LH/hCGR gene expression in human cumulus cells is linked to the expression of the extracellular matrix modifying gene TNFAIP6 and to serum estradiol levels on day of hCG administration

D. Haouzi1,2,3, S. Assou1,2,3, K. Mahmoud4, B. Hedon2, J. De Vos1,3, D. Dewailly5, and S. Hamamah1,2,3,6

1Institut de Recherche en Biothérapie, CHU Montpellier, Université Montpellier I, Hôpital Saint-Eloi, Montpellier F-34000, France
2Département de Médecine et Biologie de la Reproduction, CHU Montpellier, Hôpital Arnaud de Villeneuve, Montpellier F-34000, France
3INSERM, U847 ‘Développement embryonnaire précoce et cellules souches embryonnaires humaines’, Montpellier F-34000, France
4Centre de FIV, Clinique les Jardins, Tunis, Tunisia
5Département de Gynécologie Endocrinienne et Médecine de la Reproduction, CHU Lille, Hôpital Jeanne de Flandre, Lille F-59037, France
6Correspondence address. ART/PGD Division, Department of Reproductive Medicine, Arnaud de Villeneuve Hospital, 34295 Montpellier, France. Tel: +33-4-67-33-64-04; Fax: +33-4-67-33-62-90; E-mail: s-hamamah@chu-montpellier.fr

BACKGROUND: Recent studies suggest a role for luteinizing hormone and human chorionic gonadotrophin receptor (LH/hCGR) signaling in the regulation of the oocyte–cumulus oophorus cell interplay. The present study aimed at assessing the LH/hCGR gene expression in cumulus cells (CCs) surrounding oocytes in patients undergoing controlled ovarian hyperstimulation (COS) before ICSI and to relate the LH/hCGR expression to other COS quality parameters.

METHODS: CCs from single oocytes of normal responder patients were analysed by DNA microarrays. Concomitantly, estradiol levels on the day of hCG administration, CC morphology, total collected oocyte and metaphase II oocyte number were assessed in relation to LH/hCGR gene expression in CC.

RESULTS: The transcriptome analysis of CC indicated a variable expression of LH/hCGR among the patients and intra-patients. LH/hCGR mRNA expression was negatively correlated with serum estradiol level on the day of hCG administration. Eighty-five genes were significantly modulated between CCs from patients with a high and a low LH/hCGR expression. These genes are involved principally in steroid metabolism and in the ovulation process and include TNFAIP6, a gene expressed during CC–oocyte complex (COC) expansion. There were no significant differences in LH/hCGR gene expression profile between COS protocols.

CONCLUSIONS: LH/hCGR is expressed in CC under COS conditions. LH/hCGR expression level is associated with TNFAIP6 gene expression and negatively correlated with serum estradiol level on the day of hCG administration.

Key words: cumulus cells / LH/hCGR / TNFAIP6 gene

Introduction
The maturation and functional status of oocytes result from the bi-directional traffic between the oocyte, the cumulus cells (CCs) surrounding the egg and the floating and mural granulosa cells. It has been suggested that the cumulus–oocyte complex (COC) occupies a unique niche within the follicle and that compared with mural granulosa cells, CCs have a distinct phenotype (Erickson and Shimasaki, 2000). This difference results from a specific regulation exerted by the oocyte (Eppig et al., 1997). In particular, the expression of the plasma membrane receptor luteinizing hormone/human chorionic gonadotrophin receptor (LH/hCGR) has been reported to be low...
Materials and Methods

Patients and ICSI treatment

Normal responder patients (age 33.3 years ± 2.9, n = 46) among couples attending our centre for ICSI for male infertility factor were included in this study. This study received institutional review board approval. Patients were stimulated with a combination of GnRH agonist or antagonist protocols with the following treatments: agonist-highly purified human menopausal gonadotrophin (agonist-HP-hMG; Menopur, Ferring) (n = 7), antagonist-HP-hMG (n = 5), agonist-recombinant FSH (GonalF™, Merck-Serono) (n = 8), antagonist-recombinant FSH (n = 8), agonist-recombinant FSH with recombinant LH (Luveris™, Merck-Serono) (n = 5), antagonist-recombinant FSH with recombinant LH (n = 5). Ovarian response was evaluated by serum estradiol level (E2) and ultrasound examination to monitor follicle development. Retrieval of oocytes was performed with the puncture of many mature follicles in the same act, 36 h after hCG administration (5000 IU), under ultrasound guidance.

A third independent group of eight normal responder patients used as control was selected for floating granulosa cells collection.

