friedler S, Schenker JG, Herman A, Lewin A: The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: a critical review. *Hum Reprod Update* 1996;**2**:323-335.

Hershkovitz E, Marbach M, Bosin E, Levy J, Roberts CT Jr, LeRoith D, Schally AV, Sharoni Y. Luteinizing hormone-releasing hormone antagonists interfere with autocrine and paracrine growth stimulation of MCF-7 mammary cancer cells by insulin-like growth factors. *J Clin Endocrinol Metab*. 1993;**77**(4):963-8.

Haouzi D, Mahmoud K, Fourar M, Bendhaou K, Dechaud H, De Vos J, Rème T, Dewailly D, Hamamah S. Identification of new biomarkers of human endometrial receptivity in the natural cycle. *Hum Reprod*. 2009;**24**:198-205.

Hemler ME. Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol*. 2005;**6**(10):801-11.

Horcajadas JA, Mínguez P, Dopazo J, Esteban FJ, Domínguez F, Giudice LC, Pellicer A, Simón C. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. *J Clin Endocrinol Metab*. 2008;**93**(11):4500-10.

Horcajadas JA, Pellicer A, Simón C. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update.* 2007;**13**(1):77-86.

Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S, Simón C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. Mol *Hum Reprod*. 2005;**11**(3):195-205.

Ivetic A, Florey O, Deka J, Haskard DO, Ager A, Ridley AJ. Mutagenesis of the ezrin-radixin-moesin binding domain of L-selectin tail affects shedding, microvillar positioning, and leukocyte tethering. *J Biol Chem*. 2004;**279**(32):33263-72.

Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-beta superfamily expression and actions in the endometrium and placenta. *Reproduction*. 2006;**132**(2):217-32.

Kalma Y, Granot I, Gnainsky Y, Or Y, Czernobilsky B, Dekel N, Barash A. Endometrial biopsy-induced gene modulation: First evidence for the expression of bladder-transmembranal uroplakin Ib in human endometrium. *Fertil Steril*. 2008 Mar 18.

Kleinman D, Roberts CT Jr, LeRoith D, Schally AV, Levy J, Sharoni Y. Regulation of endometrial cancer cell growth by insulin-like growth factors and the luteinizing hormone-releasing hormone antagonist SB-75. *Regul Pept*. 1993;**48**(1-2):91-8.

Kovalevsky G, Patrizio P. High rates of embryo wastage with use of assisted reproductive technology: a look at the trends between 1995 and 2001 in the United States. *Fertil Steril*. 2005;**84**(2):325-30.

Krikun G, Schatz F, Taylor R, Critchley HO, Rogers PA, Huang J, Lockwood CJ. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. *J Clin Endocrinol Metab*. 2005;**90**(3):1812-8

Liu Y, Lee KF, Ng EH, Yeung WS, Ho PC. Gene expression profiling of human peri-implantation endometria between natural and stimulated cycles. *Fertil Steril*. 2008 Jan 11.

Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S. In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod*. 2005;**20**(8):2104-17.

Mirkin S, Nikas G, Hsiu JG, Díaz J, Oehninger S. Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. *J Clin Endocrinol Metab*. 2004;**89**(11):5742-52.

Paulson RJ, Sauer MV and Lobo RA. Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. *Fertil Steril* 1990;**53**:870–874.

Pierson RA: Imaging the endometrium: are there predictors of uterine receptivity? *J Obstet Gynaecol Can* 2003,**5**:360-368.

Rème T, Hose D, De Vos J, Vassal A, Poulain PO, Pantesco V, Goldschmidt H, Klein B. A new method for class prediction based on signed-rank algorithms applied to Affymetrix microarray experiments. *BMC Bioinformatics* 2008;**11**:9-16.

Riesewijk A, Martín J, van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S, Simón C. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol Hum Reprod*. 2003;**9**(5):253-64.

Simon C, **Oberyé J**, **Bellver J**, **Vidal C**, **Bosch E**, **Horcajadas JA**, **Murphy C**, **Adams S**, **Riesewijk A**, **Mannaerts B**, **Pellicer A**. Similar endometrial development in oocyte donors treated with either high- or standard-dose GnRH antagonist compared to treatment with a GnRH agonist or in natural cycles. *Human Reproduction* 2005;**20**(12): 3318–3327.

Simon C, Moreno C, Remohi J and Pellicer A. Cytokines and embryo implantation. *J Reprod Immunol* 1998;**39**,117–131.

Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA, Nayak NR, Giudice LC. Molecular phenotyping of

513 human endometrium distinguishes menstrual cycle phases and underlying biological
514 processes in normo-ovulatory women. *Endocrinology.* 2006;**147**(3):1097-121.
515 **Tusher VG, Tibshirani R, Chu G**. Significance analysis of microarrays applied to the
516 ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001;**98**(9):5116-21.
517 **Zhou L, Li R, Wang R, Huang HX, Zhong K**. Local injury to the endometrium in
518 controlled ovarian hyperstimulation cycles improves implantation rates. *Fertil Steril*.
519 2008;**89**(5):1166-76.
520
521

Figures legends

Table I: Number of genes significantly modulated in 6 microarray analyses comparing the pre-receptive or receptive stages between natural and stimulated cycles.
This table compares our gene lists significantly modulated between LH+2 and hCG+2, and between LH+7 and hCG+5 with five other studies using the same approach. Atg, antagonist; Ag, agonist.

