Differences in transcriptomic profiles of human cumulus cells isolated from oocytes at GV, MI and MII stages after in vivo and in vitro oocyte maturation

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BACKGROUND: Oocyte maturation and competence to development depends on its close relationship with cumulus cells (CCs). However, the maturation conditions of human cumulus–oocyte complexes (COCs) might affect gene expression in both oocyte and CCs. We thus compared the transcriptome profiles of CCs isolated from in vivo and in vitro matured COCs at different nuclear maturation stages.

METHODS: Three groups of CCs from patients who underwent ICSI were included: CCs of patients with polycystic ovary syndrome (PCOS) referred for in vitro maturation (IVM), CCs from patients with PCOS for in vivo maturation (used as controls) and CCs from normal responders referred for in vivo maturation. CCs were isolated from COCs at the germinal vesicle, metaphase I and metaphase II stages. Microarray technology was used to analyse the global gene expression and significance analysis of microarray to compare the expression profiles of CCs from COCs at different nuclear maturation stages following IVM or in vivo maturation. Selected genes were validated by RT-qPCR.

RESULTS: In CCs isolated after IVM, genes related to cumulus expansion and oocyte maturation, such as EREG, AREG and PTX3, were down-regulated, while cell cycle-related genes were up-regulated in comparison with CCs from in vivo matured COCs from PCOS and normal responder patients. Moreover, irrespective of the stage of oocyte maturation, genes involved in DNA replication, recombination and repair were up-regulated in CCs after IVM.

CONCLUSIONS: The CC transcriptomic signature varies according to both the oocyte maturation stage and the maturation conditions. Our findings suggest a delay in the acquisition of the mature CC phenotype following IVM, opening a new perspective for the improvement in IVM conditions.

Key words: cumulus cells / DNA microarray / gene expression profile / maturation condition

Introduction

Oocyte nuclear maturation depends on the resumption of meiosis in certain species, including human. During this process, oocytes complete their maturation and can support fertilization and early embryo development (Trounson et al., 2001). During IVF procedures, a large number of cumulus–oocyte complexes (COCs) are retrieved after controlled ovarian stimulation (Haouzi et al., 2009). However, ovarian stimulation might induce the ovarian hyper-stimulation syndrome and patients with polycystic ovary syndrome (PCOS), an endocrine disorder characterized by anovulation, presence of cysts in ovaries and hyperandrogenism (Adams, 1997), are at greater risk of developing this complication (Clément, 2007). In order to reduce this risk, new protocols with mild or without ovarian stimulation.
were suggested (Chian et al., 2000). Consequently, to ensure oocyte maturation and avoid the occurrence of ovarian hyper-stimulation, in vitro maturation (IVM) of immature COCs has been proposed as an alternative for these patients. However, the rate of pregnancy failure is higher following IVM than after conventional IVF (Buckett et al., 2008).

Differences in gene expression were recently reported between in vivo and in vitro matured human oocytes (Jones et al., 2008). Several genes involved in many signalling pathways, such as response to stress, cell cycle, cell proliferation, cell division and cell death, were up-regulated in in vitro matured oocytes compared with in vivo matured oocytes (Jones et al., 2008). Similarly, in bovine cumulus cells (CCs), isolated from in vitro matured COCs, genes involved in response to stress were up-regulated and genes related to cumulus expansion (such as TNFAIP6) and oocyte maturation regulation (such as INHBA and FST) were down-regulated (Tesfaye et al., 2009) compared with CCs isolated from in vivo matured COCs. These findings suggest that the expression of transcripts in oocytes and CCs is altered by the IVM conditions.

We have previously compared the gene expression profiles of CCs isolated from in vivo matured human oocytes at the germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages and observed a specific molecular CC signature for each stage of oocyte maturity (Ouandaogo et al., 2011). The aim of the present study was to establish the gene expression profile of human CCs at different stages (GV, MI and MII) of oocyte maturation following in vivo and in vitro oocyte maturation.

Materials and Methods

Experimental design

This project was approved by the Institute Review Board of the Institut de Recherche en Biothérapie. All patients were informed and gave their written consent. CC samples were collected from normal responders and patients with PCOS undergoing ICSI following in vivo or IVM. Normal responder patients were from the Arnaud de Villeneuve hospital of Montpellier, whereas patients with PCOS were recruited also from the Antoine Bécler hospital of Clamart. The ages of the patients (mean ± SEM) were 33.7 ± 4.7, 32.3 ± 3.3 and 33.8 years ± 3.1 in the normal responder, PCOS in vivo and PCOS IVM groups, respectively.