Measurement of LH, FSH and estradiol

Blood measurements for endocrine parameters (LH, FSH and E2) were done on Day 3 after starting ovarian stimulation (LH) and on the day of hCG administration (E2). We used the Cobas immunoassay test for the in vitro quantitative determination of estradiol, FSH and LH in serum.

CCs surrounding oocyte

A total of 64 CC samples provided from 64 single oocytes were analysed individually (Table I). CCs were mechanically removed shortly (≤40 h post hCG administration) before ICSI procedure, washed in culture medium and frozen at −80°C in RTL buffer (RNeasy Kit, Qiagen, Valencia, CA, USA) until analysed. For each CC sample included in this study, CC morphology and the quality parameters of the individually cultured oocyte were evaluated.

Floating granulosa cells

From control normal responder patients (n = 8), floating granulosa cells were collected (Table I). For each patient, follicular fluid from mature follicles of the same patient were pooled, after removal of the cumulus–oophorus complex and diluted in HBSS solution (BioWhittaker) in 50 ml batches, representing one sample. Floating granulosa cells purification was adapted from the protocol by Kolena et al. (1983). Following centrifugation at 500g for 20 min in swinging buckets, granulosa cells were collected on a Ficoll cushion (12 ml Lymphocyte Separation Medium, BioWhittaker). They were successively washed in HBSS and PBS, incubated 5 min in blood lysis buffer, and fixed in 4% paraformaldehyde for 10 min.

Table I Number of cumulus cell (CC) samples according to arbitrary LH/hCGR expression groups by signal intensity (microarray) and numbers of CCs and floating granulosa cell (FGC) samples analysed by QRT–PCR

<table>
<thead>
<tr>
<th>LH/hCGR expression groups</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>16</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>Microarray chips</td>
<td>16</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>QRT–PCR</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>
buffer (KHCO$_3$ 10 mM, NH$_4$Cl 150 mM, EDTA 0.1 mM) to remove red blood cells, counted and pelleted in PBS before lysis and storage at −80 °C in RLT buffer (Qiagen). The number of follicles punctured and the number of purified granulosa cells ranged from 6 to 12 and from 2 × 10$^6$ to 9 × 10$^6$, respectively.

**LH/hCGR expression analysis**

Transcriptomic gene expression profile of all CC samples from studies 1 and 2 (n = 64 CC samples) was individually analysed on microarray chips. Then, according to LH/hCGR probeset expression intensity, three arbitrary groups emerged (low, medium and high LH/hCGR expression). To analyse the relationship between the LH/hCGR probeset expression intensity and clinical data of COS, the mean LH/hCGR probeset expression intensity of multiple CC samples from individual patients in the study number 1 was used. Patients from both studies were attributed to one of the three LH/hCGR expression groups (Fig. 1).

**Complementary RNA preparation and microarray hybridization**

Cumulus and granulosa cells 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). Complementary RNA (cRNA) was prepared with two rounds of amplification according to the manufacturer’s protocol ‘double amplification’ (Two-Cycle Amplification Kit) starting from total RNA (ranging from 70 to 100 ng). cRNA obtained after the second amplification round ranged from 32 to 90 μg. Labelled fragmented cRNA (12 μg) was hybridized to oligonucleotide probes on Affymetrix HG-U133 Plus 2.0 arrays containing 54 675 sets of oligonucleotide probes (‘probeset’) which correspond to ≈30 000 unique human genes or predicted genes. Each cumulus and granulosa cell 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 and a detection call (present, marginal or absent) for each probeset, using the default analysis settings and global scaling as first normalization method, with a trimmed mean target intensity value of each array arbitrarily set to 100. Probe intensities were derived using the MASS.0 algorithm. This algorithm also determines whether a gene is expressed with a defined confidence level or not.

**Figure 1** LH/hCGR expression groups. (A) Sixty-four CC samples collected from 64 single oocytes (38 patients) were analysed by microarray. (B) The mean of LH/hCGR gene expression intensity for each patients with multiple CCs analysed (white bars, mean ± SEM, study number 1) and absolute values (black bars, study number 2) were allocated to one of the three LH/hCGR expression groups to perform correlation with the clinical parameters.
("detection call"). This ‘call’ can either be ‘present’ (when the perfect match probes are significantly more hybridized than the mismatch probes, \( P < 0.04 \)), ‘marginal’ (for \( P \)-values of \( > 0.04 \) and \( < 0.06 \)) or ‘absent’ (\( P > 0.06 \)). The microarray data were obtained in our laboratory in agreement with the Minimal Information about Microarray Experiment (MIAME) recommendations (Brazma et al., 2001).

