ARTICLE

Progesterone rise on HCG day in GnRH antagonist/rFSH stimulated cycles affects endometrial gene expression

I Van Vaerenbergh a,*, HM Fatemi b, C Blockeel b, L Van Lommel c, P In’t Veld a, F Schuit c, EM Kolibianakis d, P Devroey b, C Bourgain a

a Vrije Universiteit Brussel and UZ Brussel, Department of Pathology, Jette, Belgium; b UZ Brussel, Centre for Reproductive Medicine, Jette, Belgium; c KU Leuven, Gene Expression Unit Department of Molecular Cell Biology, Leuven, Belgium; d Aristotle University of Thessaloniki, Unit for Human Reproduction 1st Dept of Ob/Gyn, Thessaloniki, Greece

* Corresponding author. E-mail address: ivvaeren@vub.ac.be (I Van Vaerenbergh).

Inge Van Vaerenbergh graduated as a Master in Biomedical Sciences in 2006. Since November 2006, she has been affiliated as a researcher and PhD student to the Department of Experimental Pathology at Vrije Universiteit Brussel/UZ Brussel where she studies the gene expression of human endometrium in stimulated IVF cycles.

Abstract Premature progesterone rise during gonadotrophin-releasing hormone (GnRH) antagonist cycles for IVF is a frequent phenomenon and has been associated with lower pregnancy and implantation rates. This study evaluated endometrial gene expression on the day of oocyte retrieval according to the concentration of serum progesterone on the day of human chorionic gonadotrophin (HCG) administration in GnRH-antagonist/recombinant FSH IVF cycles with fresh embryo transfer. Endometrial biopsies (n = 14) were analysed with Affymetrix HG U133 Plus 2.0 Arrays. Patients were divided into three groups according to their progesterone serum concentration on the day of HCG administration: ≤0.9 ng/ml (group A), 1–1.5 ng/ml (group B) and >1.5 ng/ml (group C). Gene expression analysis showed a small number of significantly differentially expressed probe sets between groups A and B (five up/23 down in B) and a large difference between groups B and C (607 up/212 down; P ≤ 0.05, fold change >1.4). Validation was performed with quantitative real-time PCR on selected genes. As far as is known, this is the first study to demonstrate a distinct difference in endometrial gene expression profile between patients with a progesterone serum concentration above and below the threshold of 1.5 ng/ml on the day of HCG administration.

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Introduction

The actual stimulation protocols in IVF treatment induce supraphysiological serum concentrations of oestradiol during the follicular phase. However, despite the administration of gonadotrophin-releasing hormone (GnRH) analogues, many researchers have described an occurrence defined as ‘premature luteinization’ (Bosch et al., 2003; Hofmann et al., 1993; Legro et al., 1993; Ubaldi et al., 1996a). This process is defined as a rise in serum progesterone concentrations towards the end of the follicular phase above a threshold concentration, which is usually set arbitrarily. It affects about 5–30% of IVF patients (Edelstein et al., 1990; Fanchin et al., 1993; Givens et al., 1994; Schoolcraft et al., 1991; Silverberg et al., 1991; Ubaldi et al., 1995). No clear association has been reported between progesterone elevation and fertilization rates. This might be a sign of the absence of a detrimental effect of elevated progesterone on the day of human chorionic gonadotrophin (HCG) administration on oocyte quality, as previously suggested (Bosch et al., 2003; Check et al., 1994; Fanchin et al., 1996; Hofmann et al., 1993; Legro et al., 1993; Martinez et al., 2004; Moffitt et al., 1997; Shulman et al., 1996). However, a negative impact of elevated progesterone concentrations during the follicular phase on the endometrium has been described (Fanchin et al., 1997; Kyrou et al., 2009). This may affect the endometrial receptivity and embryo implantation.

Although progesterone rise has been historically associated with LH in the context of premature LH surges, more recent studies assign the elevated progesterone to FSH exposure during the follicular phase (Bosch et al., 2003; Hofmann et al., 1993; Ubaldi et al., 1996a). Changes in the paracrine regulation could explain an increase in progesterone production in the late follicular phase (Smitz et al., 2007). The pathological mechanism is probably due to the high response of the ovary to the stimulation, which results in an excess number of follicles and an excess number of proliferating granulosa cells. The high follicular progesterone concentrations could lead to an advancement of the endometrium, which disturbs the dialogue between embryo and endometrium and results in implantation failure (Kyrou et al., 2009; Papanikolaou et al., 2009).