Samples were divided into three groups according to the oocyte maturation stage: CCs isolated from COCs at GV (CCGV), MI (CCMI) and MII (CCMII) stages. To differentiate samples isolated from in vivo (patients with PCOS) or in vivo (normal responders) matured COCs, the samples from patients with PCOS were labelled as CCGV:PM, CCMI:PM and CCMII:PM, while the samples from normal responders were marked as CCGV, CCMI and CCMII. Samples were then re-grouped into three classes for transcriptomic comparison: Class I (CCGV versus CCGV:PM), Class II (CCMI versus CCMII:PM) and Class III (CCMII versus CCMII:PM).

To identify genes that were specifically deregulated by the maturation condition and not by the PCOS, another group of CCs retrieved from PCOS MII oocytes that were matured in vivo (CCMII PCOS in vivo) was added as a control for CCMII:PM. Thus, another comparison was made between CCMII:PM PCOS in vivo and CCMII:PM.

Protocol for in vitro maturation of oocytes

Patients were stimulated with a combination of GnRH agonist or antagonist protocols as follows: recombinant FSH alone or recombinant FSH with recombinant LH (Gonal™, Luveris™, Merck-Serono) at doses between 125 and 300 UI/day. FSH, LH and estradiol levels were measured 8 or 9 days after the first day of stimulation. When follicles reached a diameter of 15 mm, 5000 UI of hCG (Chonorine Endo; Organon, Paris, France) were administered. Thirty-six hours after hCG administration, COCs were retrieved by transvaginal aspiration. COCs were immediately placed at 37 °C in the commercial medium G-MOPSTM PLUS (Vitrolife) and then transferred to the GIVF PLUS culture medium (Vitrolife, Göteborg, Sweden).

Protocol for IVM of oocytes

Ovarian ultrasonography was performed on the third day of menstruation in order to make sure that cysts were absent. Estradiol, progesterone and LH serum levels were also measured. On Day 8 of ovarian stimulation (as above), a transvaginal ultrasound scan was performed to exclude the possibility of a dominant follicle. When several follicles reached a diameter of 7 mm, 10 000 UI of hCG was administered to induce ovulation. COCs were aspirated by transvaginal echography 36 h after hCG administration and collected in the saline (0.9% NaCl) medium supplemented with 2 UI/ml of heparin. Then COCs were incubated in the IVF medium (Micult, Lyon, France) supplemented with 20% inactivated maternal serum, 0.75 IU/ml FSH and 0.75 IU/ml LH (Menopur; Ferring, Gentilly, France) at 37 °C, 6% CO2 for 24 h or 48 h.

In both maturation conditions (in vivo and in vitro), the mechanical removal of CC was facilitated by exposure to hyaluronidase just before ICSI. The oocyte maturation stage was evaluated under an inverted microscope, based on morphological criteria such as the presence of GV (GV stage), absence of GV (MI stage) and presence of the first polar body (MII stage).

Cumulus cell processing

This study included 29 normal responder patients and 27 patients with PCOS.

A total of 49 and 39 CC samples from in vivo and in vitro matured COCs, respectively, were used for microarray analysis and data validation by RT-qPCR (Table I). After removal, CCs were put immediately in RLT lysis buffer (RNaseasy Micro Kit, Qiagen, Valencia, CA, USA), supplemented with 1% (v/v) 2-β-mercaptoethanol (M-3148, Sigma, Lyon, France) before storage at −80 °C until total RNA extraction.

To assess the relationship between maturation conditions and actual nuclear maturation or embryo outcome, all the CCs from the COCs retrieved from the 29 normal responders referred for in vivo and 18 patients with PCOS referred for IVM (i.e. 280 and 277 CC samples, respectively) were used.

RNA extraction and microarray technology

Total RNA from CCs was extracted using the RNeasy Micro Kit, in accordance with the manufacturer’s protocol (Qiagen). Total RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity and quality were evaluated with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA samples were stored at −80 °C until microarray analysis.

For microarray processing, the Affymetrix 3’ IVT express protocol (ref 901229) was used as previously described (Ouandaogo et al., 2011).