Figure 1: Unsupervised classification with both principal component analysis (PCA) and hierarchical clustering of 84 endometrium samples during the pre-receptive and receptive stages from natural and stimulated cycles.
(A) Unsupervised hierarchical clustering was performed, allowing a separation between the pre-receptive (LH+2 and hCG+2) and receptive samples (LH+7 and hCG+5). (B) PCA using the three dimensions was performed representing 43% of the data information, and confirmed the unsupervised cluster analysis. Pink circle, LH+2 and hCG+2 samples; grey circle, LH+7 samples; green circle, hCG+5 samples.

Figure 2: Supervised hierarchical clustering of hCG+2 and hCG+5 samples from stimulated endometrium cycles.
(A) The majority of the genes specifically modulated during endometrial receptivity in stimulated cycles (998 genes) were up-regulated (777 up-regulated genes, 221 down-regulated genes). Red, up-regulated genes; green, down-regulated genes. (B) A hierarchical clustering was performed with this gene list, allowing the separation of the two sample groups. Rhomb black outline: “Atypical” samples.

Figure 3: The two endometrium profiles during the pre-receptive and the receptive stages in stimulated cycles.

The majority of patients (18/21 patients) were characterized by a common profile, called "standard profile". Other patients (3/21) presented an "atypical profile". PR, pre-receptive; R, receptive; red, up-regulated genes; green, down-regulated genes.

Figure 4: Venn diagram of transcripts up-regulated and down regulated during endometrial receptivity in the natural cycle compared to the stimulated cycle.

Each list of genes was determined with the SAM software with a FDR < 5% and a fold change > 2.

Figure 5: Down-regulated genes related to the cell cycle function in the receptive endometrium of stimulated cycles.

Genes involved in (A) the cell cycle, (B) G1/S checkpoint regulation, and (C) the G2/M DNA damage checkpoint. All of these genes were down-regulated in stimulated cycles in comparison with natural cycles. Green: down-regulated genes.

Figure 6: Major differences in main systems involved in implantation between natural and stimulated cycles.

To identify differential distributions of KEGG pathways, we performed a FatiGO+ analysis with the two lists of genes up-regulated gene during endometrial receptivity of the natural and stimulated cycles. There were numerous differences, between the natural and stimulated cycles, in main systems involved in the implantation process, such as the TGFβ signaling

pathway, the complement and coagulation cascades, and leukocyte transendothelial migration. Red, genes up-regulated in natural cycles; pink, genes up-regulated in stimulated cycles.

Figure 7: Unsupervised classification with principal component analysis (PCA) of the hCG+2 and hCG+5 samples with the predictor list.

Our predictor list of the endometrial receptivity allows the separation of 90% of hCG+5 samples from the hCG+2 samples in this PCA using the first two dimensions, representing 39% of the data information.

Supplementary Table I: The top-twenty up-regulated genes during endometrial receptivity in natural cycles and their fold change in stimulated cycles.

This table shows the top-twenty up-regulated genes during endometrial receptivity in natural cycles (43 < Fold change < 465) and their profile in stimulated cycles (4 < Fold change < 59). Some of them are not significantly modulated in stimulated cycles (-).

Supplementary Table II: Examples of genes with expression varying significantly in four microarray comparison analysis between the pre-receptive and the receptive stages from natural and stimulated endometrium cycles.

This table was adapted from Horcajadas's review (2007); it represented a list of genes specific for the endometrial receptivity obtained from three studies (Riesewijk *et al*., 2003; Horcajadas *et al*., 2005, 2006). Our data are added in this table, allowing a comparison. The fold changes for each gene were reported in natural and stimulated cycles.

Supplementary Figure 1: Validation using quantitative PCR of selected genes identified by the microarray data from stimulated cycles in the pre-receptive (hCG+2) and receptive (hCG+5) endometrium samples.

mRNA expression level of LAMB3, MFAP5, PROK1 and NLF2 in the hCG+2 (n=1) and hCG+5 (n=1) samples were examined by quantitative PCR. The results were normalized using PGK1 expression. Gene expression levels were increased in the hCG+5 sample as compared with the hCG+2 sample. LAMB3, laminin β3; MFAP5, microfibril-associated protein 5; PROK1, prokineticin 1; NLF2, nuclear localized factor 2.

Table I

Study	Number of samples	Natural cycle (number of samples)	Stimulated cycle (number of samples)	Paired analysis	Fold change	Number of genes	
						Up	Down
Mirkin *et* al. (2004)	13	LH+8 (n=5)	hCG+9 Atg (n=5)	Partially	≥ 1.19	6	6
		LH +8 (n=5)	hCG+9 Ag (n=3)		≥ 1.2	5	1
Horcajadas *et* al. (2005)	19	LH +7 (n=14)	hCG +7 (n=5)	No	≥ 3	281	277
Simon *et* al. (2005)	28	LH +7 (n=14)	hCG +7 Atg standard dose (n=4)	No	≥ 2	22	69
		LH +7 (n=14)	hCG +7 Atg high dose (n=5)			88	24
		LH +7 (n=14)	hCG +7 Ag (n=5)			22	100
Horcajadas *et* al. (2008)	49	LH+1 (n=5)	hCG+1 (n=5)	No	-	0	0
		LH+2 (n=5)	hCG+2 (n=5)			0	0
		LH+3 (n=5)	hCG+3 (n=5)			0	0
		LH+5 (n=5)	hCG+5 (n=4)			0	0
		LH+7 (n=5)	hCG+7 (n=5)			69	73
		LH+9 (n=5)	hCG+9 (n=5)			0	0
Liu *et* al. (2008)	13	LH+7 (n=5)	hCG+7 high serum E_2 levels (n=4)	No	≥ 2	244	159
		LH+7 (n=5)	hCG+7 low serum E_2 levels (n=4)			5	2
Current study	84	LH+2 (n=21)	hCG+2 (n=21)	Yes	≥ 2	321	4
		LH+7 (n=21)	hCG+5 (n=21)			657	0