**Microarray data analysis**

Significant analysis of microarrays (SAM) (Tusher et al. 2001) was used to identify genes whose expression varied significantly between groups. SAM provides mean or median fold change (FC) values and a false discovery rate (FDR) confidence percentage based on data permutation (mean FC \( > 1.5 \) and FDR \( < 5\% \)). To perform the comparison of gene expression profile between the 64 CC samples according to their LH/hCGR expression, selection using the absent/present ‘call detection’ and a variation coefficient (\( \geq 40\% \)) between samples were performed followed by the SAM. We also performed a supervised hierarchical clustering analysis based on the expression levels of varying probes with CLUSTER and TREEVIEW software packages (Eisen et al., 1998).

**Ingenuity pathway analysis**

Genes were analysed using ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). The 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 gene/genes products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node colour indicates the degree of up-regulation.

**Quantitative reverse transcriptase–polymerase chain reaction analyses**

Labelled cRNA (1 \( \mu \)g) from CC and granulosa cell samples was used to generate first strand cDNA. These cDNAs (5 \( \mu \)l of a 1/10 dilution) were used for quantitative polymerase chain reaction (PCR) reaction according to the manufacturer’s recommendation (Applied Biosystems). The 20 \( \mu \)l reaction mixture consisted of cDNA (5 \( \mu \)l), 1 \( \mu \)M of primer and 10 \( \mu \)l of TaqMan Universal PCR Master Mix (Applied Biosystems). The amplification was measured during 40 cycles with an annealing temperature of 60°C. Phosphoglycerate kinase 1 (PGK1) was used to normalize signal intensity between samples. The amount of PCR product produced in every cycle step of the PCR reaction is monitored by a TaqMan probe. A threshold is set in the exponential phase of the amplification curve, from which the cycle number (‘Ct’ for ‘cycle threshold’) is read off. The Ct-value is used in the calculation of relative mRNA transcript levels. Effectiveness (\( E \)) of the PCR was also measured. Effectiveness is obtained by a standard curve corresponding to the primers used. Quantitative reverse transcriptase–PCR (QRT–PCR) was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems) and normalized to PGK1 for each sample using the following formula: \( e_{\text{normalisation primer}} \Delta Ct / E_{\text{PGK1}} \Delta Ct \) (\( E = 10^{-1/\text{slope}} \)). \( \Delta Ct = Ct \) control – Ct unknown, control = one CC sample of the low LH/hCGR group. Each sample was analysed in duplicate, and multiple water blanks were included in the analysis.

**Statistical analyses**

Statistical analyses for clinical values were performed with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). A reapartition difference between sample groups was considered significant when the Kruskal–Wallis non-parametric test gave a \( P \)-value of \( < 0.05 \). To relate serum estradiol level and LH/hCGR gene expression intensity, Spearman’s rank correlation coefficient was applied.

**Results**

**LH/hCGR gene expression profile in human CCs**

By microarray analysis, we analysed the expression of LH/hCGR in 64 CC samples. The LH/hCGR probeset was detected in all CC samples; however, individual CC expression levels varied up to 10-fold between patients (Fig. 1). Three arbitrary categories of LH/hCGR gene expression intensity were determined: low (\( 0 < \) signal intensity \( < 100 \), \( n = 16 \)), medium (100 \( < \) signal intensity \( < 200 \), \( n = 31 \)) and high (200 \( < \) signal intensity \( < 580 \), \( n = 17 \)) (Table I and Fig. 1).

To confirm and quantify the LH/hCGR gene expression by QRT—PCR, CC samples with an extreme difference in their LH/hCGR gene expression intensity were tested (Table I). Floating granulosa cells RNA from independent patients (\( n = 8 \)) were used as controls. QRT—PCR mean LH/hCGR transcript levels were significantly higher in CC of patients from the high LH/hCGR group when compared with the low LH/hCGR group and floating granulosa cells (12.1 \( \pm \) 0.5 versus 1.5 \( \pm \) 0.5 or 2.6 \( \pm \) 1.1, respectively, \( P = 0.001 \)). Thus, these data validated the microarray data and unambiguously showed the high expression of LH/hCGR in CCs from a subset of patients undergoing COS (Fig. 2).