Data processing

Scanned GeneChip images were processed using the Affymetrix GCOS 1.4 software. Microarray data were analysed using the Affymetrix Expression Console™ software and normalization was performed with the MASS.0 algorithm to obtain the signal intensity and the detection call.
samples were filtered with oocyte nuclear maturation. Class I (GV stage) and class III (MII stage) were also used to compare CC MII-IVM and CC MII PCOS.

Microarray processing

In vivo

Normal responders (n = 16)  CCGV  8
CCMi  8
CCMi  8
CCMi  9
CCGV-IVM  6
CCMi-IVM  9

In vitro

PCOS (n = 9)  CCGV  8
CCMi  8
CCMi  8
CCGV-IVM  8
CCMi-IVM  8

RT-qPCR

In vivo

Normal responders (n = 13)  CCGV  8
CCMi  8
CCMi  8
CCGV-IVM  8
CCMi-IVM  8

In vitro

PCOS (n = 10)  CCGV  8
CCMi  8
CCMi-IVM  8

Total number of CC samples  88

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Maturation condition</th>
<th>Patients type (number of patients)</th>
<th>Oocyte maturation stage</th>
<th>Number of CC samples</th>
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<td>Microarray processing</td>
<td>In vivo</td>
<td>Normal responders (n = 16)</td>
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CC, cumulus cell; PCOS, polycystic ovary syndrome.
CCGV, CCMi, and CCMi correspond to CCs isolated from GV, MI and MII oocytes, respectively. The in vivo CCs are isolated from normal responder and MII PCOS patients, and the IVM CCs from PCOS samples.

Microarray data analysis

MASS quality control was performed on the microarray raw data for CCs isolated from in vivo matured COCs (24 CC samples from normal responder patients and 9 from patients with PCOS) and in vitro matured COCs (23 CC samples from patients with PCOS). Before statistical comparison, CCs isolated from in vivo and in vitro matured COCs were filtered according to the ‘detection call’ absent (A)/present (P). The cut-off value for P was chosen according to the lowest number of samples in each CC class (first lists). Three analyses were performed according to the stage of oocyte nuclear maturation. Class I (GV stage) and class III (MII stage) samples were filtered with P > 0.05. Class II (MI stage) samples were filtered with P > 0.06.

Reverse transcriptase-quantitative polymerase chain reaction

Independent cohorts of CC samples from in vivo or in vitro matured oocytes were used for the validation of some of the genes that were found to be over-expressed in CCGV (n = 8 samples), CCMi-IVM (n = 8), CCMi (n = 8) and CCMi-IVM (n = 8; Table I). Validation of the CCMi expression data was not performed due to the rarity of these samples.

RT-qPCR screening was performed on 23 transcripts (5 and 6 transcripts from the CCGV and CCMi-IVM lists, respectively, and 6/each for the CCMi and CCMi-IVM lists). The primer sequences (Sigma) are listed in Supplementary data, Table SI. RT-qPCR amplification was carried out by using the Superscript First Strand Synthesis System kit (Invitrogen, Saint Aubin, France) according to the manufacturer’s recommendations. First-strand cDNA was generated starting from 250 ng total RNA and then used (dilution 1:10) to assess gene expression by qPCR in 384-well plates on a Light Cycler 480 (Roche, Mannheim, Germany). Each well contained 8 µl of Master SYBR green mix (Roche) with 1.625 µM of each primer (final concentration) and 2 µl of diluted DNA. To check reproducibility, each qPCR reaction was carried out in duplicate, and water was used as a negative control. The amplification was a 45-cycle run with annealing temperature at 60°C. The PCR products generated by each cycle were monitored with the SYBR green probe. To normalize the expression level, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used because its expression was stable in all CC groups. PCR efficiency (E) was obtained by a standard curve that varies in function of the primers used. Moreover, at the end of the amplification, a melting curve for each sample was generated to assess the absence of primer dimers or contamination. The cycle number (Ct’ for cycle threshold) value was used to calculate the relative amount of mRNA transcripts. The qPCR data were analysed using the followed.

<i>http://babelomics3.epfl.ch</i> web tool Fatigo. This application allows the extraction of gene ontology (GO) terms by applying the Fisher’s exact test on two lists of genes (Al-Shahrour et al., 2004). The ingenuity pathways analysis (IPA) was also used to identify the different network connections in each list of genes.