Figure 1

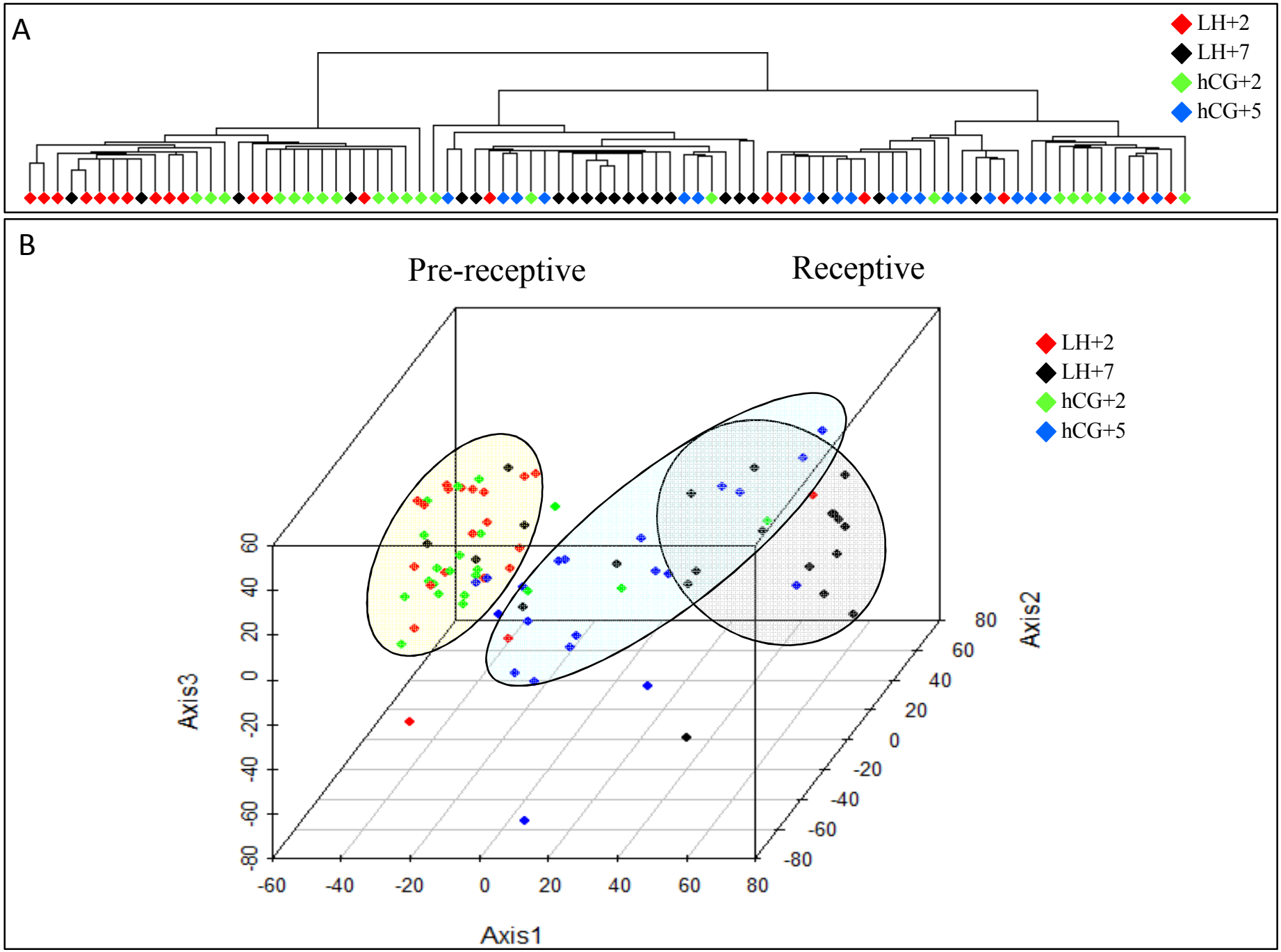

A
LH+2
LH+7
hCG+2
hCG+5
B
Pre-receptive
Receptive
LH+2
LH+7
hCG+2
hCG+5
Axis3
Axis1
Axis2
-60
-40
-20
0
20
40
60
80
-80