Earlier studies (Bosch et al., 2003; Fanchin et al., 1993; Harada et al., 1995; Schoolcraft et al., 1991; Shulman et al., 1996; Silverberg et al., 1991) suggested that in ovarian stimulation protocols for IVF a modest increase in progesterone serum concentrations before ovulation is associated with lower pregnancy rates and higher pregnancy loss. However, this issue is still debatable and additional studies have failed to show this negative effect (Saleh et al., 2009; Venetis et al., 2007). Most of the published studies were performed in agonist cycles (Edelstein et al., 1990; Li et al., 2008; Martinez et al., 2004; Ou et al., 2008; Ozcakir et al., 2004; Silverberg et al., 1991) as compared with antagonist cycles (Bosch et al., 2003; Ubaldi et al., 1996b).

A recent study (Kyrou et al., 2009) on recombinant FSH/GnRH antagonist cycles demonstrated that a rise in progesterone on the day of HCG administration impairs pregnancy outcome. Moreover, it was recently shown that a significantly lower probability of clinical pregnancy was present in patients with progesterone rise above 1.5 ng/ml as compared with patients with normal progesterone, when embryo transfer was performed on day 3 of embryo culture (Papanikolaou et al., 2009). Progesterone rise in the same study did not affect pregnancy outcome, when embryo transfer was performed on day 5. However, the threshold of progesterone on the day of HCG administration, used to classify patients with or without premature progesterone rise, varies considerably in the literature. Several values have been proposed, such as 0.9 ng/ml (Edelstein et al., 1990; Fanchin et al., 1993; Hofmann et al., 1996; Martinez et al., 2004; Miller et al., 1996; Moffitt et al., 1997; Schoolcraft et al., 1991; Silverberg et al., 1991; Ubaldi et al., 1996b; Urman et al., 1999), 1.0 ng/ml (Check et al., 1993, 1994; Shechter et al., 1994) or 1.2 ng/ml (Bosch et al., 2003).

In this study, endometrial gene expression on the day of oocyte retrieval according to the concentration of serum progesterone on the day of HCG administration was assessed.

Materials and methods

Stimulation protocol and biopsy

Endometrial biopsies from 14 patients were taken with a Pipelle (Pipelle de Cornier, Prodimed, Neuilly-en-Thelle, France) on the day of oocyte retrieval in a GnRH antagonist/recombinant FSH stimulated cycle with fresh embryo transfer, as previously described (Van Vaerenbergh et al., 2009). Histological dating was performed according to Noyes et al. (1950) by a pathologist, blinded for clinical outcome. Ovarian stimulation was performed with a median starting dose of 200 IU recombinant FSH (Puregon; Schering-Plough, Oss, The Netherlands), from day 2 until day 6 of the cycle. From day 7 onwards, the dose was adjusted individually. To inhibit premature LH surge, daily GnRH antagonist (Orgalutran 0.25 mg; Schering-Plough) was used from the morning of day 6 of stimulation. Final oocyte maturation was achieved by administration of 10,000 IU of HCG (Pregnyl; Schering-Plough) as soon as three or more follicles ≥17 mm were present. Oocyte retrieval was carried out 36 h after HCG administration. The luteal phase was supported with 600 mg micronized progesterone (Utrogestan; Piette, Brussels, Belgium). IVF and intracytoplasmic sperm injection procedures have been described in previous studies in detail (Van Landuyt et al., 2005).

Hormonal measurements

Serum progesterone and serum oestradiol were measured with the automated Elecsys immunoanalyser (Roche Diagnostics, Mannheim, Germany). Intra-assay and interassay coefficients of variation (CV) were <3% and <5% for progesterone and <5% and <10% for oestradiol, respectively.

Gene expression

Endometrial biopsies from 14 patients were analysed for gene expression: patients were divided into three different
groups according to their progesterone serum concentration on the day of HCG administration: ≤0.9 ng/ml (group A; \(n = 3\)), 1–1.5 ng/ml (group B; \(n = 6\)) and >1.5 ng/ml (group C; \(n = 5\)).

Gene expression profiling was performed with Affymetrix Human Genome (HG) U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) and data were analysed with Affymetrix GeneChip operating software version 1.4, as previously described (Van Vaerenbergh et al., 2009). In summary, transcripts were considered as differentially expressed when all of the following criteria were met: (i) present in all samples of the selected group; (ii) consistent decreased or increased change in expression for pair-wise comparisons between the two selected groups; (iii) a mean absolute value of the signal log ratio ≥0.5; this equals a fold change ratio (FC) of 1.4 or higher; and (iv) a two-tailed unpaired Student’s t-test with a significant result (\(P < 0.05\)). The data files have been deposited in NCBI’s Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; Edgar et al., 2002) and are accessible through GSE19959.