(i.e. the expression of a gene with a defined confidence level) for each probe set. The detection call can be ‘present’ [when the perfect match probes are significantly more hybridized than the mismatch probes, FDR < 0.04 (FDR, false discovery rate)], ‘marginal’ (for FDR ≥ 0.04 and ≤ 0.06) or ‘absent’ (FDR > 0.06).

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Then, a significant analysis of microarrays (SAM; Tusher et al., 2001) was performed to identify the genes that were significantly over- or under-expressed in in vivo versus in vitro matured CCs, which were classified according to the oocyte nuclear maturation stage: CCGV versus CCMi-IVM, CCMi versus CCMi-IVM and CCMi versus CCMi-IVM. SAM was also used to compare CCMi and CCMi PCOS in vivo. SAM provides score values and an FDR confidence percentage based on data permutation. Two conditions were used for SAM, based on the false discovery rate (FDR < 5%) and fold change (FC > 2). The set of genes identified by SAM was used to explore the biological functions that were over-represented in class I (CCGV versus CCMi-IVM), II (CCMi versus CCMi-IVM) and III (CCMi versus CCMi-IVM) CC samples with the Babelomics (http://babelomics3.epfl.ch) web tool Fatigo. This application allows the extraction of gene ontology (GO) terms by applying the Fisher’s exact test on two lists of genes (Al-Shahrour et al., 2004). The ingenuity pathways analysis (IPA) was also used to identify the different network connections in each list of genes.

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Clinical outcome
The percentage of samples isolated from oocytes at GV, MI and MII stages, after in vivo and IVM, was calculated as well as the percentage of MII oocytes that reached the embryo stage at Day 3.

Statistical analysis
The expression data for the CC GV, CC GV-IVM, CC MI and CC MI-IVM classes obtained by RT-qPCR was analysed with the two tailed t-test using the GraphPad Prism version 5.00 software (GraphPad, San Diego, CA, USA; www.graphpad.com). Differences between groups were considered significant when the P-value was <0.05.

For the percentage of samples that reached MII and embryo stages, the P-values were calculated using the chi-squared test (comparison of two percentages).

Results
Microarray internal quality controls and global gene expression in human CCs
The optical density 260/280 of all CC RNA samples varied between 1.64 and 2.26. The RIN (RNA integrity number) values ranged from 5.4 to 9.6. Microarray data quality was monitored by checking all the Affymetrix controls (for hybridization, labelling and internal control genes) for each sample. The ratio between the 3' probe set and the 5' probe set of GAPDH and ACTB, the two housekeeping genes adapted to the present study, was ≤3 for all arrays. The distribution of signal values for each probe set on each array was compared with the median array for the group using MASS and showed a good distribution among the samples.

Based on the detection call, we found that 16 749 genes were expressed in the CC GV and 16 271 in the CC GV-IVM group, 16 742 in the CC MI and 16 694 in the CC MI-IVM group and 16 752 in the CC MII and 17 081 in the CC MII-IVM samples.

At each stage of oocyte maturity, unsupervised clustering showed that CC groups obtained from in vivo and in vitro matured COCs displayed specific molecular signatures (Fig. 1). As observed in each unsupervised cluster, a first branch allows a clear segregation between the two types of maturation whatever the oocyte maturity stage. Then, the length of the branches reflects the degree of similarity between gene expressions of samples.

Comparison between gene expression profiles of CCs isolated from in vivo and in vitro matured COCs
Using SAM, the three classes (based on the oocyte maturation stage) of CCs from in vivo and in vitro matured COCs were compared (Fig. 2).

For genes that were over-expressed in class I samples (CC GV versus CC GV-IVM, Fig. 2A), the FC ranged from 2.0 to 31.1 and for down-regulated genes from 2.0 to 28.3. In class II (CC MI versus CC MI-IVM, Fig. 2A), the FC of up-regulated genes varied between 2.0 and 43.8 and for under-expressed genes between 2.0 and 45.6. In class III (CC MII versus CC MII-IVM), the FC range of values for over-expressed genes was between 2.0 and 102.4 and for under-expressed genes between 2.0 and 53.1. For complete lists, see Supplementary data, Tables SII-GC, SII-MI and SIII-MII.