Figure 2

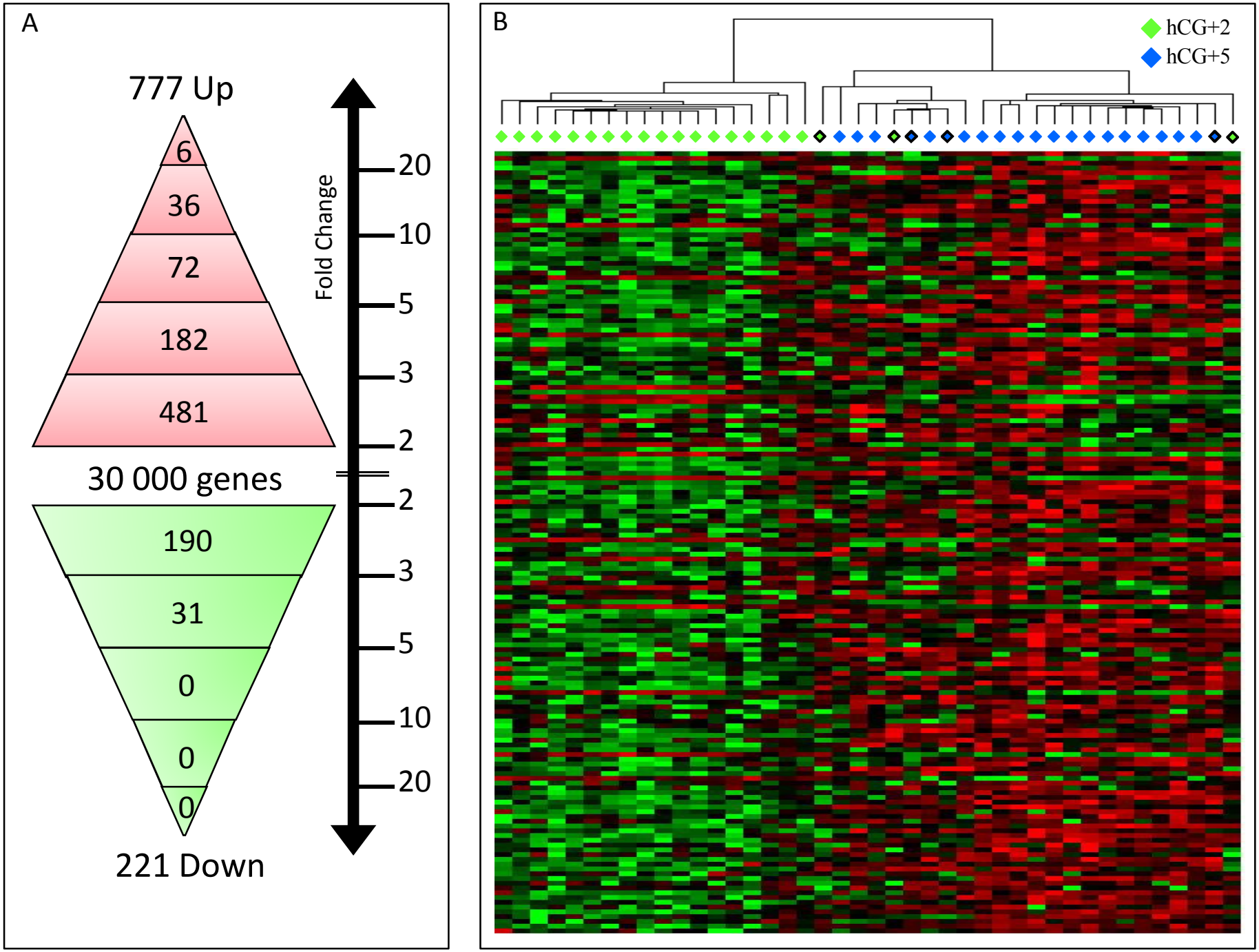

A
777 Up
6
36
72
182
481
30 000 genes
190
31
0
0
0
221 Down
Fold Change
20
10
5
3
2
2
3
5
10
20
B
hCG+2
hCG+5