In addition, unsupervised principal component analysis and hierarchical clustering analysis with a condition tree was performed with GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA, USA), as previously described (Van Vaerenbergh et al., 2009).

### Quantitative real-time PCR

Selected differentially expressed genes from the microarray data were technically validated with quantitative real-time PCR (Q-PCR).

Genes were selected for validation by Q-PCR because of their highly significant P-value or FC and/or because redundant probe sets for the same gene were differentially expressed. Some of these genes have been described in the literature before as fertility-related genes. A two-step Q-PCR was performed on the microarray samples from groups B and C (\(n = 11\) in total). Therefore, a reverse-transcription reaction from total RNA was achieved with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol. Q-PCR was performed with the appropriate TaqMan Gene Expression Assay (Applied Biosystems), containing two unlabelled primers and one TaqMan FAM dye-labelled MGB probe. *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) was chosen as the control housekeeping gene using the TaqMan Endogenous Control Assay (Applied Biosystems). Both assays are cDNA specific, since the probes span an exon junction. All Q-PCR assays were run using the TaqMan Universal PCR Master Mix (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-Time PCR System. Thermal cycling parameters were set as follows: 2 min at 50 °C (AmpErase UNG activation), 10 min at 95°C (AmpliTaq Gold activation), followed by 40 cycles of denaturation, annealing and extension (15 s at 95°C and 1 min at 60°C, respectively). No-template and no-reverse-transcriptase controls were included to verify the quality and cDNA specificity of the primers. All samples were analysed in triplicate. The relative quantification was performed by the standard curve method. For each sample, the amount of target gene and endogenous control (GAPDH) was determined from their respective standard curves. First, the target gene amount was divided by the endogenous control amount to obtain a normalized value. In a second step, the samples were normalized again to the sample with the lowest normalized expression, the calibrator sample or 1× sample. Therefore, each of the normalized values was divided by the calibrator normalized value to generate the relative expression levels (Applied Biosystems). Real-time PCR data were analysed using t-tests with a statistical significance level of \(P < 0.05\).

### Pathway analysis

To gain more information about the biological functions and pathways involved, the gene list with significant differentially expressed genes between groups B and C was analysed with Ingenuity Pathways Analysis 8.5 (Ingenuity Systems, Redwood City, CA, USA), as previously described (Van Vaerenbergh et al., 2009).

### Results

#### Gene expression

Patients (\(n = 14\)) were divided into three different groups, according to progesterone serum concentration on the day of HCG administration: (A) ≤0.9 ng/ml, (B) 1–1.5 ng/ml, and (C) >1.5 ng/ml (Table 1). These cut-offs were based on the literature (Papanikolaou et al., 2009; Venetis et al., 2007), where 0.9 ng/ml was established as the lowest threshold for premature progesterone rise and 1.5 ng/ml as the highest described threshold for premature progesterone rise. The mean oestradiol concentrations on the day of HCG and the mean number of oocytes obtained were also calculated. The serum oestradiol concentrations were on average 1367.7 ± 345.1 pg/ml in group A, 1935.5 ± 809.9 pg/ml in group B and 2431.8 ± 1276.9 pg/ml in group C. There were no significant differences in oestradiol concentrations on the day of HCG between groups (one-way ANOVA). The average number of cumulus–oocyte–complexes obtained was also calculated. The serum oestradiol concentrations were on average 8.0 ± 2.0 in group A, 9.17 ± 4.1 in group B and 11.8 ± 1.9 in group C (not significantly different).

No ongoing pregnancies occurred in group C; all pregnant patients were observed in group A (\(n = 2\)) and B (\(n = 2\)). Biochemical pregnancy was defined as the presence of jHCG in serum 12 days after embryo transfer, which failed to progress to ultrasonographic detection of a gestational sac. Pregnancies were considered as ongoing after 12 weeks of gestation (Kolibianakis et al., 2002). Histological dating showed an advanced endometrial maturation for the majority of patients (13 out of 14 or 92.9%), ranging from +2 to 4 days (Table 1).

Endometrial gene expression analysis showed a small number of differentially expressed probe sets between groups A and B (five up-regulated and 23 down-regulated in group B) and a large amount of differentially expressed probe sets between groups B and C (607 up-regulated in group B and 212 down-regulated; 819 differentially expressed probe sets in total; Figure 1).

Clustering analysis with hierarchical clustering (condition tree) and principal component analysis showed a separate
cluster of the samples with premature progesterone rise above 1.5 ng/ml (group C; Figure 2).