Among the up-regulated transcripts, the gene encoding VLDLR, a low-density lipoprotein receptor, was over-expressed in CC MII (versus CC MI-IVM; FC = 6.8), whereas the genes encoding the heat shock proteins (HSP90AB1 (FC = 2.9) and DNAJC9 (FC = 4.2) were over-expressed in CC MI-IHM (compared with CC MI) and CC MII-IHM (compared with CC MII), respectively.

Moreover, the luteinizing hormone/choriogonadotrophin receptor (LH) gene was over-expressed in CC MII (compared with CC MI-IHM; FC = 20.2) and CC MI (compared with CC MI-IHM; FC = 4.3). Epiregulin (EREG), betacellulin (BTC) and tumor necrosis factor alpha-induced protein 6 (TNFAIP6) were significantly over-expressed in CC GV (compared with CC GV-IHM; FC = 6.0, 4.5, 4.1, respectively), CC MI (compared with CC MI-IHM; FC = 10.3, 3.6, 4.3, respectively) and CC MII (compared with CC MII-IHM; FC = 17.8, 2.8, 7.1, respectively). Other cumulus expansion-related genes, such as pentraxin 3 (PTX3), amphiregulin (AREG), prostaglandin-endopeptidase synthase 2 (PTGS2) and follistatin (FST) were also over-expressed in CC GV (compared with CC GV-IHM; FC = 11.1, 5.3, 3.1, 3.0, respectively), CC MI (compared with CC MI-IHM; FC = 2.5, 11.1, 5.3, 2.8, respectively) and CC MII (compared with CC MII-IHM; FC = 2.8, 12.7, 25.9, 2.3, respectively). Conversely, the over-expression of different cyclins and cyclin-dependent kinases (CDK), including CCNA2, CCNB1, CDK2, CDK6 and CCNE2, was observed in CC GV(IHM) (compared with CC GV; FC = 12.6, 7.2, 4.0, 5.8, 5.6, respectively), CC GV-IHM (compared with CC GV; FC = 16.3, 12.8, 4.5, 4.4, 4.9, respectively) and CC GV-IHM (compared with CC GV; FC = 36.7, 20.4, 3.5, 7.5, 9.9, respectively).

To understand if the differential expression of these genes was associated with the maturation conditions or with the impacts of PCOS, the list of genes obtained from the class III comparison (CC GV versus CC GV-IHM) was compared with the one obtained by comparing the transcriptome of the CC GV-IHM with that of CCs that were isolated from MII oocytes of patients with PCOS following in vivo maturation (CCs PCOS in vivo; Fig. 2B, Supplementary data, Table SIII). More than 65% of the genes identified in the class III comparison were also present in the list of genes obtained from the CCs PCOS in vivo CC GV-IHM comparison (Fig. 2C, Supplementary data, Table SIV). All genes reported in the section Results are part of this common list, thus reinforcing the notion that the differential expression of these genes was associated with the maturation conditions.

Functional annotation of genes that are differentially expressed in human CCs
Fatigo was used to assess functional enrichment for specific genes in the three CC classes (GV, MI and MII) following in vivo or in vitro oocyte maturation. Only significant functions (adjusted P-value < 5%) were reported. The comparison of the CC GV and CC GV-IHM gene expression profiles showed that genes over-represented in CC GV were involved in steroid metabolic processes (5.3 versus 0.37%), lipid biosynthesis (5.9 versus 1.85%), inflammatory response (3.95 versus 0.89%), signal transduction (29.61 versus 21%) and cell adhesion (8.42 versus 4.55%); Fig. 3A). Conversely, CC GV-IHM were enriched in genes involved in the M phase of the cell cycle (11.56 versus 0.47%), DNA replication (9.54 versus 0.62%),
recombination (3.12 versus 0.16%) and repair (7.89 versus 0.93%), response to endogenous stimuli (8.36 versus 1.6%) and cytoskeleton organization and biogenesis (8.47 versus 3.07%; Fig. 3A).