Figure 3

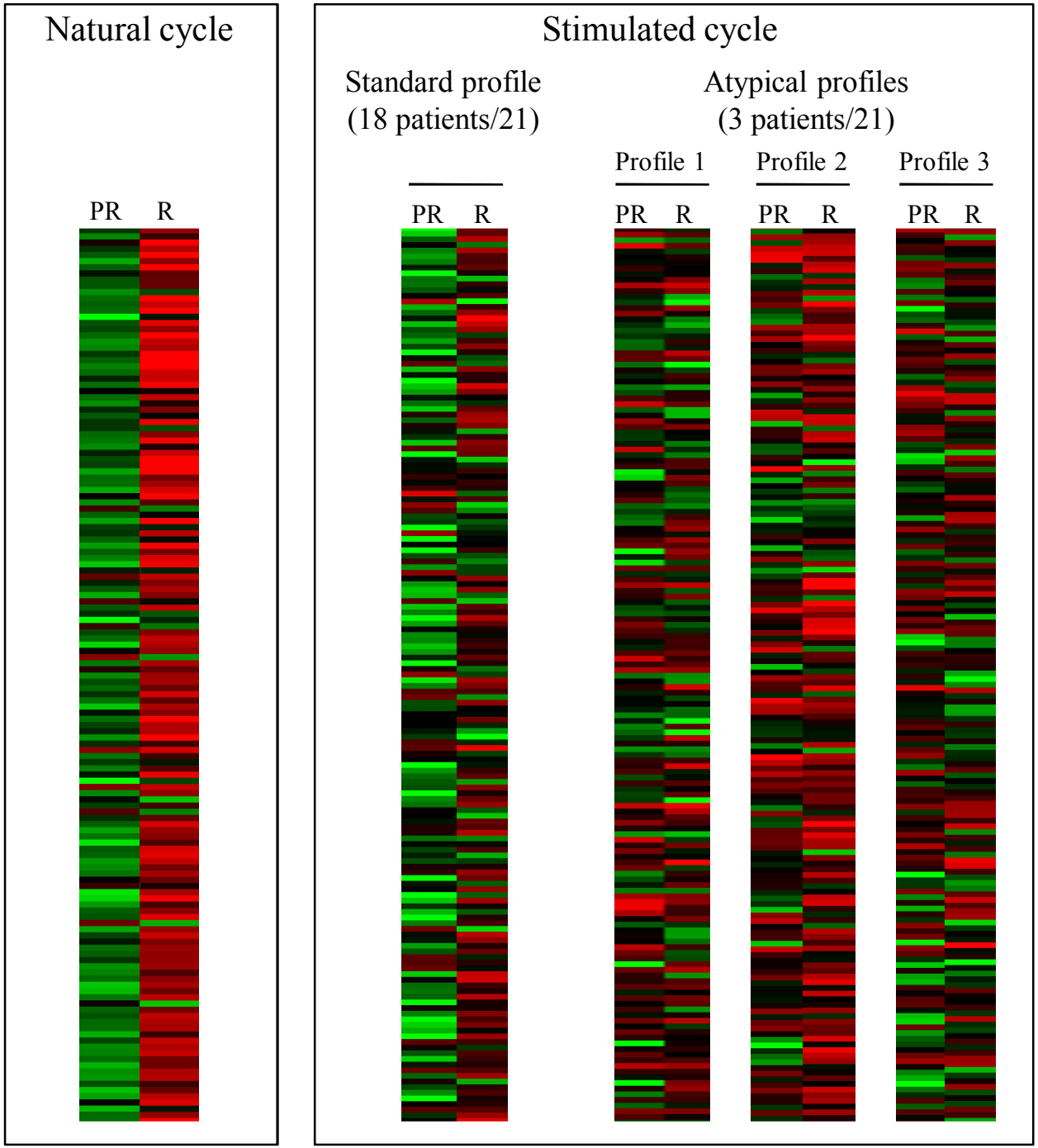

Natural cycle
PR
R
Stimulated cycle
Standard profile
(18 patients/21)
Atypical profiles
(3 patients/21)
Profile 1
Profile 2
Profile 3
PR
R
PR
R
PR
R
PR
R