Quantitative real-time PCR

The most recent and highest cut-off value for progesterone serum concentration on the day of HCG administration was set at 1.5 ng/ml (Papanikolaou et al., 2009). Therefore, a number of genes out of the list of probe sets, differentially expressed between groups B and C, were validated. Validation was performed with the Q-PCR technique (n = 11; group B n = 6, group C: n = 5) on the following selected genes: pregnancy-associated plasma protein-A (PAPP-A; pappalysin 1; Hs01032307_m1), protease, serine, 23 (PRSS23; Hs00359912_m1), interleukin 17 receptor B (IL17RB; Hs00914532_m1), thrombospondin type I domain containing 4 (THSD4; Hs00388232_m1) and Dickkopf homolog 3 (Xenopus laevis) (DKK3; Hs00247429_m1). This resulted in significant fold changes (t-test with P < 0.05, consistent with the fold changes from the microarray analysis; Table 2 and Figure 3).

Pathway analysis

To focus on the biological functions and related canonical pathways, the list of significant differentially expressed genes between groups B and C was analysed. This analysis demonstrated that the most relevant functions were related to cellular growth and proliferation, cellular movement, development, cell-to-cell signalling and cell death. Furthermore, one of the most relevant canonical pathways was the Wnt/β-catenin signalling pathway. DKK3 was identified as one of the up-regulated genes (FC = 2.9 and 2.7 for two microarray probe sets) in this pathway.

Discussion

As has been suggested previously (Kolibianakis et al., 2002; Ubaldi et al., 1997), an endometrial biopsy on the day of oocyte retrieval may be used to assess endometrial receptivity in patients with serum progesterone rise. In the present study, endometrial gene expression analysis from 14 patients on the day of oocyte retrieval, categorized into three different groups according to their serum progesterone concentration on day of HCG administration, generated lists of differentially expressed genes. A major difference in endometrial gene expression was observed between groups

Table 1 Clinical outcome and histological dating results for patients grouped according to progesterone serum concentration on day of human chorionic gonadotrophin administration.

<table>
<thead>
<tr>
<th>Patient’s progesterone concentration (ng/ml)</th>
<th>Clinical outcome</th>
<th>Histological datinga</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (progesterone ≤0.9 ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.68</td>
<td>Pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>0.69</td>
<td>Not pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>0.9</td>
<td>Pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>Group B (progesterone 1–1.5 ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Not pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>1.1</td>
<td>Not pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>1.44</td>
<td>Pregnant</td>
<td>+3 days</td>
</tr>
<tr>
<td>1.1</td>
<td>Pregnant</td>
<td>+3 days</td>
</tr>
<tr>
<td>1.05</td>
<td>Not pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>1.3</td>
<td>Not pregnant</td>
<td>+4 days</td>
</tr>
<tr>
<td>Group C (progesterone &gt;1.5 ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.61</td>
<td>Not pregnant</td>
<td>+4 days</td>
</tr>
<tr>
<td>1.8</td>
<td>Not pregnant</td>
<td>+4 days</td>
</tr>
<tr>
<td>1.54</td>
<td>Not pregnant</td>
<td>+2 days</td>
</tr>
<tr>
<td>1.93</td>
<td>Not pregnant</td>
<td>Late proliferative</td>
</tr>
<tr>
<td>1.7</td>
<td>Biochemical pregnancy</td>
<td>+3 days</td>
</tr>
</tbody>
</table>

a(Advanced) endometrial maturation as compared with the chronological cycle day.
B (progesterone 1–1.5 ng/ml) and C (progesterone >1.5 ng/ml). Moreover, clustering analysis showed a separate cluster for patients in group C. This clustering is possibly also related to the endometrial maturation, as one sample is more closely situated near the group C cluster and this sample has also been histologically dated as +4 days. Another sample from group C dated as ‘late proliferative’ was situated closely to samples from group B. The morphology of the majority (>75%) of this biopsy sample corresponded to the late proliferative phase. However, several glands showed more secretory activity. Nevertheless, this biopsy could not be confidently classified as ‘secretory phase’ because of heterogeneity in glandular maturation. One has to acknowledge the fact that the biological secretory activity could have been underestimated. In addition, in a previous paper by Ubaldi et al. (1997), it was demonstrated that, although the endometrial maturation was advanced in patients with elevated progesterone in the follicular phase, the morphology could not be predicted from the number of days of premature progesterone rise and cumulative exposure to this.

Following this analysis, several genes were selected for confirmation of the microarray data with Q-PCR. PRSS23 is an extracellular serine protease expressed in the mouse.