The comparison of the class II (CC MI and CC MI-IVM) expression profiles (Fig. 3B) showed that, in CC MI, many genes playing a role in steroid metabolic processes (4.64 versus 0.16% in CC MI-IVM), cellular lipid metabolism (8.79 versus 2.28%), alcohol metabolism (4.39 versus 1.48%), membrane organization and biogenesis (2.02 versus 0.3%) and vesicle-mediated transport (4.21 versus 1.07%) were over-represented (Fig. 3B). Conversely, genes involved in intracellular transport (9.49 versus 4.62%), nucleic acid transport (1.74 versus 0%), DNA replication (9.49 versus 0.92%), recombination (3.48 versus 0.26%) and repair (8.39 versus 1.32%) were enriched in the CC MI-IVM group.
The investigation of biological processes in class III (CC MII and CCMII-IVM; Fig. 3C) showed that, in CC MII, genes involved in response to external stimuli (6.61 versus 2.55%), small GTPase-mediated signal transduction (4.94 versus 1.87%), lipid biosynthesis (6.11 versus 1.99%), steroid metabolic processes (5.46 versus 0.82%) and monocarboxylic acid metabolism (5.46 versus 0.82%) were over-represented.

On the other hand, genes highly represented in the CCMII-IVM group were found to regulate DNA replication (7.62 versus 0.78%), recombination (2.93 versus 0.26%) and repair (7.15 versus 0.78%) processes. Moreover, genes involved in the M phase of the cell cycle (8.32 versus 0.52%) and microtubule-based processes (5.04 versus 0.78%) were also over-represented.

Finally, the IPA functional analysis tool showed that the ATM signaling pathway was specifically activated in CCMII and the BRCA1 signalling cascade in CCGV-IVM (Supplementary data, Fig. S1 A and B).

### Validation of selected genes by RT-qPCR

For validation of the DNA chip data by RT-qPCR, five to six genes that were differentially regulated in class I and II groups (total n = 23 genes) were chosen. The differential expression of 20 transcripts was validated (P-value between 0.0011 and 0.0458). Conversely, SPP1 and TIA1 did not show significant differences in the expression between CCs from in vitro and in vivo matured COCs. Moreover, DNAJB8 could not be amplified, possibly due to the primer design (Fig. 4).

### Immature CC phenotype following IVM

We have previously described the specific gene expression profiles of CCs isolated from in vivo matured COCs at different stages of oocyte maturation (Ouandaogo et al., 2011). We used these specific molecular signatures that included 10 (GV), 4 (MI) and 11 (MII) genes to check the maturity status of PCOS CCs isolated from in vitro (CCMII-IVM) and in vivo (CCMII PCOS in vivo) matured COCs at MII. The expression profile of CCMII-IVM was strongly altered in comparison with that of CCMII PCOS in vivo and also of CCMII (normal responders; Fig. 5). Specifically, the CCMII-IVM group was characterized by over-expression of the CC GV and CC MI signatures, whereas genes that belong to the CC MII in vivo signature were not detected, thus suggesting an immature phenotype in CCs following IVM.

### Effects of the oocyte maturation conditions on embryo outcome

Finally, we found a significant difference in the clinical outcome between in vivo and in vitro oocyte maturation. Following in vivo maturation (n = 280 samples), 7.86% CCs were isolated from GV oocytes, 8.21% from MI oocytes and 82.14% from MII oocytes, and 1.78% samples presented empty zona pellucida. Moreover, 85.22% of MII oocytes reached the embryo stage (P value = 10^{-6} when compared with the percentage from in vivo matured oocytes).
Discussion

Acquisition of oocyte competence is a complex process, which depends on the follicular microenvironment. Here, we report the down-regulation of genes involved in cumulus expansion (TNFAIP6, PTGS2 and PTX3) as well as of genes related to oocyte maturation, including several EGF-like growth factors (EREG, AREG and BTC) in CCs from in vitro matured oocytes in comparison with CCs from in vivo matured oocytes. In addition, many cell cycle-related genes, such as cyclins and CDKs, were up-regulated in CCs from in vitro matured oocytes suggesting that in vitro matured CCs are still proliferating and that CCs surrounding mature (MII) oocytes, as defined in Ouandaogo et al. (2011), are not fully mature yet. Moreover, the functional annotation of genes expressed in CCs at different stages of oocyte maturity (GV, MI and MII) showed that CCs from in vivo matured oocytes were mainly enriched in genes associated with lipid metabolism, whereas in CCs from in vitro matured oocytes, genes involved in DNA replication, recombination and repair were predominant.