![Figure 2](image-url)  
**Figure 2** Relative LH/hCGR mRNA expression level in CCs by QRT—PCR. LH/hCGR mRNA expression in CCs was examined, and granulosa cells were added as positive control. The results were normalized using PGK1 expression. LH/hCGR mRNA expression is stronger in CC belonging to the group with a high LH/hCGR expression than in granulosa cells. Significant reparation between groups for LH/hCGR mRNA (\( P = 0.01 \)) with the Kruskal–Wallis test. Bars are mean \( \pm \) SEM.
Gene expression profile of CCs according to LH/hCGR expression levels

To analyse the relationship between the LH/hCGR expression and transcriptome in CCs, we compared the signal intensity of all 54,675 probes between CCs of the low, medium and high LH/hCGR expression groups using SAM. Only seven genes were significantly modulated between the low and the medium LH/hCGR groups (data not shown), whereas 85 genes were significantly modulated between the high LH/hCGR group and the two other groups (Fig. 3). Interestingly, the majority of the modulated genes (81%) were up-regulated in CCs with high LH/hCGR expression, with an FC > 2 and an FDR < 0.05, of which 22 displayed an FC between 3 and 12 (Supplementary Table S1).

Pathways associated with high LH/hCGR expression in CCs

To identify biological processes associated with a high LH/hCGR expression, Ingenuity and PubMed databases were used to carry out pathway and function analyses on the genes modulated between the high and low/medium LH/hCGR expression. The 85 modulated transcripts of the high LH/hCGR group involved primarily metabolic pathways, mostly steroid and androgen/estrogen metabolisms. As shown in Fig. 4, these transcripts encoded enzymes for cortisol/testosterone biosynthesis (CYP11A1, HSD3B2 and CYB5B) and estradiol catabolism (CYP1B1). Lastly, CYP1B1 mRNA, an aromatase metabolizing 17β-estradiol (Hayes et al., 1996; Lee et al., 2003), was strongly increased (×4.8, \( P = 0.0001 \)) (Fig. 4). Although the expression of

![Supervised hierarchical clustering of cumulus samples with 85 genes. Eighty-five genes were significantly modulated in CCs between the high LH/hCGR group and the two other groups (\( P < 0.05 \)). The group of CCs characterized by a high LH/hCGR expression formed a distinct group from CCs of the low and medium LH/hCGR group. Red, genes up-regulated; green, genes down-regulated in the high LH/hCGR group; *CCs of the high LH/hCGR expression group.](image-url)
these genes in CCs was confirmed by QRT–PCR, significant distribution between groups was observed only for HSD3B2 (Fig. 4).

The tumour necrosis factor alpha-induced protein 6 (TNFAIP6), a hyaluronan binding protein involved in CC expansion, was exclusively increased ($/2.96, P = 0.046$) in the high LH/hCGR group in comparison with the two other groups.

Other signalling pathways were modulated in the high LH/hCGR group, including calcium signalling. RYR3, a ryanodine receptor...
involved in calcium pathway, was increased (×2.7, \( P = 0.0001 \)) in CC as well as PKCα mRNA (×1.5, \( P = 0.03 \)) (Fig. 4). The two major networks of the 85 modulated genes from the high LH/hCGR group identified by IPA software are shown in the Fig. 5.

Serum estradiol level on the day of HCG administration under COS according to LH/hCGR expression in CCs

The mean of LH/hCGR gene expression intensity level of CCs for each patient was analysed in several CC samples and then attributed...
to one of the three LH/hCGR groups. The microarray gene expression intensity of LH/hCGR was 48 ± 7, 135 ± 23 and 347 ± 55 in low, medium and high LH/hCGR groups, respectively (Fig. 6). No differences were observed among the three LH/hCGR groups in relation to patient age, body mass index, serum-FSH, LH at Day 3 and rFSH or HP-hMG dose/duration of stimulation (data not
in COS (Lee et al., 2003, 2005; Fu et al., 2007). However, in this study, we did not show significant association between LH/hCGR expression level and CC morphology. Expansion of CC was induced by the pre-ovulatory surge of LH in vivo, or by epidermal growth factor or FSH treatment in vitro, and involved TNFAIP6 up-regulation (Yoshioka et al., 2000; Fulop et al., 2003; Ochsner et al., 2003; Diaz et al., 2006, 2007; Sayasith et al., 2007). It was recently reported that up-regulated expression of LH/hCGR allowed LH to induce expression of genes involved in porcine COC expansion in vitro, including TNFAIP6 (Kawashima et al., 2008). In the present study, we report that TNFAIP6 gene expression was exclusively up-regulated in the high CC LH/hCGR group, suggesting a possible role of human LH/hCGR in human CC expansion in stimulated cycles. Although TNFAIP6 has been shown to be expressed in granulosa cells after the LH surge (Yoshioka et al., 2000), our data revealed that significantly more TNFAIP6 mRNA transcripts were detectable in CCs with both low and high LH/hCGR expression than in floating granulosa cells, where TNFAIP6 mRNA level was almost undetectable. This finding reinforces hypothesis that TNFAIP6 is expressed in COC undergoing mucification.