Figure 4

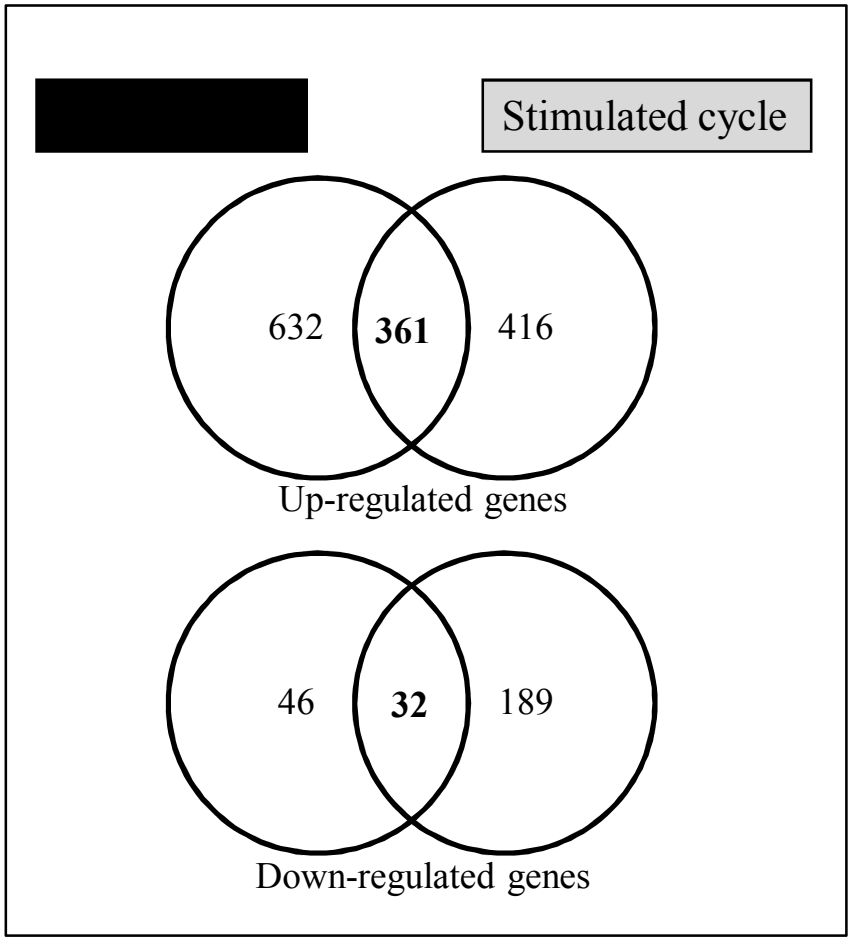

Stimulated cycle
632
361
416
Up-regulated genes
46
32
189
Down-regulated genes

Figure 6

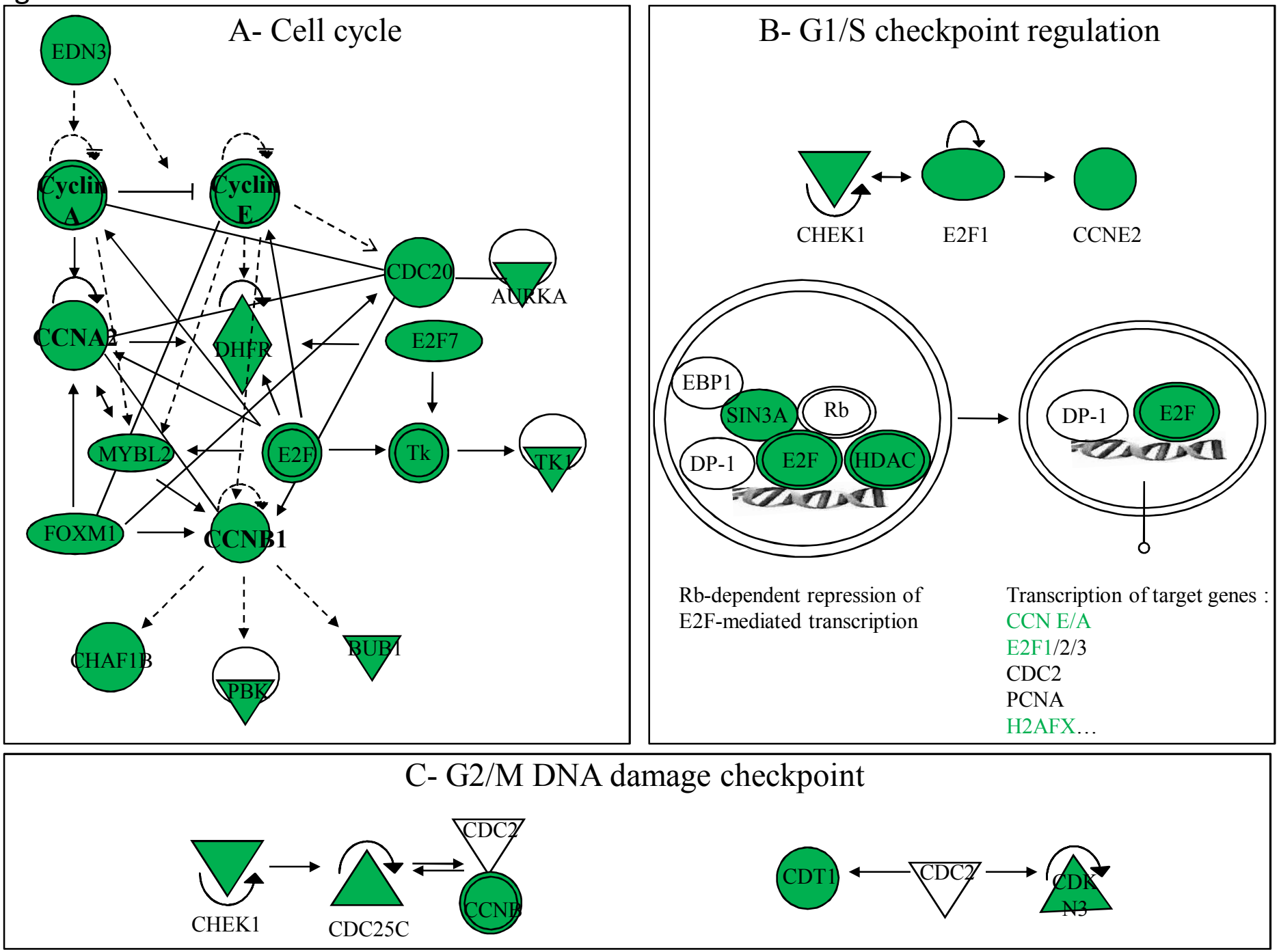

A- Cell cycle
EDN3
Cyclin A
Cyclin E
CDC20
AURKA
CCNA2
DHFR
E2F7
MYBL2
E2F
Tk
TK1
FOXM1
CCNB1
CHAF1B
PBK
BUB1
B- G1/S checkpoint regulation
CHEK1
E2F1
CCNE2
EBP1
SIN3A
Rb
DP-1
E2F
HDAC
DP-1
E2F
Rb-dependent repression of
E2F-mediated transcription
Transcription of target genes :
CCN E/A
E2F1/2/3
CDC2
PCNA
H2AFX…
C- G2/M DNA damage checkpoint
CHEK1
CDC25C
CDC2
CCNB
CDT1
CDC2
CDK
N3