Down-regulation of genes involved in cumulus expansion and oocyte maturation

It is well known that LH action on ovarian follicles is mediated by multiple signalling molecules, including EGF-like growth factors (Hsieh et al., 2007; Reizel et al., 2010; Zamah et al., 2010; Romero et al., 2011). Among the factors accumulated in the follicular fluid of mature follicles from hCG-stimulated patients, AREG is the most abundant and plays a central role in the gonadotrophin-dependent stimulation of cumulus expansion and oocyte maturation (Zamah et al., 2010). Moreover, EREG and BTC are up-regulated in both
cumulus and granulosa cells after LH ovulatory stimulus. Several studies reported the importance of the EGF pathway during oocyte meiosis reinitiation as the inhibition of this signalling pathway can limit CC expansion and meiotic resumption (Park et al., 2004; Nyholt de Prada et al., 2009; Reizel et al., 2010; Romero et al., 2011). In the present study, regardless of the oocyte maturity stage, we observed down-regulation of LHCGR, EREG, AREG and BTC, as well as of other cumulus expansion-related genes, such as TNFAIP6, PTGS2 and PTX3, in CCs from in vitro matured oocytes compared with CCs isolated from in vivo matured oocytes from patients with PCOS and normal responder patients. These findings are in agreement with previous studies showing that the oocyte developmental capacity after in vitro or in vivo maturation is accompanied by distinct differences in CC gene expression profile (Nyholt de Prada et al., 2009; Tesfaye et al., 2009). The regulation of LHCGR expression in CCs has been previously reported to depend on oocyte-secreted factors and also on the FSH dose (Kawashima et al., 2008; Romero et al., 2011). Due to the profile of patients with PCOS, differences in LHCGR expression in CCs may thus be related to differences in administered doses of FSH.

Other genes involved in oocyte maturation, such as FST which inhibits FSH release, were also down-regulated in CCs isolated from COCs matured in vitro COCs in comparison with CCs from in vivo matured COCs. These findings are in agreement with a previous study (Tesfaye et al., 2009).

**Proliferative and immature status of CCs under in vitro condition**

Several data suggest that CCs continue to proliferate following the ovulatory stimulus thereby leading to cumulus expansion (Cannon et al., 2005; Hernandez-Gonzalez et al., 2006; Kawashima et al., 2008; Nyholt de Prada et al., 2009). In the mouse, CCs are highly mitotic for a few hours after hCG administration and then stop dividing after 16–24 h concomitantly with maturation (Hernandez-Gonzalez et al., 2006). Cyclin D (CCND)/CDK4, 6 complexes are involved in the early transition through G1 of the cell cycle, and the Cyclin E (CCNE)/CDK2 complex in the progression through the G1/S phase. We report for the first time the over-expression of CDK2, CDK6, CCNE2, CCNA2 and CCNB1 mRNAs in human CCs matured in vitro compared with in vivo matured CCs, whatever the oocyte nuclear maturity stage. CCNA2 and CCNB1 play an essential role in cell proliferation through promotion and maintenance of the mitotic state, respectively (Gong and Ferrell, 2010). These findings suggest that in vitro matured CCs are highly mitotic in comparison with those matured in vivo and show a delay in their complete maturation as suggested also by their specific gene expression profiles at different stages of oocyte nuclear maturation (Ouandaogo et al., 2011).

**Major difference in lipid metabolism and DNA recombination/repair**

Lipids and fatty acids constitute the main energetic source for protein synthesis during oocyte nuclear maturation and embryonic development (Loos et al., 1989; Sturmy and Leese, 2003; Cui et al., 2009; Murphy et al., 2009). Our results show that genes involved in the cholesterol biosynthesis pathway (DHCR7, LDLR and VLDLR) are down-regulated in in vitro matured CCs in comparison with in vivo matured CCs. These data suggest that the IVM conditions alter lipid metabolism in CCs, thus affecting their ability to provide the oocyte with lipid products and consequently guarantee oocyte nuclear maturation (Kim et al., 2001; Yamashita et al., 2003; Su et al., 2008).