**Figure 2** (A) Principal component analysis (PCA) and (B) hierarchical clustering (condition tree), displayed as heatmap.
ovary and may play a role in follicular development in the mouse. Its dynamic expression pattern suggests that PRSS23 is involved in different tissue remodelling processes in the ovary, possibly by allowing extracellular matrix degradation and/or regulating growth factor availability. (Miyakoshi et al., 2006; Wahlberg et al., 2008)

The receptor IL17RB is found to be a prognostic marker for breast cancer (Jerevall et al., 2008; Ma et al., 2008).

Table 2  Real-time PCR was performed for validation of microarray results on selected genes, which resulted in fold changes consistent with the fold changes from the microarray data. Microarray fold changes represent the different significantly regulated probe sets.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Microarray results</th>
<th>QPCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold change</td>
<td>P-value</td>
</tr>
<tr>
<td>DKK3 (up in group B)</td>
<td>2.7, 2.9</td>
<td>0.009, 0.012</td>
</tr>
<tr>
<td>THSD4 (down in B)</td>
<td>-2.23, 3.21, 2.15</td>
<td>-0.026, 0.038, 0.049</td>
</tr>
<tr>
<td>PAPP-A (up in group B)</td>
<td>4.59, 3.21, 2.15</td>
<td>0.04, 0.038, 0.049</td>
</tr>
<tr>
<td>PRSS23 (up in group B)</td>
<td>4.51, 3.97</td>
<td>0.01, 0.01</td>
</tr>
<tr>
<td>IL17RB (up in group B)</td>
<td>4.67, 4.62</td>
<td>0.02, 0.006</td>
</tr>
</tbody>
</table>

DKK3 = Dickkopf homolog 3 (Xenopus laevis); IL17RB = interleukin 17 receptor B; PAPP-A = pregnancy-associated plasma protein-A; PRSS23 = protease, serine, 23; THSD4 = thrombospondin type I domain containing 4.

Figure 3  Quantitative PCR results for pregnancy-associated plasma protein-A (PAPP-A), protease, serine, 23 (PRSS23), interleukin 17 receptor B (IL17RB), thrombospondin type I domain containing 4 (THSD4) and Dickkopf homolog 3 (Xenopus laevis) (DKK3), shown as mean ratio ± SE of the mean for patients in groups B (n = 6) and C (n = 5). GAPDH = glyceraldehyde-3-phosphate dehydrogenase. P < 0.05.
Premature progesterone rise affects endometrial gene expression

The Wnt/β catenin signalling pathway has been described before in different implantation and endometrial receptivity studies (Tulac et al., 2003; Van Vaerenbergh et al., 2009). Another member of the Dickkopf family, DKK1, has been demonstrated as one of the genes with a significant change observed in the mid-secretory phase (LH+7 days) of a natural cycle in different microarray endometrial receptivity studies (Carson et al., 2002; Haouzi et al., 2009; Riesewijk et al., 2003; Talbi et al., 2006).

All four ongoing pregnancies ensued in patients from groups A and B. The differences in gene expression profiles between pregnant and non-pregnant patients in stimulated cycles for IVF have been studied before (Van Vaerenbergh et al., 2010). Furthermore, two patients in group C showed an advanced endometrial maturation of +4 days. Previous studies (Kolibianakis et al., 2002; Ubaldi et al., 1997) with endometrial biopsies on the day of oocyte retrieval and following embryo transfer demonstrated that an endometrial advancement of more than 3 days never resulted in an ongoing pregnancy.

The endometrial biopsies of the patients analysed with microarrays were as comparable as possible within the three groups, with regard to good RNA quality (RIN (RNA Integrity Number) > 8.0; Van Vaerenbergh et al., 2009) and stimulation protocols, to reduce the interpatient bias. Although the number of patients analysed in the present study was limited, the progesterone rise on the day of HCG administration seems to have an instant effect on the endometrial maturation, as shown by histological dating on a biopsy sample of only 36 h later, on the day of oocyte retrieval. Further study on larger patient groups is necessary to define a threshold for premature progesterone increase, with only a minimal effect on endometrial maturation.

As far as is known, this is the first study to investigate endometrial gene expression on the day of oocyte retrieval in GnRH antagonist cycles with fresh embryo transfer, according to the concentration of serum progesterone on the day of HCG administration. The current data demonstrate a distinct difference in endometrial gene expression profile between patients with a progesterone serum concentration above and below the threshold of 1.5 ng/ml on the day of HCG administration. These significant changes observed at the gene expression level between endometrial samples exposed to different concentrations of progesterone on the day of HCG administration may explain the impairment of endometrial receptivity in the presence of elevated progesterone, reflected in the lower pregnancy rates reported in the literature (Bosch et al., 2003).

References


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