Figure 7

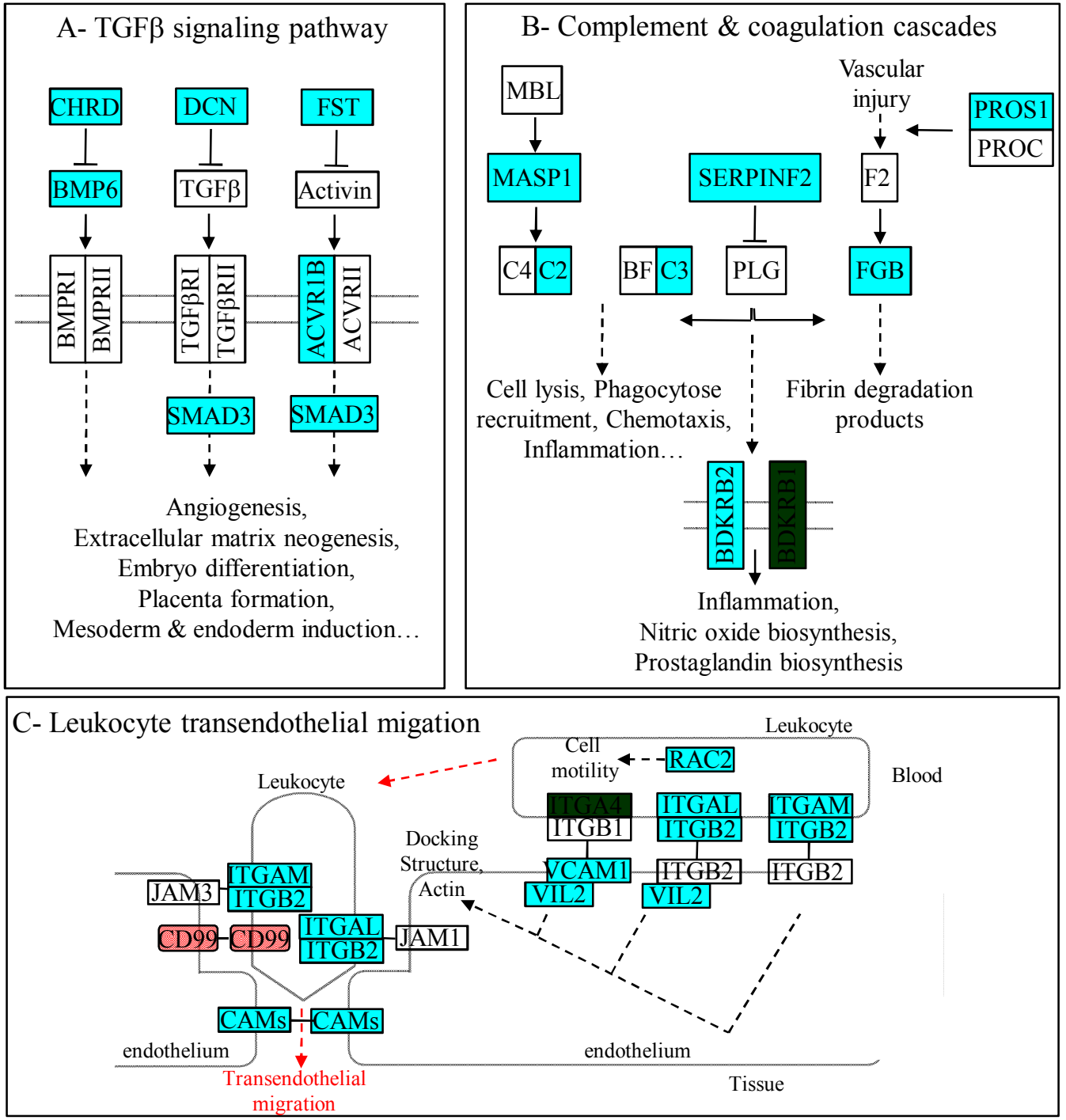
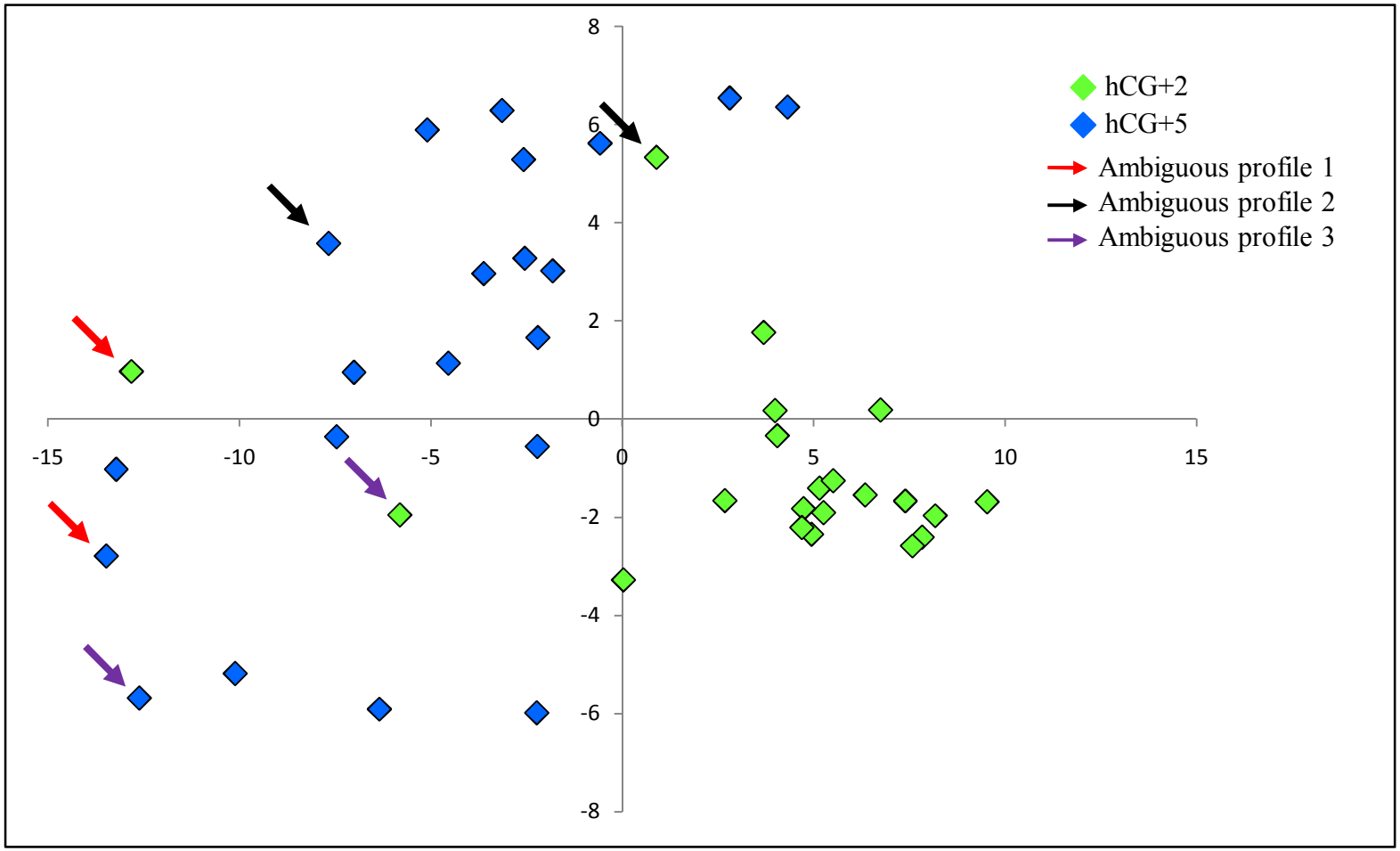