Conversely, many genes that are involved in DNA repair, such as FANC2D and BIRC5 (Tanguchi et al., 2002; Deng, 2006), were over-expressed in CCs isolated from in vitro matured COCs. BIRC5 regulates the spindle checkpoint and cell division and FANC2D is essential for the S-phase checkpoint (Garcia-Higuera et al., 2001; Joenje and Patel, 2001; Tanguchi et al., 2002; Lens et al., 2003). The high accuracy of chromosome segregation is ensured by the spindle checkpoint as it inhibits cells with non-aligned chromosomes from exiting mitosis (Deng, 2006). Furthermore, CCs recovered from pre-ovulatory follicles are markedly mitotic (Hernandez-Gonzalez et al., 2006), suggesting that the maturation conditions may induce more spindle checkpoints in CCs. Moreover, the ATM and BRCA1 signalling pathways, which play a role in the DNA damage response, were over-represented in in vitro matured CCs, suggesting higher activity of DNA repair genes during IVM.

CCs play an essential role in protecting the oocyte from temperature changes by inducing the synthesis of thermoprotector proteins, such as HSPs, in the oocyte (Edwards and Hansen, 1996, 1997; Schöfl et al., 1998; Lånskå et al., 2006; Wang et al., 2009). Here, we report the over-expression of several members of the HSP family in CCs isolated from in vitro matured versus in vivo matured COCs, including HSP90AB1, DNAJB8, DNAJC12 and DNAJC9. The precise role of such HSPs during oocyte maturation is not well understood. However, previous reports in animal models suggest that HSP transcripts are affected by the follicular environmental conditions (Camargo et al., 2007; Tesfaye et al., 2009), reinforcing the hypothesis that their presence is probably linked to the maturation conditions. This hypothesis is strengthened by our results as HSP up-regulation was observed only in CC\textsubscript{MII,IVM}. More studies are required to confirm these results.

In the present study, we cannot exclude the possibility that the differences in CC gene expression profiles were not only due to the different oocyte nuclear maturation conditions (in vitro and in vivo), but also to other effects. For example, gonadotrophin doses can also affect CC gene expression profiles (Doozier et al., 2008; Adriaenssens et al., 2010; Sánchez et al., 2010). Moreover, the PCOS profile could affect gene expression in CCs. Indeed, the irregular intrafollicular environment in patients with PCOS can alter the cytoplasmic and/or nuclear maturation of the oocyte through direct or indirect effects via cumulus cell-oocyte signalling. This alteration has a negative impact on the activation of essential genes expressed in oocytes during early embryonic stages (Dumesic and Abbott, 2008). The altered LH and FSH responsiveness in patients with PCOS could also affect the expression profile of CCs. However, the comparison of PCOS CCs from in vivo matured MII oocytes (as controls) with CC\textsubscript{MII,IVM} in the current study suggests that the reported differences in gene expression profiles could be associated with the maturation conditions. As it was not possible to perform the same comparison with CCs from GV and MI stages, further investigations are required to be sure that the observed differences in CC gene expression profiles are only associated with the in vivo or IVM conditions.

In conclusion, our data suggest that the CC transcriptomic profile is affected not only by the degree of oocyte nuclear maturation, but also by the oocyte maturation conditions. Many genes involved in cumulus...
expansion and oocyte nuclear maturation were down-regulated, while genes related to proliferation were over-expressed in CCs following IVM, suggesting a delay in the acquisition of the mature CC phenotype in comparison with those isolated from in vivo matured COCs. However, according to the different transcriptomic analyses performed in the present study, our results suggest that the alteration observed in CC gene expression profile at MI stage is the consequences of the IVM conditions rather than the PCOS profile. These findings can help understand the less favorable IVM outcome since CC gene expression profile has been related to nuclear maturation, fertilization, embryo quality and pregnancy outcome (Assou et al., 2006, 2008, 2010; Filali et al., 2009; Ouandaogo et al., 2011). Consequently, this study provides crucial data for the improvement in IVM conditions.

Supplementary data

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

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Authors’ roles

O.Z.G. performed the microarray and qRT-PCR experiments, analyses of the data and contributed to the paper redaction; F.N. performed sample recruitment from the in vitro condition maturation and contributed to data interpretation; H.L. performed sample recruitment from the in vitro condition maturation; A.S. and D.H. contributed to data interpretation and participation to the paper redaction; H.D. contributed to data interpretation and wrote the paper; F.R. was involved in patient recruitment for IVM and participation to data interpretation; H.S. conceived the experiments, participation in data interpretation and in paper redaction and final approval.

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Conflict of interest

None declared.

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