Our transcriptome analyses of the CCs show a similar LH/hCGR expression in CC of patients stimulated with GnRH agonist long or antagonist protocols and between HP-hMG or rFSH treatments. However, recently, it was reported that the LH/hCGR gene was expressed at lower levels in floating granulosa cells when patients were stimulated with a combination of the long GnRH agonist protocol with HP-hMG when compared with stimulation with rFSH (Grøndahl et al., 2009). In the present study, no difference in LH/hCGR expression was observed between rFSH supplemented or not with rLH on Day 8 of COS (data not shown). This is probably due to the delay in the supplementation with rLH with the consequence that all LH/hCGR may already be occupied. Although LH/hCGR undergoes down-regulation in response to ligand exposure under pharmacological (high hCG doses) conditions, it has been shown that 24–48 h after hCG administration, the LH/hCGR reappears and is abundantly present in the luteinized granulosa cells (Peegel et al., 1994; Kash and Menon, 1998). Our data suggest that a similar regulation of LH/hCGR expression occurs in CC, which were removed <40 h post-hCG administration. However, as FSH is known to stimulate LH/hCGR expression (Shi and Segaloff, 1995), the difference observed in LH/hCGR gene expression between the three groups could be reflected by differences in FSH responsiveness. Moreover, saturation ratio receptor assays revealed that FSH up-regulates CC LH/hCGR receptors in the absence of LH, and subsequently LH stimulates COC expansion and oocyte maturation upon binding to these newly formed receptors (Chen et al., 1994; Kawashima et al., 2008). However, in the present study, there was no difference in the total FSH stimulation dose between the three LH/hCGR groups (low, medium, high), suggesting that the difference in FSH responsiveness is not associated with the modulation of LH/hCGR.
LH/hCGR expression in cumulus cells and TNFAIP6 gene expression

gene expression. Furthermore, there was no significant difference in serum LH on Day 3 after starting ovarian stimulation between the three LH/hCGR expression groups.

Several splice variants of LH/hCGR have been identified and were shown to be able to modulate the expression and the functional property of the wild-type (full length) LH/hCGR (Muller et al., 2003; Nakamura et al., 2004). Therefore, a difference in the splice variant pattern between patients could explain the difference in LH/hCGR expression variability of CCs obtained from different patients in this study.

Microarray and QRT–PCR analysis of CCs showed an increased expression of mRNA species involved in steroid metabolism in the high LH/hCGR group (HSD3B2, CYB5B), more particularly aromatases (CYP1B1, CYP11A1) that were previously described in theca and mural granulosa cells (Cheng et al., 2001; Penning et al., 2004; Luo and Wiltbank, 2006; Grundahl et al., 2009). Interestingly, CYP11A1 is an enzyme controlling the first and limiting step of steroid biosynthesis. HSD3B2 (3β hydroxysteroid dehydrogenase) gene expression controls the production of progesterone, and CYB5B is a cytochrome facilitating the activity of the enzyme catalysing pregnenolone to dehydroepiandrosterone conversion. Our data suggest therefore that CCs under COS also play a key role in follicular androgenic and estrogenic microenvironment after gonadotropin stimulation. This concept is supported by the fact that several studies reported that CC in vitro were able to secrete estradiol during COCs culture from patients undergoing stimulated cycles, probably as a consequence of the action of gonadotrophins (Chian et al., 1999; Mingoti et al., 2002; Shirazi and Moalemian, 2007). Therefore, it is possible that regardless of their origin, stimulation of CC by an LH or an hCG surge during ovulation induction leaves them with the same capability as mural granulosa cells for steroidogenesis.

In conclusion, this study unambiguously reports LH/hCGR mRNA expression in human CC surrounding the oocyte. Under COS, high LH/hCGR expression is associated with increased TNFAIP6 gene expression, a gene specially expressed in COC undergoing mufication, and inversely correlated with serum estradiol level on the day of hCG administration.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank the University Hospital of Montpellier for its support and the ART team for their assistance during this study. We thank Prof. Ilpo Huhtaniemi and Dr Masoud Afnan for their critical reading of the manuscript.

Funding

Funding was provided by the Ferring and Merck-Serono, Schering-Plough Pharmaceutical Companies.

References


Kawashima I, Okazaki T, Noma N, Nishibori M, Yamashita Y, Shimada M. Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and