A- TGFβ signaling pathway
CHRD
DCN
FST
BMP6
TGFβ
Activin
BMPRI
BMPRII
TGFβRI
TGFβRII
ACVR1B
ACVRII
SMAD3
SMAD3
Angiogenesis,
Extracellular matrix neogenesis,
Embryo differentiation,
Placenta formation,
Mesoderm & endoderm induction…
B- Complement & coagulation cascades
MBL
Vascular injury
PROS1
PROC
MASP1
SERPINF2
F2
C4
C2
BF
C3
PLG
FGB
Cell lysis, Phagocytose recruitment, Chemotaxis, Inflammation…
Fibrin degradation products
BDKRB2
BDKRB1
Inflammation,
Nitric oxide biosynthesis,
Prostaglandin biosynthesis
C- Leukocyte transendothelial migation
Leukocyte
Cell motility
RAC2
Blood
ITGA4
ITGB1
ITGAL
ITGB2
ITGAM
ITGB2
Docking Structure, Actin
VCAM1
VIL2
ITGB2
VIL2
ITGB2
Leukocyte
ITGAM
ITGB2
JAM3
CD99
CD99
ITGAL
ITGB2
JAM1
CAMs
CAMs
endothelium
endothelium
Transendothelial migration
Tissue

Figure 8


hCG+2
hCG+5
Ambiguous profile 1
Ambiguous profile 2
Ambiguous profile 3
8
6
4
2
0
-2
-4
-6
-8
-15
-10
-5
0
5
10
15

Supplementary table I

Natural cycle				Stimulated cycle			
Gene ID	**Gene Name**	**Fold Change**	**FRD (%)**	**Gene ID**	**Gene Name**	**Fold Change**	**FRD (%)**
204351_at	S100P	465	0	204351_at	S100P	17	0
206859_s_at	PAEP	457	0	206859_s_at	PAEP	59	0
205713_s_at	COMP	403	0	-	-	-	-
203716_s_at	DPP4	165	0	203717_at	DPP4	3	1
208161_s_at	ABCC3	140	0	208161_s_at	ABCC3	18	0
205302_at	IGFBP1	139	0,5	-	-	-	-
218002_s_at	CXCL14	132	0	218002_s_at	CXCL14	15	0
241031_at	NLF1	128	0	241031_at	NLF1	15	0
219140_s_at	RBP4	95	0	-	-	-	-
203824_at	TSPAN8	93	0	203824_at	TSPAN8	7	0,7
1553809_a_at	C9orf71	85	0	231426_at	C9orf71	22	0
243483_at	TRPM8	66	0	243483_at	TRPM8	33	0
208335_s_at	DARC	58	0	208335_s_at	DARC	4	1
201348_at	GPX3	57	0	201348_at	GPX3	9	0
206378_at	SCGB2A2	50	0	206378_at	SCGB2A2	20	0
204988_at	FGB	46	0,5	-	-	-	-
203256_at	CDH3	45	0	-	-	-	-
235988_at	GPR110	45	0	-	-	-	-
205654_at	C4BPA	43	0	205654_at	C4BPA	17	0
236489_at	Hs.72307	43	0	236489_at	Hs.72307	12	0

Supplementary table II

	other studies		current study	
Gene symbol	Natural cycle (LH+2 *vs* LH+7)	COS (LH+7 *vs* hCG+7)	Natural cycle (LH+2 *vs* LH+7)	COS (hCG+2 *vs* hCG+5)
IL15	3	-	2.6	-
SPP1	11	-	4.4	3.5
GPX3	25.9	-11,9	57	9
PAEP	81.6	-9.8	457	59
FXYD2	4.1	4.5	7.3	2.3
DPP4	31.4	-37.1	165.1	2.5
LIF	36.6	-23	19.8	-
IGFBP3	4	-4.3	3.4	-
GADD45A	8	-3	6.8	2.2
HABD2	5.9	-6.4	8.6	3.5
EDNRB	8.2	-3.2	3.6	2.3
LMOD1	29.7	-4.1	10	2.3
CNN1	10.3	-9.3	-	-
CLU	28.8	-7.1	7.9	2.1
TAGLN	8.4	-3.7	5.2	-
CYP2C9	2.5	-4.7	8.3	4.9
CAPN6	-4.6	10.3	-	-
TFPI2	-3.5	5.4	-	-
MAP2K6	-4.9	8.6	-	2.6
CTNNA2	-8.5	7.3	-	4
SORD	-2.4	11.6	-	4.1
BCKDHB	-3.3	10.3	-	-
ALDH3B2	-3.1	4.3	-	3.8
NDRG2	-6	5.9	-	-
FOLR1	-10.5	9.3	-	3.7

Supplementary figure1